

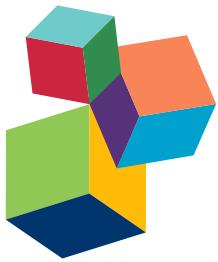
ROLE OF STEM CELLS IN SKELETAL MUSCLE DEVELOPMENT, REGENERATION, REPAIR, AGING AND DISEASE

EDITED BY: Pura Muñoz-Cánoves, Jaime J. Carvajal,

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ROLE OF STEM CELLS IN SKELETAL MUSCLE DEVELOPMENT, REGENERATION, REPAIR, AGING AND DISEASE

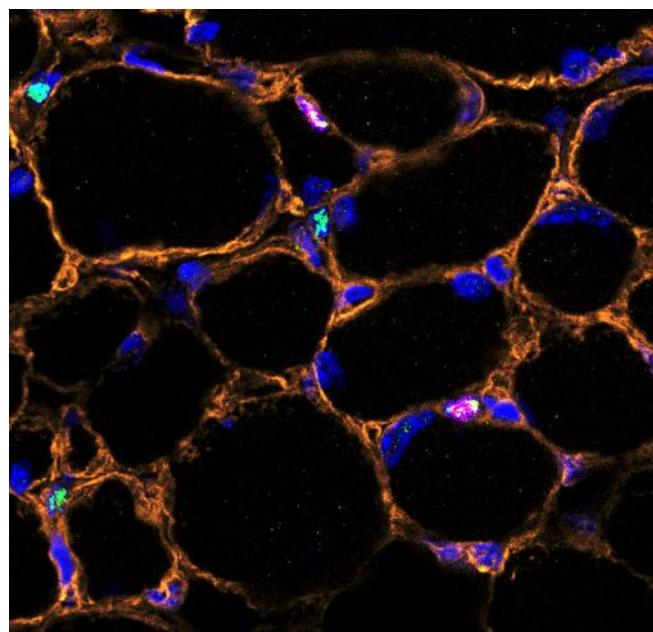
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Transversal sections of extraocular muscles from C57Bl6 mice stained for PW1 (green), Pax7 (red), Laminin (orange) and DAPI (blue). For details, see Formicola et al. (page 161).

Adult stem cells are responsible for tissue regeneration and repair throughout life. Their quiescence or activation are tightly regulated by common signalling pathways that often recapitulate those happening during embryonic development, and thus it is important to understand their regulation not only in postnatal life, but also during foetal development. In this regard, skeletal muscle is an interesting tissue since it accounts for a large percentage of body mass (about 40%), it is highly amenable to intervention through exercise and it is also key in metabolic and physiological changes underlying frailty susceptibility in the elderly. While muscle-resident satellite cells are responsible for all myogenic activity in physiological conditions and become

senescent in old age, other progenitor cells such as mesoangioblasts do seem to contribute to muscle regeneration and repair after tissue damage. Similarly, fibro-adipogenic precursor cells seem to be key in the aberrant response that fills up the space left from atrophied muscle mass and which ends up with a dysfunctional muscle having vast areas of fatty infiltration and fibrosis. The complex interplay between these stem/progenitor cell types and their niches in normal and pathological conditions throughout life are the subjects of intense investigation. This eBook highlights recent developments on the role of stem cells in skeletal muscle function, both in prenatal and postnatal life, and their regulation by transcriptional, post-transcriptional and epigenetic mechanisms. Additionally, it includes articles on interventions associated with exercise, pathological changes in neuromuscular diseases, and stem cell aging.

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Table of Contents

- 06 Editorial: Role of Stem Cells in Skeletal Muscle Development, Regeneration, Repair, Aging, and Disease**
Pura Muñoz-Cánoves, Jaime J. Carvajal, Adolfo Lopez de Munain and Ander Izeta
- CHAPTER 1. Role of stem cells in skeletal muscle development**
- 10 A population of Pax7-expressing muscle progenitor cells show differential responses to muscle injury dependent on developmental stage and injury extent**
Stefanie Knappe, Peter S. Zammit and Robert D. Knight
- 27 The emergence of Pax7-expressing muscle stem cells during vertebrate head muscle development**
Julia Meireles Nogueira, Katarzyna Hawrot, Colin Sharpe, Anna Noble, William M. Wood, Erika C. Jorge, David J. Goldhamer, Gabrielle Kardon and Susanne Dietrich
- 49 Conditional Cripto overexpression in satellite cells promotes myogenic commitment and enhances early regeneration**
Carolina Prezioso, Salvatore Iaconis, Gennaro Andolfi, Lorena Zentilin, Francescopaolo Iavarone, Ombretta Guardiola and Gabriella Minchiotti
- CHAPTER 2. Role of stem cells in skeletal muscle regeneration and repair**
- 60 Chromatin signaling in muscle stem cells: interpreting the regenerative microenvironment**
Arianna Brancaccio and Daniela Palacios
- 77 DNA methylation dynamics in muscle development and disease**
Elvira Carrió and Mònica Suelves
- 89 Pericytes: multitasking cells in the regeneration of injured, diseased, and aged skeletal muscle**
Alexander Birbrair, Tan Zhang, Zhong-Min Wang, Maria L. Messi, Akiva Mintz and Osvaldo Delbono
- 107 Potential of adipose-derived stem cells in muscular regenerative therapies**
Sonia-V Forcales
- CHAPTER 3. Role of stem cells in skeletal muscle aging**
- 119 Exceptional longevity and muscle and fitness related genotypes: a functional in vitro analysis and case-control association replication study with SNPs THRH rs7832552, IL6 rs1800795, and ACSL1 rs6552828**
Noriyuki Fuku, Zi-hong He, Fabian Sanchis-Gomar, Helios Pareja-Galeano, Ye Tian, Yasumichi Arai, Yukiko Abe, Haruka Murakami, Motohiko Miyachi, Hirofumi Zempo, Hisashi Naito, Thomas Yvert, Zoraida Verde, Letizia Venturini, Carmen Fiuza-Luces, Alejandro Santos-Lozano, Gabriel Rodriguez-Romo, Giovanni Ricevuti, Nobuyoshi Hirose, Enzo Emanuele, Nuria Garatachea and Alejandro Lucia

- 128 *Forever young: rejuvenating muscle satellite cells***
Luca Madaro and Lucia Latella
- 131 *Myogenic-specific ablation of Fgfr1 impairs FGF2-mediated proliferation of satellite cells at the myofiber niche but does not abolish the capacity for muscle regeneration***
Zipora Yablonka-Reuveni, Maria E. Danoviz, Michael Phelps and Pascal Stuelsatz
- CHAPTER 4. Role of stem cells in diseases of the skeletal muscle**
- 147 *A muscle stem cell for every muscle: variability of satellite cell biology among different muscle groups***
Matthew E. Randolph and Grace K. Pavlath
- 161 *The extraocular muscle stem cell niche is resistant to ageing and disease***
Luigi Formicola, Giovanna Marazzi and David A. Sassoon
- 169 *The quasi-parallel lives of satellite cells and atrophying muscle***
Stefano Biressi and Suchitra D. Gopinath
- 186 *Muscle wasting in myotonic dystrophies: a model of premature aging***
Alba Judith Mateos-Aierdi, María Goicoechea, Ana Aiastui, Roberto Fernández-Torrón, Mikel García-Puga, Ander Matheu and Adolfo López de Munain
- 202 *MicroRNAs modulated by local mIGF-1 expression in mdx dystrophic mice***
Laura Pelosi, Angela Coggi, Laura Forcina and Antonio Musarò
- 213 *Feasibility of resistance training in adult McArdle patients: clinical outcomes and muscle strength and mass benefits***
Alfredo Santalla, Diego Munguía-Izquierdo, Lidia Brea-Alejo, Itziar Pagola-Aldazábal, Jorge Díez-Bermejo, Steven J. Fleck, Ignacio Ara and Alejandro Lucía



Editorial: Role of Stem Cells in Skeletal Muscle Development, Regeneration, Repair, Aging, and Disease

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The Editorial on the Research Topic

Role of Stem Cells in Skeletal Muscle Development, Regeneration, Repair, Aging, and Disease

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Skeletal muscle is a highly dynamic and plastic tissue, able to modify its intrinsic size or strength following electric impulse, mechanical loading, or diet. Several muscular dystrophic disorders have been characterized but the development of therapies, although promising, is still at an early phase. Muscle dysfunction is not restricted to dystrophic patients; during aging, there is a gradual loss of muscle function that results in a significant negative impact on the individual's health, increasing fall and lesion risks, loss of mobility and independence, and associated elevation of morbidity and mortality. This loss of muscle has an estimated prevalence between 5 and 13% among 60–70 year old individuals, increasing to 11–50% in those over the age of 80 (Morley, 2008). According to the WHO, the expected number of individuals over 65 years old by the year 2050 will be around 1.5 billion (WHO, 2015); by extrapolation, this suggests that over 150 million patients will suffer from muscle wasting disorders associated with aging.

The vertiginous development of the stem cell therapy and cellular reprogramming fields will eventually result in the emergence of cell replacement therapies to treat a wide range of pathologies. In skeletal muscle, the satellite cell—long regarded as a heterogeneous cell population—is intimately linked to muscle physiology and regeneration processes (Chang and Rudnicki, 2014; Dayanidhi and Lieber, 2014; Snijders et al.). The activation of these muscle-specific stem cells is essential for injury healing, maintenance of muscle strength and tone, and delayed onset of age-related sarcopenia.

In this issue, eight review and eight original research articles on this Research Topic summarize recent progress and current challenges of the muscle biology field in aging and disease.

ROLE OF STEM CELLS IN SKELETAL MUSCLE DEVELOPMENT

Stem cells have a key role during the sequential stages of embryonic muscle development, as demonstrated through many decades of work using a multitude of animal models (Brand Saberi, 2015). In this issue, Knight and colleagues develop a novel *pax7a:eGFP* transgenic zebrafish injury-model in which they demonstrate quantitatively that migration of GFP+ cells upon injury depends on the specific characteristics of the induced damage and on the embryonic developmental stage (Knappe et al.). This suggests that muscle-resident *pax7a*-expressing cells may function differently depending on experimental conditions; unraveling these diverse functions represents a challenge and has important implications for muscle repair and regeneration studies.

Muscular dystrophies target head and trunk muscles differently, indicating that satellite cells, which drive muscle regeneration, may have molecular functional differences in each muscle tissue. Dietrich and colleagues report on the regulation of Pax7-expressing head muscle stem cells by comparing chicken, mouse, frog and zebrafish models (Nogueira et al.). They postulate a somewhat counterintuitive mechanism for the emergence of craniofacial muscle stem cells, whereby *de novo* expression of Pax7 is acquired later than the cellular commitment to the myogenic fate, which is established by expression of the myogenic regulatory factors (MRFs). Guardiola and colleagues generate a novel mouse model to better understand the importance of Cripto—a known regulator of vertebrate embryogenesis—during postnatal skeletal muscle regeneration (Prezioso et al.). When overexpressed in adult satellite stem cells, Cripto promotes myogenic commitment and fusion/differentiation in a time-dependent manner, improving muscle regeneration upon acute injury. Availability of the *Tg:Cripto* model will be instrumental for the future development of pharmacological approaches that target this important pathway.

ROLE OF STEM CELLS IN SKELETAL MUSCLE REGENERATION AND REPAIR

A number of epigenetic modifications are known to be key in the regulation of satellite cell function (Giordani and Puri, 2013; Dumont et al., 2015; Parker; Segales et al., 2015; Sousa-Victor et al., 2015). Epigenetic marks on chromatin can be modified, modulated, written and/or erased by the action of growth factors, inflammatory signals, cellular redox status, developmental pathway switches, and/or mechanical stimuli. The potential reversibility of most of these epigenetic modifications provides an attractive targeting approach for pharmacological manipulation. Palacios and colleagues exhaustively review what is known about the epigenetic impact of the signaling microenvironment upon satellite cell chromatin during adult regenerative myogenesis (Brancaccio and Palacios). Complementarily, Suelves and colleagues review DNA methylome (DNA methylation signature) dynamic changes during the processes of commitment and

differentiation of skeletal muscle, driven by the activation of the MRFs, and address what is known about the methylome status in pathological and aging processes (Carrio and Suelves).

Satellite cells are not the only stem cells residing in skeletal muscle, and other progenitors and immune cell infiltrates can influence this tissue's regenerative potential (Burzyn et al., 2013; Mozzetta et al., 2013; Pannerec et al., 2013; Farup et al., 2015; Kostallari et al., 2015). Two articles in this issue summarize the role of non-satellite stem cells in muscle biology as well as their potential use in cell-based therapies. Delbono and colleagues review the different roles of muscle-resident perivascular cells in skeletal muscle physiology, defining two distinctive types of pericytes. Their effects are linked—but not limited—to positive outcomes, such as support for muscle regeneration, reinnervation, and *de-novo* vessel formation and negative outcomes, including unwanted differentiation, fibrosis, fat accumulation, and heterotopic ossification (Birbrair et al.). The article serves as a powerful warning for those eager to push the early implementation of stem cell therapies, and highlights the need to deepen our knowledge on the activation and integration of transplanted stem cells, as these will not contribute to the regeneration processes of the targeted organ in all therapeutic approaches and could indeed be detrimental for the patient.

In a distinct contribution, Forcales puts forward the case for the closely related adipose-derived mesenchymal stem cell (ASC) to be used for muscle regenerative therapies. The article presents a collective view of ASC's myogenic differentiation, engraftment and functional assessment protocols as reported by different research groups. Since these cells have shown good safety records but poor efficacy when transplanted, the author advocates for enhanced enrichment, expansion and manipulation of ASC-derived myogenic progenitors to improve clinical outcomes.

ROLE OF STEM CELLS IN SKELETAL MUSCLE AGING

Centenarians are the paradigm of healthy aging, and their exceptional longevity and physical fitness must somehow be related to genetic make-up. The identification of genetic polymorphisms linked to muscle mass and function maintenance in the old age should be advantageous for the prevention of age-related sarcopenia. In this long road, discarding many potential candidates is also important. Lucia and colleagues study the functional significance (i.e., whether they affect the muscle transcriptional levels of the gene) of three candidate SNPs in independent human cohorts. Although, they detect correlation with transcriptional levels of the genes in close proximity to the SNPs, they do not find association with exceptional longevity (Fuku et al.). These findings are relevant and intriguing because these SNPs, which are associated with lean body mass, cardiorespiratory fitness and better physical performance, nevertheless do not seem to be related necessarily to an increase in longevity.

Another theoretical therapeutic approach gaining momentum is directed toward the rejuvenation of the stem cell niche through

its exposure to a “younger environment.” The identity of the particular systemic factors mediating this promising therapeutic possibility is under debate (Li and Izpisua Belmonte, 2014; Sinha et al., 2014; Sousa-Victor et al., 2014, 2015; Brun and Rudnicki, 2015; Egerman et al., 2015; Rodgers and Eldridge, 2015). Latella and colleagues provide an interesting review of some recent breakthrough studies on cell-intrinsic vs. cell-extrinsic mechanisms underlying satellite cell regenerative decline with aging (Madaró and Latella). They advocate for new studies to further explore the relationship between DNA damage, p38 and p16^{INK4a} signaling pathways on this age-associated decline.

The control of fibroblast growth factor (FGF) signaling by Spry1 is altered with aging and affects satellite cell quiescence maintenance (Chakkalakal et al., 2012; Tajbakhsh, 2013). Yablonka-Reuveni et al. show that only Fgfr1 and Fgfr4 receptors are expressed in satellite cells; to better understand their role in adult muscle regeneration upon injury, the authors analyse the phenotype after Fgfr1 ablation. They show that although FGF2-driven mitogenic response in satellite cells is virtually absent, this does not affect muscle regeneration, possibly due to the existence of compensatory mechanisms.

ROLE OF STEM CELLS IN DISEASES OF THE SKELETAL MUSCLE

Muscular dystrophies are among the most common and severe diseases affecting skeletal muscle homeostasis and function. While dystrophies share many common characteristics—most being linked to the dystrophin-associated protein complex—a distinct feature, used in diagnosis, is the differential effect of the particular mutation on the various skeletal muscle groups. Satellite stem cells are considered a notoriously heterogeneous cell population (Motohashi and Asakura; Dumont et al., 2015). Pavlath and colleagues review what is known on skeletal muscle stem cell biology and compare satellite cells from eight different muscle groups and the distinct extent of affection of the same muscles in muscular dystrophies (Randolph and Pavlath). The authors highlight the importance of further advancing our knowledge on the biology of muscle derived satellite cells from non-limb muscles, which have somehow been neglected until now. Following this theme, Marazzi and colleagues analyze variations on the support role provided by interstitial progenitor cells (“PICs”) to muscle regeneration in an effort to further characterize the satellite stem cell niche in extraocular muscles (Formicola et al.). Interestingly, they observe an increase in the number of PICs in both aged and dystrophic animals. Because the maintenance of a high number of PICs positively supports myogenesis, they conclude that the extraocular-specific increase of PIC numbers might underlie the resistance to dystrophy by this muscle subset.

Biressi and Gopinath challenge the current thinking on the connections between satellite stem cells and loss of muscle mass, otherwise known as muscle wasting or atrophy. They review the signaling pathways implicated and conclude that multiple cause-related atrophies coexist within a single clinical entity

and that this may hinder the precise targeting required for the development of novel therapeutic approaches.

Fittingly with the proposed topic, Lopez de Munain and colleagues review the available evidence to classify Myotonic dystrophy type 1 as a progeroid syndrome (Mateos-Aierdi et al.). A number of the so-called “hallmarks of aging” (Lopez-Otin et al., 2013) are detected in these patients whose disease is possibly associated with satellite cell dysfunction. The article proposes some of the research avenues that might be worth exploring in the future.

Several microRNAs (miRs) have been implicated in muscle stem cell function and homeostasis; their role on muscle dysfunction and their associated disease has also been extensively investigated (Rosales et al., 2013; Dey et al., 2014; Hindi and Kumar, 2016). Musaró and colleagues locally overexpressed the anabolic growth factor insulin-like growth factor 1 (IGF-1) in Duchenne muscular dystrophy (*mdx*) muscle and studied modulation of the miR signature, finding major changes in miR-206 and miR-24 levels, that contributed to muscle differentiation (Pelosi et al.). This research links the activation of anabolism by the IGF-1 pathway in skeletal muscle with changes in essential miRNA molecules never implicated in this process before.

Finally, in McArdle disease, a genetic disorder of skeletal muscle metabolism, patients struggle to perform resistance exercise due to increased risk of severe rhabdomyolysis, which may develop into acute tubular necrosis, renal failure, and severe low blood pressure. In a clinical study, Lucia and colleagues propose a novel resistance-training program in adult McArdle patients that was well-tolerated according to clinical assessments. The preliminary data support the possibility of a therapeutic intervention involving regular participation in strength training programs, with the potential to improve muscle mass, strength and clinical outcome of McArdle patients (Santalla et al.).

FUTURE PROSPECTS

The field of skeletal muscle stem cell biology has exploded in the past decade, although the advance experienced in our knowledge of the function, origin, renewal, gene regulation, epigenetics, aging, and senescence of these remarkable cells, only represents a small step toward our full understanding of their abilities and therapeutic potential. Many aspects of these cells are still puzzling and would require a combined effort to unravel their many secrets. We still lack comparative metabolomic and transcriptomic analysis between cells of various origins and muscle groups that may provide a more focused view on the inherent properties of different muscles and their resistance to disease; the explosion of single-cell “omic” approaches will provide an unexpected wealth of new data and insight into the biology of these cells. Another key area of present and future development is to develop pharmacological or cell-based therapeutic approaches to transcriptionally regulate satellite cells so that they become amenable for clinical manipulation and therapeutic deployment. In this quick journey from development to adulthood, from regeneration to disease, we hope the reader

finds some enlightenment on the multiple facets of satellite cell biology, which is not free of discrepancies. In all, an increased understanding of muscle biology makes us even more conscious of the limits of our knowledge. Or, as Walt Whitman wrote “*Do I contradict myself? Very well, then, I contradict myself; (I am large-I contain multitudes.).*”

AUTHOR CONTRIBUTIONS

AI drafted the manuscript that was amended and approved by all authors, acting as co-Editors of the Research Topic.

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A population of Pax7-expressing muscle progenitor cells show differential responses to muscle injury dependent on developmental stage and injury extent

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Skeletal muscle regeneration in vertebrates occurs by the activation of quiescent progenitor cells that express *pax7* to repair and replace damaged myofibers. We have developed a mechanical injury paradigm in zebrafish to determine whether developmental stage and injury size affect the regeneration dynamics of skeletal muscle. We found that both small focal injuries, and large injuries affecting the entire myotome, lead to expression of *myf5* and *myogenin*, which was prolonged in older larvae, indicating a slower process of regeneration. We characterized the endogenous behavior of a population of muscle-resident Pax7-expressing cells using a *pax7a:eGFP* transgenic line and found that GFP+ cell migration in the myotome dramatically declined between 5 and 7 days post-fertilization (dpf). Following a small single myotome injury, GFP+ cells responded by extending processes, before migrating to the injured myofibers. Furthermore, these cells responded more rapidly to injury in 4 dpf larvae compared to 7 dpf. Interestingly, we did not see GFP+ myofibers after repair of small injuries, indicating that *pax7a*-expressing cells did not contribute to myofiber formation in this injury context. On the contrary, numerous GFP+ myofibers could be observed after an extensive single myotome injury. Both injury models were accompanied by an increased number of proliferating GFP+ cells, which was more pronounced in larvae injured at 4 dpf than 7 dpf. This indicates intriguing developmental differences, at these early ages. Our data also suggests an interesting disparity in the role that *pax7a*-expressing muscle progenitor cells play during skeletal muscle regeneration, which may reflect the extent of muscle damage.

Keywords: regeneration, stem cells, myogenesis, zebrafish, satellite cells, skeletal muscle, wound healing

Introduction

Skeletal muscle is a complex tissue consisting of multinuclear, contractile muscle fibers, yet it has a robust regenerative capacity through the action of muscle stem cells (muSCs). In mouse, the principle cells responsible for repairing or replacing damaged muscle are satellite cells (SCs). These were first described in frog and rat in 1961 (Mauro, 1961), and have since been described in most vertebrate species, including fish and other rodents

(Moss and Leblond, 1970; Schultz et al., 1978; Koumans et al., 1990; Stoiber and Sänger, 1996). Generally, these tissue-resident stem cells are located beneath the basal lamina of muscle fibers and show a characteristically large, heterochromatic nucleus in their quiescent state. Genetically, quiescent muSCs are characterized by expression of the paired homeobox transcription factor Pax7 (Seale and Rudnicki, 2000). It has been shown that Pax7-deficient muSCs show a loss of heterochromatin in their nucleus, suggesting the transcription factor is essential for maintaining quiescence (Gunther et al., 2013).

Pax7-expressing muSCs originate in the dermomyotome during embryogenesis, which contributes to the formation of skeletal muscle tissue in the trunk (Kassar-Duchossoy et al., 2004; Gros et al., 2005; Relaix et al., 2005). Their activation in post-natal animals can be induced by many stimuli, most notably mechanical injury. Activated and proliferating muSCs are characterized by the expression of the myogenic regulatory factors (MRFs) *Myf5* and *MyoD* and later express *Myogenin* upon differentiation. A subset of these cells can undergo asymmetric cell division, giving rise to one progenitor and one differentiating daughter cell (Zammit et al., 2004; Shinin et al., 2006; Conboy et al., 2007). These cells are essential for maintenance and repair of skeletal muscle tissue and loss of Pax7 results in an impaired regenerative response after injury (Lepper et al., 2011; Sambasivan et al., 2011; Von Maltzahn et al., 2013). They regenerate injured muscle predominantly by either fusing to damaged muscle fibers, or to each other to form *de novo* muscle fibers. In mammals, newly formed myofibers generally have a smaller diameter and show myonuclei located centrally, as opposed to their usual location at the periphery of the myofiber.

Much of our understanding of how skeletal muscle regenerates comes from studies performed in the mouse. In fish, the presence of muSCs has been demonstrated in adult muscle tissue in a number of species including salmon, carp, and electric fish (Nag and Nursall, 1972; Akster, 1983; Weber et al., 2012). Extraction of muSCs from adult zebrafish also reveals that these cells show immunoreactivity for Pax7 and can form muscle fibers in culture (Alexander et al., 2011; Zhang and Anderson, 2014). Tissue regeneration in adult zebrafish has been described to occur within 28 days and involves the formation of regenerative fibers in conjunction with BrdU labeling, indicating proliferating progenitor cells (Rowlerson et al., 1997).

Investigations into the developmental origin of *pax7*+ cells in zebrafish larvae revealed that they originate from the dermomyotome, similarly to amniotes (Hollway et al., 2007; Marschallinger et al., 2009). These cells are mitotically inactive and express several typical muSC markers, such as *cxcr4* genes (Hollway et al., 2007) and Syndecan-4 (Froehlich et al., 2013). Further, muscle regeneration occurs through *de novo* formation of new fibers and not, as previously assumed, by dedifferentiation in larval animals (Rodrigues et al., 2012). Further, muSCs have also been shown to respond to injury stimuli by migrating to, and proliferating at, the site of injury in zebrafish larvae (Seger et al., 2011; Otten et al., 2012).

The majority of studies examining muSC function have been performed in mouse using models, such as cardiotoxin or barium chloride, inducing fairly major injuries. Considering

recent evidence from the skin, which indicates that the response of hair follicle stem cells differs depending on the magnitude of injury (Chen et al., 2015), we aimed to investigate whether this could also be true for muSCs. We have therefore investigated how Pax7-expressing cells respond to muscle injury using a transgenic zebrafish line in which the *pax7a* promoter drives eGFP expression. We have defined two protocols for creating precise muscle damage and characterized the process of injury healing using immunohistochemistry, *in situ* hybridization and *in vivo* imaging. We find that, although *pax7a*-expressing (GFP) cells in this line respond to injury, it is the extent of damage that determines whether they form new muscle fibers. Furthermore, we find that the developmental stage may have an impact on the speed of regeneration and the response of *pax7a*-expressing cells to injury.

Materials and Methods

Zebrafish

Adult zebrafish were maintained according to standard procedures (Westerfield, 2000) in a 12 h light/dark cycle. All animal work was carried out in accordance with the Animals (Scientific Procedures) Act 1986. Embryonic fish were maintained in E3 medium at 28°C. After 5 dpf, E3 medium was changed and larvae fed with Gemma75 (Skretting) daily.

The *Tg[pax7a:eGFP]* transgenic line was a kind gift from Christiane Nüsslein-Volhardt (Max-Planck Institute for Developmental Biology, Tübingen, Germany) and has been described previously (Mahalwar et al., 2014). This line was maintained in a homozygous *pfeffer* (*pfe*, allele tm36b) mutant background (Odenhal et al., 1996). *pfe* fish form fewer *pax7a:eGFP* transgene-expressing xanthophore pigment cells in the skin due to a mutation in the *fms/csf1r-1* gene (Parichy et al., 2000; Maderspacher and Nüsslein-Volhardt, 2003). *pax7a:eGFP;pfe* were crossed with double mutant *roy;mitfa* (*caspar*) mutants (White et al., 2008). The F2 offspring were maintained as homozygous *pfe;mitfa* mutants carrying the *pax7a:eGFP* transgene (subsequently referred to as *pax7a:eGFP*).

Mechanical Injury

Larvae were anesthetized in 0.01% MS-222 (Sigma) in E3 embryo medium and embedded laterally in 1.5% low melting agarose (Sigma) as previously described (Westerfield, 2000).

For small injuries, tungsten wire (diameter 0.125 mm, GoodFellow) was sharpened by electrolysis in potassium hydroxide. Wire was then cleaned and polished using a pair of fine forceps, inserted into a needle holder and fixed into a microinjection rig. Injuries were targeted to the center of the 12th ventral myotome. Approximate depth of injuries was 0.1 mm within a single myotome.

For extensive injuries, tungsten wire was replaced by an unsharpened steel manipulation needle (diameter 1 mm). Three adjacent puncture wounds were targeted to the 12th ventral myotome and the needle was agitated from a medial to distal position in order to damage as many muscle fibers as possible.

For recovery, free-swimming larvae were maintained as above. To assess proliferation, larvae were incubated in 10 mM BrdU

(Sigma) 1% DMSO in E3 embryo medium for the duration of recovery.

Immunohistochemistry

For GFP/phalloidin staining, larvae were anesthetized in 0.02% MS-222 and fixed in 4% paraformaldehyde (PFA, Sigma) for 1 h at room temperature. Samples were washed in 0.1% Tween20 in phosphate-buffered saline (PBT, Sigma) and permeabilized in 100% acetone for 30 min at -20°C . After several washes in PBT, samples were blocked in 5% goat serum (Life Technologies) in PBT for at least 2 h, then incubated with primary antibody diluted in 5% goat serum/PBT over night at 4°C . Samples were washed for at least 4 h in 0.1% PBT the following day, then incubated with secondary antibodies diluted in 5% goat serum/PBT for 2 h at room temperature. After several washes in PBT, samples were taken through glycerol series and mounted in VectaShield with DAPI (Vector Laboratories).

For Pax7/GFP staining, larvae were fixed in 2% PFA for 30 min at room temperature. Samples were washed in 1% Triton-X (Sigma) in PBS (1% PBTx) and blocked in 5% goat serum in 1% PBTx for at least 2 h. Incubation with primary antibodies, diluted in 5% goat serum/1% PBTx, was carried out for 48 h at room temperature. Samples were washed in 1% PBTx and immunostained with secondary antibody diluted in 5% goat serum/1% PBTx over night at room temperature. Larvae were then washed in 1% PBTx, taken through glycerol series and mounted in VectaShield with DAPI.

For BrdU/GFP staining, larvae were fixed for 30 min in 2% PFA at room temperature and subsequently stored in 100% methanol (Fisher) over night at -20°C . The next day, larvae were taken through a re-hydration series of methanol/PBS and washed in 1% PBTx. Following, the samples were permeabilized in 10 $\mu\text{g}/\text{ml}$ of proteinase K in 0.1% PBT for 1 h at room temperature. After several washes in 0.1% PBT, samples were incubated in 2 N HCl (Sigma) in H_2O for 1 h. Samples were subsequently washed with 1% DMSO/0.1% Tween20 in PBS (PBDT) and blocked in 5% goat serum in PBDT for at least 2 h at room temperature. Incubation with primary antibodies, diluted in 5% goat serum in PBDT, was carried out over night at 4°C . After several washes in PBDT, larvae were incubated in secondary antibodies, diluted in 5% goat serum in PBDT, for 2 h at room temperature. Samples were then washed, taken through glycerol series and mounted in VectaShield with DAPI.

Primary antibodies used: rabbit polyclonal anti-GFP (1:500; Life Technologies), mouse polyclonal anti-Pax7 (1:5, DSHB), rat monoclonal anti-BrdU (1:200, Abcam).

Secondary antibody used: goat anti-rabbit IgG AlexaFluor488 conjugated (1:500; Life Technologies), goat anti-mouse IgG AlexaFluor568 conjugates (1:500, Life Technologies), goat anti-rat IgG AlexaFluor568 conjugated (1:500, Life Technologies), fluorophore-conjugated phalloidin555 (1:300; PromoKine).

In situ Hybridization

In situ hybridization was performed as described previously (Thisse and Thisse, 2008) with the following modifications. Larvae were permeabilized in a 100 $\mu\text{g}/\text{ml}$ solution of collagenase (Sigma, stock solution of 1 mg/ml in Ringer's solution, diluted

1:10 in 0.1% PBT) for 2 h at room temperature prior to hybridization with riboprobe. For hybridization, DIG-conjugated riboprobes to *myf5* (Groves et al., 2005) and *myogenin* (Weinberg et al., 1996) were used, which were detected using alkaline phosphatase conjugated FAB fragments (Roche). After detection, samples were developed in 0.25% NBT/BCIP in PBT (Sigma) for 7 days, then post-fixed in 4% PFA for 30 min, taken through glycerol series and mounted for analysis

Expression was quantified by eye and expression classified as either present or absent in the injured myotome. For all experiments, 10 larvae were used per condition and animals showing poor health after injury excluded from subsequent analyses. We then calculated the number of animals showing expression per condition as a percentage to compensate for any differences in overall number.

Injury Volume Measurements

Samples were scanned using a Leica TCS SP5 microscope equipped with a Leica CTR 6500 laser and LAS AF software and subsequently analyzed using ImageJ/Fiji (Schindelin et al., 2012). The area of injured muscle and resulting gaps between myofibers was selected using the Fiji ROI tool for each slice in a z-stack and measured using “ROI manager.” The area of each slice was then multiplied by the slice thickness and summed to obtain the total volume of injury in μm^3 .

Individual Cell Tracking and Counting

For time-lapsed recordings, larvae were embedded laterally in 1.5% low-melting agarose. A Nikon D-Eclipse C1 microscope with 488 nm argon laser, EZ-C1 3.70 software and x40 water dipping objective was used. Z-stacks were acquired in 1 μm steps with upper limit at the skin and lower limit at the neural tube. Z-stacks were acquired every 30 min and were 8–14 h in total duration. Time-lapsed data was processed entirely in ImageJ/Fiji. Drift was adjusted using the “Correct 3D drift” plugin, single cells were tracked manually using the “Mtrack” plugin.

For cell counting, immunostained samples were scanned at the level of the 12th ventral myotome using a Leica confocal microscope and analyzed using Image J/Fiji. Individual DAPI+ cells in z-stacks were counted using the “Cell counter” plugin. For control larvae, cells in the uninjured 12th ventral myotome were counted.

Data Analysis

All plots and statistical analyses were generated using IBM SPSS Statistics 21.

Results

Small Single Myotome Injury: A Reproducible Mechanical Injury Paradigm in Zebrafish Larvae

Previous approaches to model muscle injury in zebrafish larvae involved the injection of cardiotoxin (chemical injury; Seger et al., 2011) and tail transection (large mechanical injury; Rodrigues et al., 2012). We were interested in investigating regeneration dynamics in response to a targeted mechanical injury to a

small number of muscle fibers, and used a sharpened tungsten wire fixed into a microinjection rig. We analyzed injuries using fluorophore-conjugated phalloidin to stain F-actin in muscle fibers and so provide the means to visualize the injury. This revealed that several myofibers in the targeted myotome showed signs of tearing and hyper-contraction (indicated by brighter phalloidin staining), while all muscle fibers in neighboring myotomes were unaffected (**Figure 1A**). Furthermore, the injury was precisely targeted to avoid damaging the vertical or central myosepta (see asterisk in the orthogonal view in **Figure 1A**).

To analyze the extent and reproducibility of our small single myotome injury paradigm, we measured the total volume of the injury at 1 hours post injury (hpi) ($n = 14$ larvae; **Figure 1B**). As shown in (**Figures 1C,D**), most injury volumes were between 5×10^4 and $1.5 \times 10^5 \mu\text{m}^3$ (11/14 larvae). Thus, a controlled injury using a sharpened tungsten wire results in a reproducible muscle wound contained within a single myotome, which can be used for evaluating the process of muscle regeneration *in vivo*. We termed this injury model a “small single myotome injury.”

Small Single Myotome Injuries Are Rapidly Repaired

We next analyzed the size of the small single myotome injury in phalloidin-stained larvae at defined intervals after injury. Injury was performed on larvae at 4 or 7 dpf to also examine whether muscle repair was affected by developmental stage (**Figure 2A**). At 1 hpi, the injured myotome in 7 dpf larvae resembled that in 4 dpf fish, with hyper-contracted, and torn myofibers easily distinguishable from undamaged muscle fibers. After 24 h, large gaps were visible between myofibers in superficial and deep portions of the myotome, regardless of larva age at injury. Such gaps were never observed in adjacent, un-targeted ventral myotomes. While these gaps largely disappeared by 48 hpi in the superficial myotome of larvae injured at 4 dpf, they occasionally persisted in larvae injured at 7 dpf. Furthermore, it was noticeable that the gaps were still present in the more central regions of the myotome in larvae injured at either age. By 96 hpi, gaps in the central portion of the myotome were no longer present. Interestingly, in larvae injured at 7 dpf, the

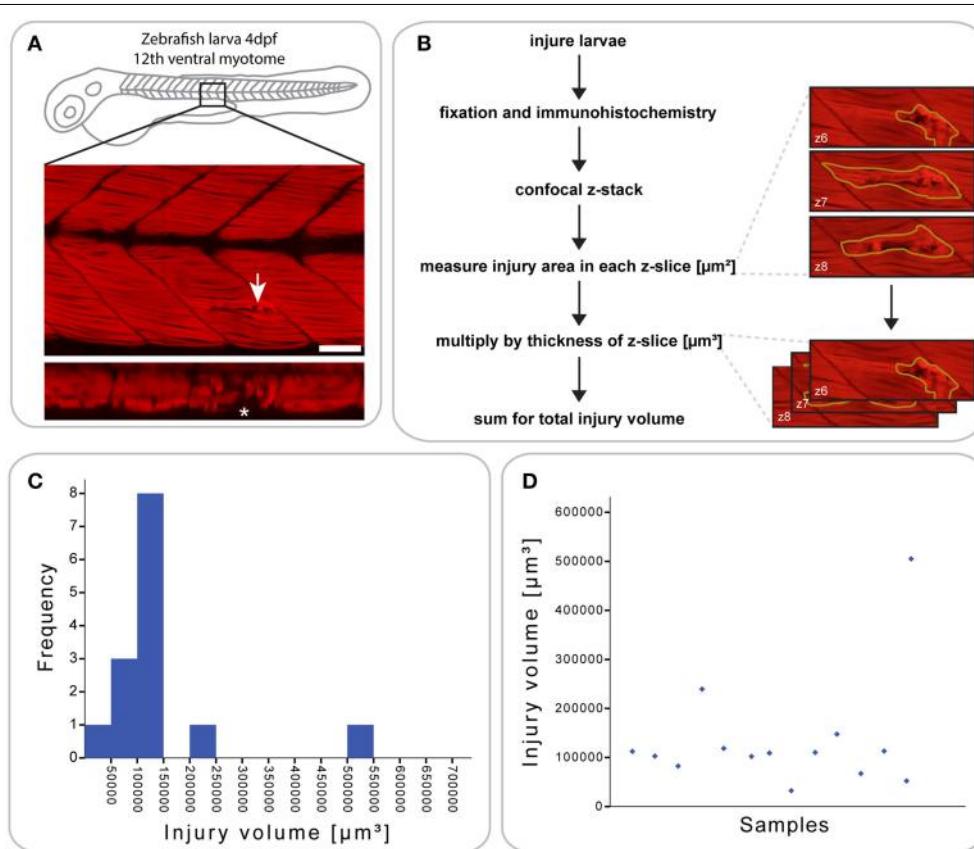


FIGURE 1 | Defining a reproducible mechanical injury paradigm in the zebrafish larva. **(A)** Confocal slice of larvae injured in the 12th ventral myotome at 4 days post-fertilization (dpf) and immunostained for phalloidin (F-actin) at 1 h post-injury (hpi). Arrowhead indicates site of injury. Panel of orthogonal XZ view centered on the injury site is shown beneath and injury site is indicated by an asterisk. Left is anterior, top is dorsal, scale bar is 50 μm . **(B)** Workflow for the quantification of injury size. After immunostaining and confocal imaging of injured larvae, the injured muscle, and resulting gaps between myofibers were circled

using Fiji ROI tool on each slice and the area measured. This is illustrated by the images shown on the right, which present the phalloidin-stained site of injury with ROI contours in yellow. The area of each slice was then multiplied by the slice thickness and all slices summed to obtain the total volume of injury in μm^3 , illustrated by the images on the right. **(C)** Histogram showing the distribution of injury volume at 1 hpi for $n = 14$ larvae injured at 4 dpf. Most samples cluster around an injury volume of $100,000 \mu\text{m}^3$. **(D)** Scatter plot for the same dataset, plotting the injury volume for each of the 14 samples.

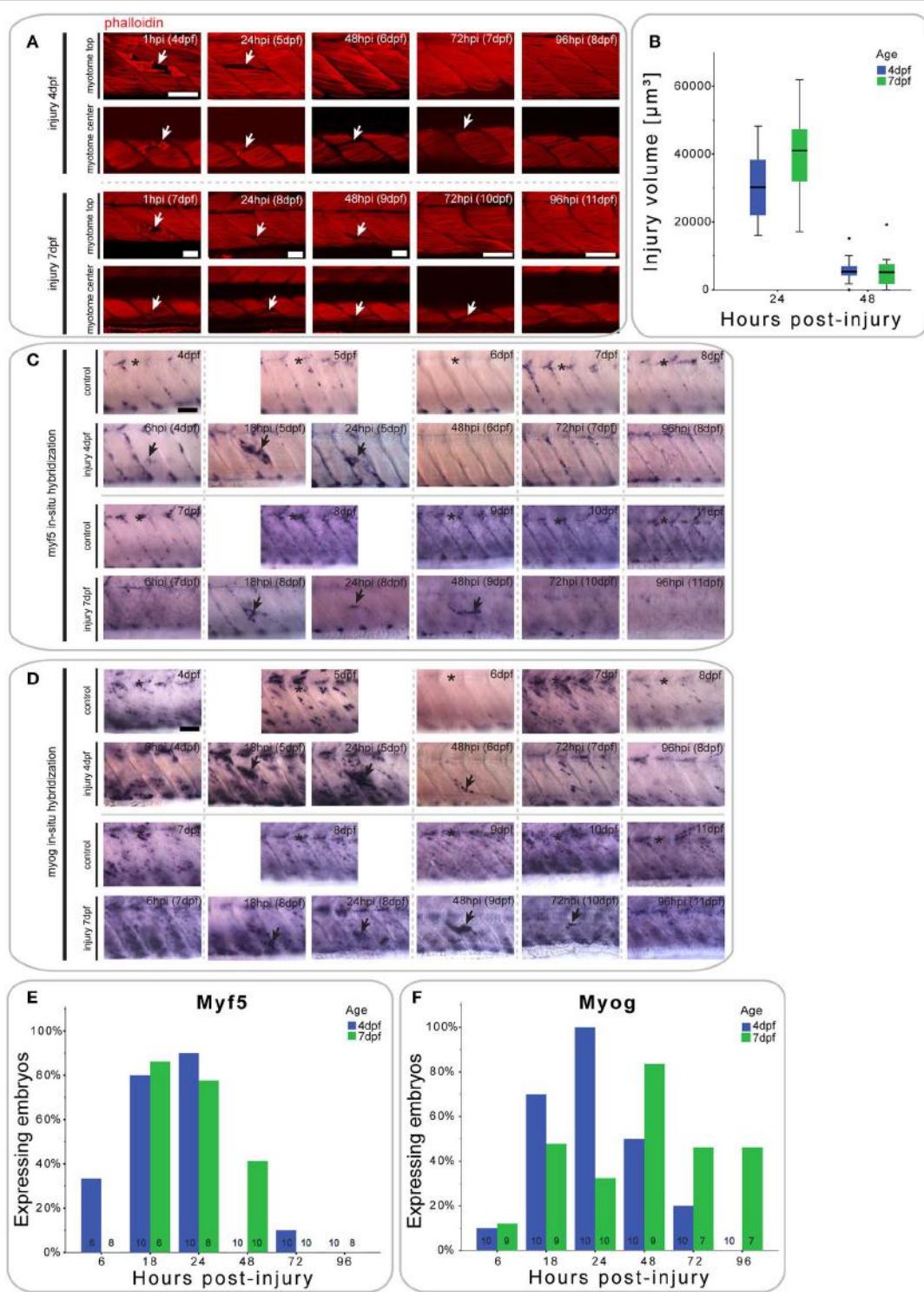


FIGURE 2 | Small single myotome injuries are rapidly repaired and result in characteristic expression of MRFs. **(A)** Confocal slices of the superficial and deep 12th ventral myotome of larvae injured at 4 or 7 dpf and stained for phalloidin (f-actin) after injury. Arrowheads indicate site of injury. **(B)** Quantification of injury volume at varying time-points post-injury in larvae injured at 4 or 7 dpf. Injury volume was measured as described in Figure 1B. $p < 0.005$ for differences in injury volume between 24 and 48 hpi independent of age; $p = 0.223$ for differences in injury volume between 4 and 7 dpf independent of hpi; $p = 0.158$ for differences in injury volume

dependent on age and hpi. **(C,D)** Representative images of *in situ* hybridization with *myf5* (**C**) or *myogenin* (**D**) anti-sense probe of uninjured controls and larvae injured in the 12th ventral myotome at 4 or 7 dpf at various time-points post-injury. The 12th myotome is marked by an asterisk in control images. Arrowheads indicate expression patterns of note in injured samples. Left is anterior, top is dorsal, scale bar is 50 μm for all images. **(E,F)** Bar graph showing the proportion of embryos expressing *myf5* (**E**) and *myogenin* (**F**) at the site of injury at different time-points post-injury. Small numbers on bars indicate the total number of larvae.

upper myotome was largely repaired by 72 hpi, but still exhibited slight distortion. To identify whether there were differences in muscle repair initiated at 4 or 7 dpf, we measured the volume of injury at 24 and 48 hpi (**Figure 2B**). Even though there was no significant difference between larvae injured at 4 or 7 dpf, the average injury volume of 4 dpf larvae was smaller. At 48 hpi, the injury volume of 7 dpf larvae showed a larger distribution.

Expression Profile of *myf5* during Repair Following Small Single Myotome Injury

Despite the visible reduction in injury size and apparent repair of the muscle, regenerating myofibers were not observed close to the original site of injury (**Figure 2A**). To understand how myogenesis is regulated during muscle repair, we characterized expression of MRFs using *in situ* hybridization.

Larvae injured at 4 dpf showed a small increase in *myf5* expression in the injured myotome at 6 hpi (2/6 larvae), which peaks between 18 hpi (8/10 larvae) and 24 hpi (9/10 larvae) (**Figure 2C**, quantified in **Figure 2E**). By 48 hpi, *myf5* was no longer expressed in the injured myotome (0/10 larvae).

In larvae injured at 7 dpf, *myf5* expression was not detected in the injured myotome at 6 hpi (0/10 larvae), but was elevated at 18 hpi (5/6 larvae) (**Figure 2C**, quantified in **Figure 2E**). *myf5* expression further increased at 24 hpi (6/8 larvae), though seemed less pronounced than in larvae injured at 4 dpf. Expression persisted at 48 hpi (4/10 larvae) in 7 dpf larvae, which was not the case in larvae injured at 4 dpf. After 72 hpi, *myf5* was no longer expressed in the injured myotome of 7 dpf larvae (1/10 larvae).

Expression Profile of *Myogenin* during Repair Following Small Single Myotome Injury

To identify cells initiating myogenic differentiation, we also investigated the expression of *myogenin* after injury. Six hours after injury, there was no noticeable *myogenin* expression in the injured myotome of 4 dpf larvae (1/10 larvae) (**Figure 2D**, quantified in **Figure 2F**). *myogenin* became detectable at 18 hpi (7/10 larvae) and was subsequently up-regulated at 24 hpi (10/10 larvae). Expression then gradually decreased between 48 hpi (5/10 larvae) and 72 hpi (3/10 larvae) and was back to background levels at 96 hpi (0/10 larvae).

Similarly, *myogenin* expression was not elevated in the targeted myotome of 7 dpf larvae at 6 hpi (1/9 larvae) (**Figure 2D**, quantified in **Figure 2F**). Interestingly, expression of *myogenin* appeared at a slower rate in the older larvae, with only slightly elevated expression at 24 hpi (3/10 larvae). Expression peaked only at 48 hpi (7/9 larvae) and persisted until 72 hpi (3/10 larvae) and 96 hpi (3/7 larvae).

Thus, repair of muscle fibers in zebrafish larvae with small single myotome injury involves an initial expression of *myf5* at the injury site, followed by *myogenin*, with *myf5* and *myogenin* persisting longer in repairing muscle in larvae injured at 7 dpf, compared to those injured at 4 dpf.

The Extensive Single Myotome Injury Model Reveals That Muscle Regeneration Dynamics Are Affected by Injury Extent

To test whether the size of injury affects muscle regeneration, we also developed an extensive single myotome injury paradigm, using an unsharpened steel manipulation needle which damaged at least 50% of myofibers in the targeted myotome. Muscle injuries were analyzed by staining for F-actin using fluorophore-conjugated phalloidin. Following injury at 4 dpf, no damaged muscles fibers remained in the injured myotome after 24 h (**Figure 3A**). The injured myotome was filled with cells as shown by DAPI labeling (data not shown). Small diameter regenerating myofibers appeared between 72 and 144 hpi. Despite regeneration of muscle fibers, the injured myotome remained mildly deformed and smaller in size than adjacent myotomes 6 days after injury.

In larvae injured at 7 dpf, most damaged myofibers were cleared by 24 hpi (**Figure 3A**). Regenerating myofibers first become visible between 72 and 144 hpi, though they were visibly less numerous than in larvae injured at 4 dpf. Six days post-injury, the injured myotome was still visibly distorted.

Expression Profile of *myf5* during Regeneration Following Extensive Single Myotome Injury

In larvae injured at 4 dpf, *myf5* expression was undetected at the site of injury at 6 hpi (0/10 larvae) (**Figure 3B**, quantified in **Figure 3D**). Expression was greatly up-regulated at 18 hpi (10/10 larvae) and remained at high levels through 24 hpi (10/10 larvae) and 48 hpi (10/10 larvae). It was also apparent in bright-field images that the size of the injured myotome was reduced at 48 hpi relative to adjacent uninjured myotomes. Expression of *myf5* decreased at 72 hpi (9/10 larvae), although it remained above endogenous levels at 96 hpi in the injured myotome (10/10 larvae). Finally, *myf5* further decreased at 120 hpi (7/10 larvae) and was reduced to levels comparable to controls by 144 hpi (4/10 larvae).

A similar profile of *myf5* expression was observed in larvae that underwent extensive single myotome injury at 7 dpf. There was no *myf5* detectable in the injured myotome at 6 hpi (0/6 larvae) (**Figure 3B**, quantified in **Figure 3D**). At 18 hpi, *myf5* expression was observed in the injured myotome, particularly around the edges (9/10 larvae). Expression increased by 24 hpi (8/8 larvae) and was robust at the site of injury at 48 hpi (9/9 larvae). As in larvae injured at 4 dpf, the size of the myotome was visibly reduced at 48 hpi. Subsequently, expression levels of *myf5* decreased but remained detectable between 72 hpi (9/9 larvae) and 96 hpi (5/5 larvae). In contrast to larvae injured at 4 dpf, larvae injured at later stages maintain higher levels of *myf5* expression at the injury site at 120 hpi (8/10 larvae) and 144 hpi (6/8 larvae).

Expression Profile of *Myogenin* during Regeneration Following Extensive Single Myotome Injury

The differentiation marker *myogenin* is not expressed in the injured myotome at 6 hpi in 4 dpf larvae (0/10 larvae) (**Figure 3C**, quantified in **Figure 3E**). Slight expression was visible at 18 hpi

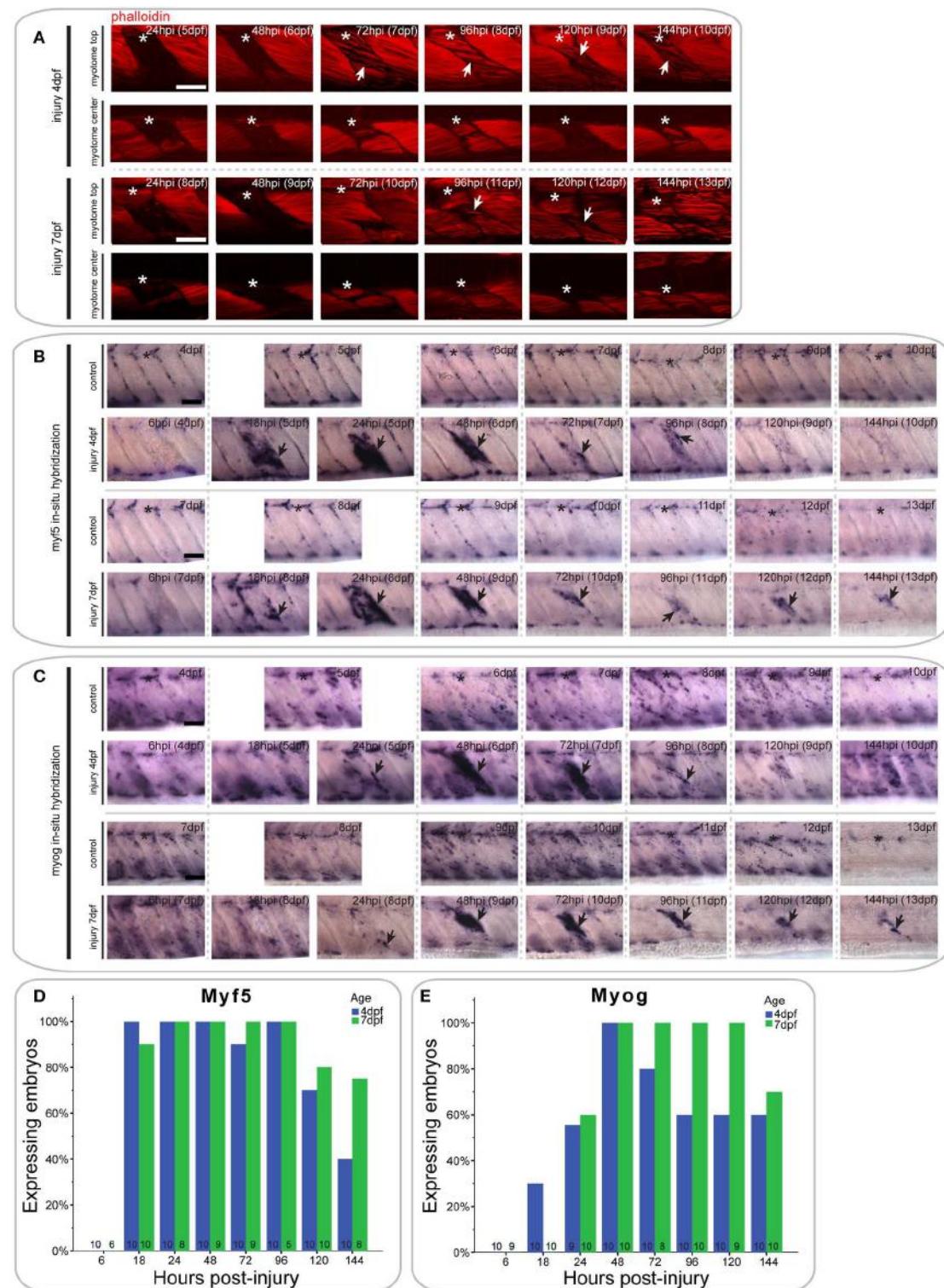


FIGURE 3 | Extensive single myotome injuries are rapidly regenerated and result in characteristic expression of MRFs. **(A)** Confocal slices of the superficial and deep 12th ventral myotome of larvae with an extensive single myotome injury at 4 or 7 dpf and stained for phalloidin (f-actin) after injury. Asterisk indicates injured myotome, arrowheads indicate structures of note. **(B,C)**. Representative images of *in situ* hybridization using *myf5* **(B)** or *myogenin* **(C)** anti-sense probe of uninjured controls and larvae injured

extensively in the 12th ventral myotome at 4 or 7 dpf at various time-points post-injury. The 12th myotome is marked by an asterisk in control images. Arrowheads indicate expression patterns of note in injured samples. Left is anterior, top is dorsal, scale bar is 50 μ m for all images. **(D,E)** Bar graph showing the proportion of embryos expressing *myf5* **(D)** or *myogenin* **(E)** at the site of injury at different time-points post-injury. Small numbers on bars indicate the total number of larvae.

(3/10 larvae) and 24 hpi (5/9 larvae). *myogenin* expression was then greatly increased at 48 hpi (10/10 larvae), and remained at a high level through to 72 hpi (8/10 larvae). *Myogenin* expression was reduced at 96 hpi (6/10 larvae) and then remained at a constant level in the regenerating myotome through 120 hpi (6/10 larvae) and 144 hpi (6/10 larvae).

Larvae injured at 7 dpf had a very similar *myogenin* expression profile to those injured at 4 dpf. There was little detectable expression in the injured myotome at 6 hpi (0/9 larvae) and 18 hpi (0/10 larvae) (**Figure 3C**, quantified in **Figure 3E**). Small groups of cells started to express *myogenin* around the site of injury at 24 hpi (6/10 larvae). Expression then strongly increased between 48 hpi (10/10 larvae) and 72 hpi (8/8 larvae). Compared to larvae injured at 4 dpf, *myogenin* expression was stronger at 96 hpi and still prominent around the wound site (10/10 larvae). Similarly, expression levels of *myogenin* were still markedly elevated at 120 hpi (9/9 larvae) and 144 hpi (7/10 larvae) in larvae injured at 7 dpf.

Thus, larvae injured at 4 or 7 dpf are both able to partially regenerate muscle following an extensive single myotome injury, which damages the majority of a myotome. However, larvae injured at 7 dpf appear to regenerate fewer myofibers than those injured at 4 dpf and show prolonged expression of MRFs throughout the course of regeneration.

pax7a-expressing Cells in the Myotome Display Developmental-Dependent Changes in Their Behavior

We hypothesized that the delayed regenerative response in 7 dpf larvae relative to that in 4 dpf larvae could be caused by a developmental change in muSC behavior. To investigate this, we used a transgenic line in which eGFP is expressed under control of a *pax7a* promoter (*pax7a:eGFP*; Mahalwar et al., 2014). In this line, we note that there were many GFP+ cells localized at the myoseptum, where Pax7+ cells have previously been described during zebrafish development and have been shown to be recruited to sites of muscle injury (Devoto et al., 2006; Hollway et al., 2007; Seger et al., 2011).

To first understand how *pax7a*+ cells behave during different stages of muscle development, we characterized GFP+ cell movement in *pax7a:eGFP* larvae between 3 and 7 dpf using time-lapsed microscopy (**Figures 4A–C**, **Videos S1–S3**).

At 3 dpf, GFP+ cells in *pax7a:eGFP* larvae were located at the horizontal and vertical myosepta as previously described for Pax7+ cells (Hollway et al., 2007; Seger et al., 2011). GFP+ cells at the vertical myosepta could occasionally be observed moving into the middle of the myotome, although the majority of the cells remained at the myosepta (**Figure 4A**). This movement was preceded by the extension of processes toward the middle of the myotome (**Figure 4A**, blue arrow), followed by cell migration (**Figure 4A**, yellow arrow). Cell division events could be observed at this stage (mean = 0.375 divisions/h, stddev = 0.177 for $n = 2$ larvae) and predominantly occurred at the myosepta (**Figure 4A**, green arrow).

In 5 dpf *pax7a:eGFP* larvae, there was a more extensive movement of GFP+ cells (**Figure 4B**). Fewer cells appeared to

be present at the myosepta and cells could be observed moving from this area toward the middle of the myotome (**Figure 4B**, yellow arrow). In the middle of the myotome, cells appeared to be moving along, or adjacent to, muscle fibers (**Figure 4B**, blue arrow). Further, cell division events occurred at 5 dpf (mean = 0.5 divisions/h, stddev = 0.303 for $n = 2$ larvae), but in the middle of the myotome (**Figure 4B**, green arrow; also in the upper left portion of the myotome at 5 h).

GFP+ cell behavior differs entirely in *pax7a:eGFP* larvae at 7 dpf (**Figure 4C**). Cells were located both at the myosepta and in the middle of the myotome, but did not show extensive movement, unlike at 3 or 5 dpf. Cells at the myosepta were generally of a rounded morphology and occasionally extended processes toward the middle of the myotome, but did not move from their myoseptal location (**Figure 4C**, green and blue arrows). On the contrary, cells located in the middle of the myotome had an elongated morphology, aligned in the same orientation as muscle fibers. These cells did not move extensively along the myofibers, but occasionally their cell morphology changed (**Figure 4C**, yellow arrow). Furthermore, in contrast to 3 and 5 dpf, no cell divisions could be observed in 7 dpf larvae (mean = 0 for $n = 2$ larvae).

To quantify GFP+ cell behavior during development in *pax7a:eGFP* larvae, cell movement in the myotome was analyzed in 3, 5, and 7 dpf larvae through manual tracking (**Figures 4D–F**). This revealed that the average cell speed did not differ significantly between 3 and 5 dpf larvae ($p = 0.959$), but was significantly reduced in 7 dpf larvae (3 dpf vs. 7 dpf: $p = 0.024$; 5 dpf vs. 7 dpf: $p = 0.007$). Furthermore, there was less variability in the average cell speed of GFP+ cells at 7 dpf relative to 3 or 5 dpf.

In summary, *pax7a*+ cells display different cell behaviors at 3, 5, and 7 dpf, with changes in cell migratory behavior and location.

To confirm that GFP expression in cells of the myotome of *pax7a:eGFP* larvae occurs in Pax7-expressing cells, we performed immunohistochemistry on 4 and 7 dpf larvae to detect GFP and Pax7 (**Figures 4G–H'**) and counted the number of labeled cells in the myotome (**Figure 4I**). This analysis shows that approximately 70% of Pax7+ cells also express the GFP protein in both 4 and 7 dpf larvae (**Figure 4I**). Further, we note that approximately 92% of GFP+ cells also express Pax7 at 4 dpf, with the remaining 8% accounting for freshly divided or differentiating cells (data not shown). We are thus confident that the *pax7a:eGFP* line is an accurate reporter of Pax7 protein expression in the myotome.

***pax7a*+ Cells Show Differential Responses to Muscle Injury Dependent on Age**

Pax7+ SCs are the principal cell type that regenerates muscle in mammals (Relaix and Zammit, 2012), but it is not clear if this is true for zebrafish. To characterize the response of *pax7a*+ cells to muscle injury in zebrafish, we generated a small single myotome injury in 4 and 7 dpf *pax7a:eGFP* larvae and observed GFP+ cell responses by time-lapsed recordings from 30 min after injury (**Videos S4, S5**).

In *pax7a:eGFP* larvae injured at 4 dpf, the first GFP+ cell responses (extension of processes) were observed as soon as

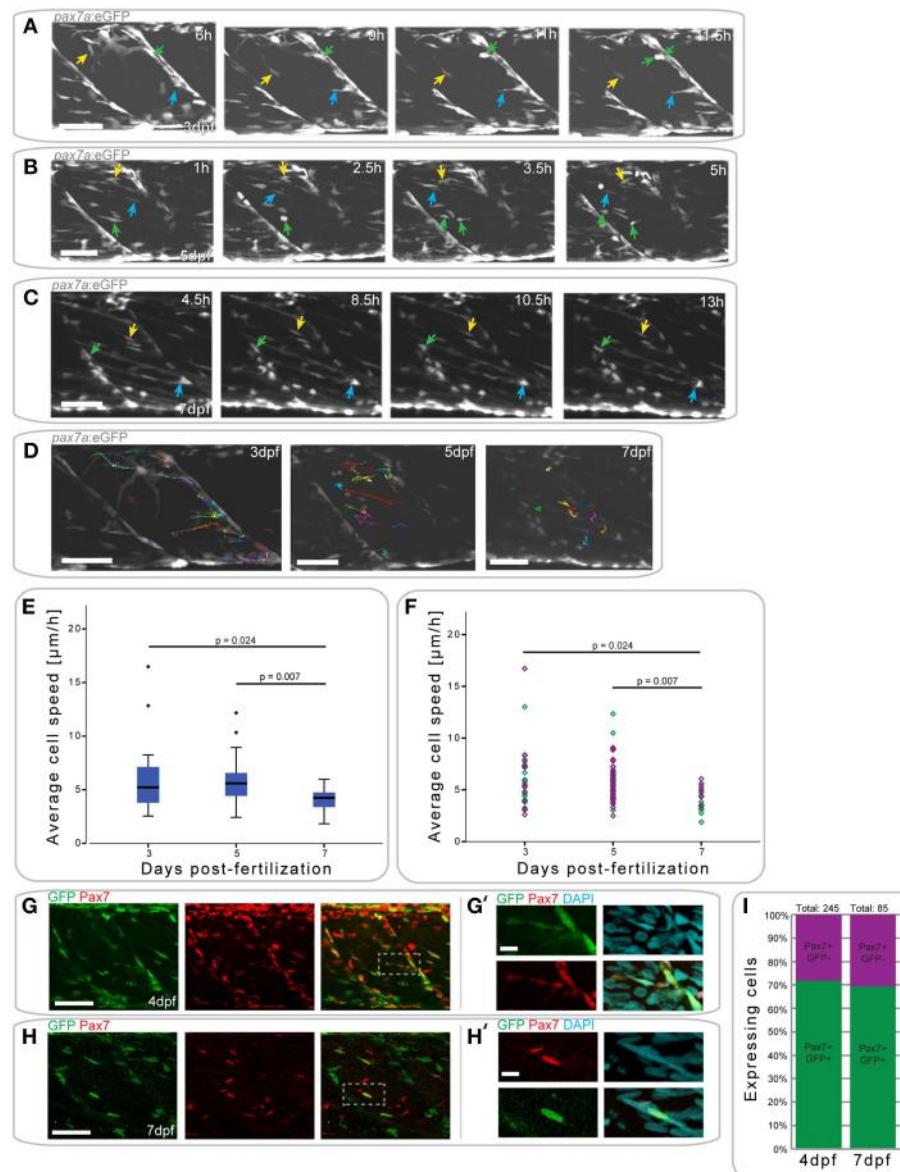


FIGURE 4 | *pax7a*+ cells in the myotome display changes in their behavior depending on developmental stage. **(A)** Z-projections of selected time-points captured every 30 min after the start of a confocal time-lapse stack of an uninjured *pax7a:eGFP* larva at 3 dpf. Arrowheads of different colors indicate cells which show characteristic cell movement throughout the time-lapse. **(B,C)** Z-projections of selected time-points after the start of a confocal time-lapse stack of an uninjured *pax7a:eGFP* larva at 5 dpf **(B)** and 7 dpf **(C)**. Arrowheads of different colors indicate cells which show characteristic cell movement throughout the time-lapse. **(D)** Z-projections of the last frame of time-lapse recordings shown in **(A–C)**. Individual cells were manually tracked throughout the duration of time-lapse recordings using Fiji MtrackJ. Colored and numbered lines indicate the movement of individual cells through the entire duration of the time-lapse for 3, 5, and 7 dpf larvae. **(E)** Box plot indicating the

average velocity of cells tracked in $n = 2$ time-lapse recordings in 3, 5, and 7 dpf larvae. P -values were calculated using One-Way ANOVA with Tukey post-hoc test. **(F)** Scatter plot indicating the average velocity of individual cells in $n = 2$ timelapse recordings in 3, 5, and 7 dpf larvae. Each diamond represents one cell; different colors indicate the n-number, thus different individual larvae. P -values were calculated using One-Way ANOVA with Tukey post-hoc test. **(G,H)** Z-projections of 4 dpf **(G)** or 7 dpf **(H)** *pax7a:eGFP* larvae immunostained for GFP (green) and Pax7 (red). **(G',H')** Magnified images of boxed region in **(G)** or **(H)**, respectively with addition of DAPI image (cyan) and GFP/Pax7/DAPI merge. Left is anterior, top is dorsal, scale bar is 50 μm for images of myotome, 10 μm for magnified images. **(I)** Percentage bar chart indicating the proportion of *pax7a*-expressing cells which are GFP+ in un-injured 4 ($n = 12$) and 7 dpf ($n = 12$) larvae.

4 hpi (**Figure 5A**); cells moved toward the injury from both vertical and horizontal myosepta (**Figure 5A**, orange and purple arrows). At the site of injury, GFP+ cells then elongated in the same orientation as existing fibers between 9 and 14 hpi. GFP+

cells located in the middle of the myotome responded to the injury in the same manner as those at the myosepta, displaying a migratory response to the injury (**Figure 5A**, turquoise arrow).

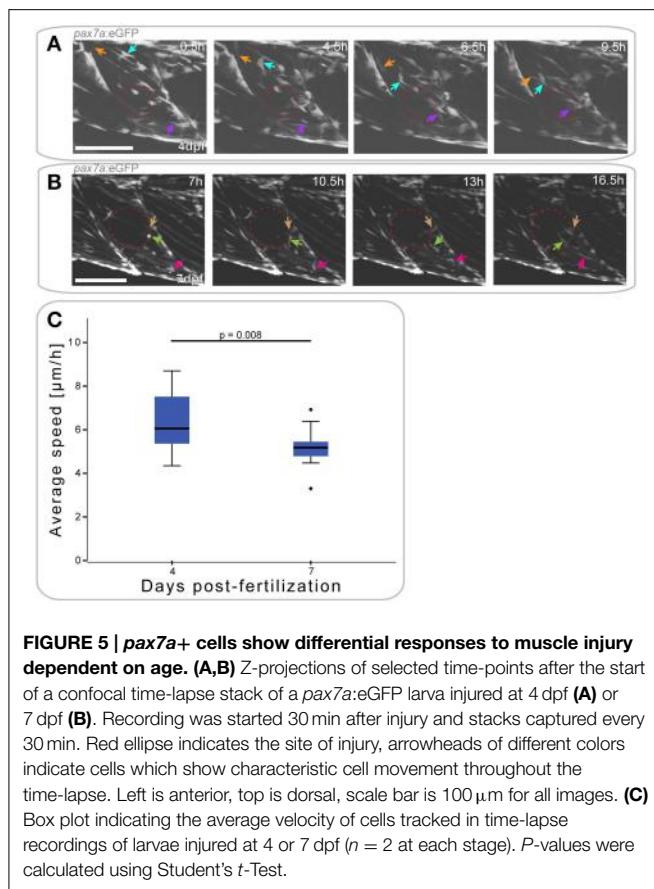


FIGURE 5 | *pax7a*+ cells show differential responses to muscle injury dependent on age. **(A,B)** Z-projections of selected time-points after the start of a confocal time-lapse stack of a *pax7a:eGFP* larva injured at 4 dpf **(A)** or 7 dpf **(B)**. Recording was started 30 min after injury and stacks captured every 30 min. Red ellipse indicates the site of injury, arrowheads of different colors indicate cells which show characteristic cell movement throughout the time-lapse. Left is anterior, top is dorsal, scale bar is 100 μ m for all images. **(C)** Box plot indicating the average velocity of cells tracked in time-lapse recordings of larvae injured at 4 or 7 dpf ($n = 2$ at each stage). P -values were calculated using Student's *t*-Test.

Muscle injury in 7 dpf *pax7a:eGFP* larvae resulted in GFP+ cells extending thin processes toward the injury around 7 hpi (**Figure 5B**, maroon arrow). Cell movement toward the site of injury was not visible until approximately 10 hpi. At this stage, cells adjacent to the injury site (**Figure 5B**, light green arrow) and at more distal locations (**Figure 5B**, pink arrow) adopted an elongated morphology.

To determine whether cell responses to injury are affected by developmental stage, we again tracked GFP+ cells. Average cell speed of only those cells that responded to the injury was measured and plotted (**Figure 5C**). This revealed that GFP+ cells responding to muscle injury in 7 dpf larvae were significantly slower than those in 4 dpf larvae ($p = 0.008$).

***pax7a*-expressing Cells Do Not Contribute to Fiber Formation after Small Single Myotome Injury**

The response of GFP+ cells to muscle injury in *pax7a:eGFP* larvae suggests that they may contribute to muscle repair. To test this, we investigated whether GFP+ cells can contribute to myofiber formation after injury, by assessing whether GFP was localized to F-actin+ muscle fibers labeled with fluorophore-conjugated phalloidin.

In *pax7a:eGFP* larvae with a small single myotome injury at 4 dpf, GFP+ cells were clearly localized to the site of injury by 24 hpi (**Figures 6A,A'**). Interestingly, these cells were clustered

in the gaps between myofibers observed after injury, although many GFP- cells were also present (data not shown). However, no GFP+/F-actin+ striated muscle fibers were detected at the injury site, which would be indicative of myofibers derived from cells expressing the *pax7a:eGFP* transgene. Despite this absence of GFP+ F-actin+ fibers, GFP+ cells could be seen in close proximity to each other and sometimes with multiple DAPI labeled nuclei at 24 hpi (7/9 samples), which may indicate fusion (**Figure S1A**). GFP+ cells were no longer present in clusters by 48 hpi, coincident with loss of the spaces between myofibers. Again, no GFP+/F-actin+ muscle fibers were detected at this stage. By 72 hpi, GFP+ cells were found between myofibers, similar to those in uninjured myotomes.

In *pax7a:eGFP* larvae injured at 7 dpf, fewer GFP+ cells were observed at the wound site at 24 hpi, relative to those injured at 4 dpf. Clusters of GFP+ cells could be observed at the wound site at both 24 and 48 hpi when larvae were injured at 7 dpf (**Figures 6B,B'**). Similarly to 4 dpf larvae, GFP+ cells lay close to each other and could sometimes be identified as multinuclear (4/5 samples; **Figure S1B**). At 72 hpi, these GFP+ cells were still present in the deep portions of the myotome, but disappeared in the superficial layers. We were not able to observe GFP+/F-actin+ fibers at any of these time-points in 7 dpf larvae, similar to our findings from 4 dpf larvae.

We confirmed these findings using GFP/Pax7 co-immunostaining at 24 hpi in larvae injured at 4 or 7 dpf (**Figures 6C–D'**). This shows that many cells that are localized to the site of injury also express the Pax7 protein.

***pax7a*-expressing Cells Contribute to Fiber Formation after Extensive Single Myotome Injury**

Since we were unable to identify GFP+ muscle fibers in small single myotome injured *pax7a:eGFP* larvae, we wondered whether the size of injury could influence the ability of *pax7a*+ cells to contribute to muscle regeneration. We tested this by inducing an extensive single myotome injury in 4 and 7 dpf larvae and evaluated the ability of GFP+ cells to form myofibers at different times after injury.

Following injury of 4 dpf *pax7a:eGFP* larvae, we observed numerous GFP+ cells around the site of injury at 24 hpi (**Figures 7A,A'**). Elongated GFP+ cells were first observed at 48 hpi, and GFP+/F-actin+ fibers were first observed at 72 hpi (**Figure 7A'**). Interestingly, we noted that some of the regenerating fibers in the injured myotome were not GFP+ (**Figure 7A**, see 72 hpi arrow). GFP+ cells were still present within the injured myotome at 144 hpi, but were less numerous than at earlier stages of injury.

A similar response of GFP+ cells was observed in *pax7a:eGFP* larvae injured extensively at 7 dpf (**Figure 7B**). GFP+ cells were present in the injured myotome at 24 and 48 hpi and seemed to lie in the same orientation as existing muscle fibers (**Figure 7B'**). The first GFP+/F-actin+ fibers appeared by 72 hpi and were detectable until 120 hpi in the injured myotome. By 144 hpi, GFP+ cells were still present within the myotome, though GFP+ fibers could no longer be observed (**Figure 7B**).

Again, we confirmed the presence of Pax7-expressing cells at the site of injury at 48 hpi using GFP/Pax7

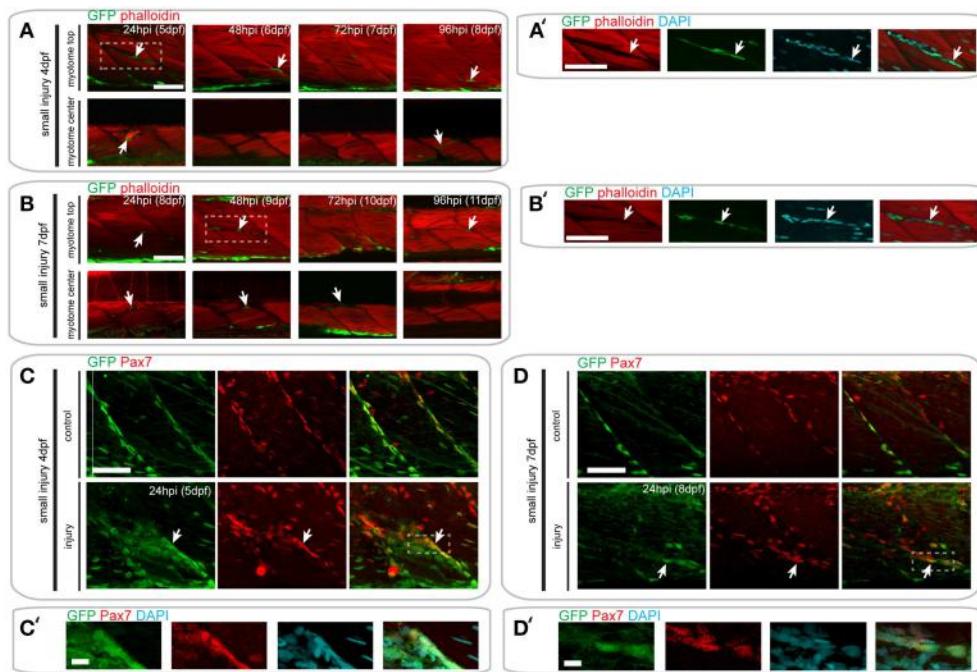


FIGURE 6 | *Pax7a*-expressing cells do not contribute to muscle fiber formation after small single myotome injury. **(A,B)** Confocal slices of the superficial and deep 12th ventral myotome of *pax7a:eGFP* larvae injured at 4 dpf **(A)** or 7 dpf **(B)** and stained for phalloidin (f-actin, red) and GFP (green) after small single myotome injury. Arrowheads indicate cells of interest. **(A',B')** Magnified images of boxed regions in **(A)** or **(B)**, respectively. Images show staining for

phalloidin (f-actin, red), GFP (green) and DAPI (cyan). Arrowheads indicate cells of interest. **(C,D)** Z-projections of 4 dpf **(C)** or 7 dpf **(D)** *pax7a:eGFP* larva immunostained for GFP (green) and Pax7 (red) at 24 hpi after small single myotome injury. **(C',D')** Magnified images of boxed region in **(C)** or **(D)**, respectively with addition of DAPI image (cyan) and GFP/Pax7/DAPI merge. Scale bar is 10 μ m. For all other images, scale bars are 50 μ m. Left is anterior, top is dorsal.

immunohistochemistry (**Figures 7C–D'**). This staining shows that there is a large number of Pax7-expressing cells present at the site of injury in larvae injured at 4 and 7 dpf. Many of these cells also expressed GFP, indicating that the *pax7a*+ cells contributing to regeneration are expressing Pax7 protein.

A Pool of *pax7a*-expressing Cells is Recruited for Repair and Regeneration in 7 dpf, but Not 4 dpf Animals

To assess whether *pax7a*-expressing cells proliferated in response to injury, similar to mammals, we pulsed *pax7a:eGFP* larvae after injury with BrdU and counted GFP+ and BrdU+ cells after immunostaining.

At 24 h after creation of a small single myotome injury in 4 and 7 dpf *pax7a:eGFP* larvae, there was clearly observable cell proliferation (**Figures 8A,B**). Upon injury, the number of GFP+BrdU+ cells increased 0.7-fold in 4 dpf larvae, whereas the number of GFP+BrdU– cells did not obviously change. In 7 dpf larvae we observed a significant increase in the number of proliferating GFP+ cells after injury ($p = 0.006$), which was coincident with a sharp decrease in the number of BrdU– cells ($p < 0.001$).

Similar trends can be observed 48 h after extensive single myotome injury (**Figures 8C,D**). In *pax7a:eGFP* larvae injured at 4 dpf, there was a significant increase in the number of

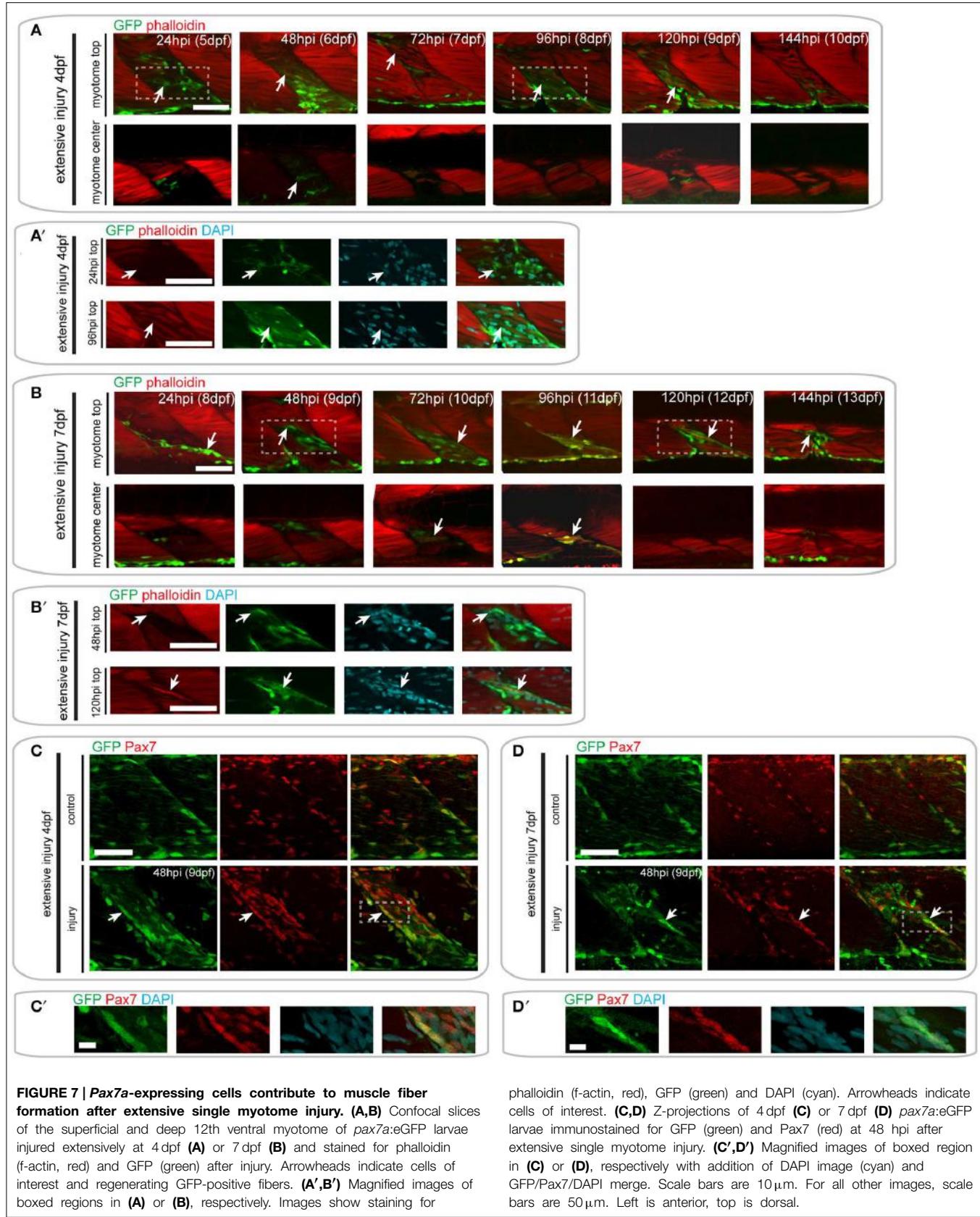
proliferating GFP+ cells compared to controls ($p = 0.003$). We also observed a slight increase in the number of GFP+BrdU– cells at this stage. In 7 dpf larvae, the increase in the number of proliferating GFP+ cells was even more pronounced than for small single myotome injuries ($p < 0.001$). Further, we observed the same reduction in number of GFP+BrdU– cells as seen occurring after small injuries ($p < 0.001$).

When comparing the average number of counted cells in 4 and 7 dpf *pax7a:eGFP* larvae, it is notable that there were consistently more GFP+BrdU+ cells present in 4 dpf larvae compared to 7 dpf. Of further interest, we found that most GFP+ cells were proliferative at 4 dpf in uninjured larvae, whereas no GFP+ cells showed BrdU incorporation in uninjured 7 dpf larvae.

Thus, we observe significant differences in the proliferation patterns of GFP+ cells in response to small and extensive single myotome injury between 4 and 7 dpf *pax7a:eGFP* larvae.

Discussion

Skeletal muscle regeneration is a complex process, involving the activation and differentiation of stem cells and their integration into existing muscle tissue or formation of *de novo* myofibers. In this study, we characterize the response of a population of muscle-resident *pax7a*+ cells during muscle injury and repair in zebrafish. We describe two reproducible mechanical injury



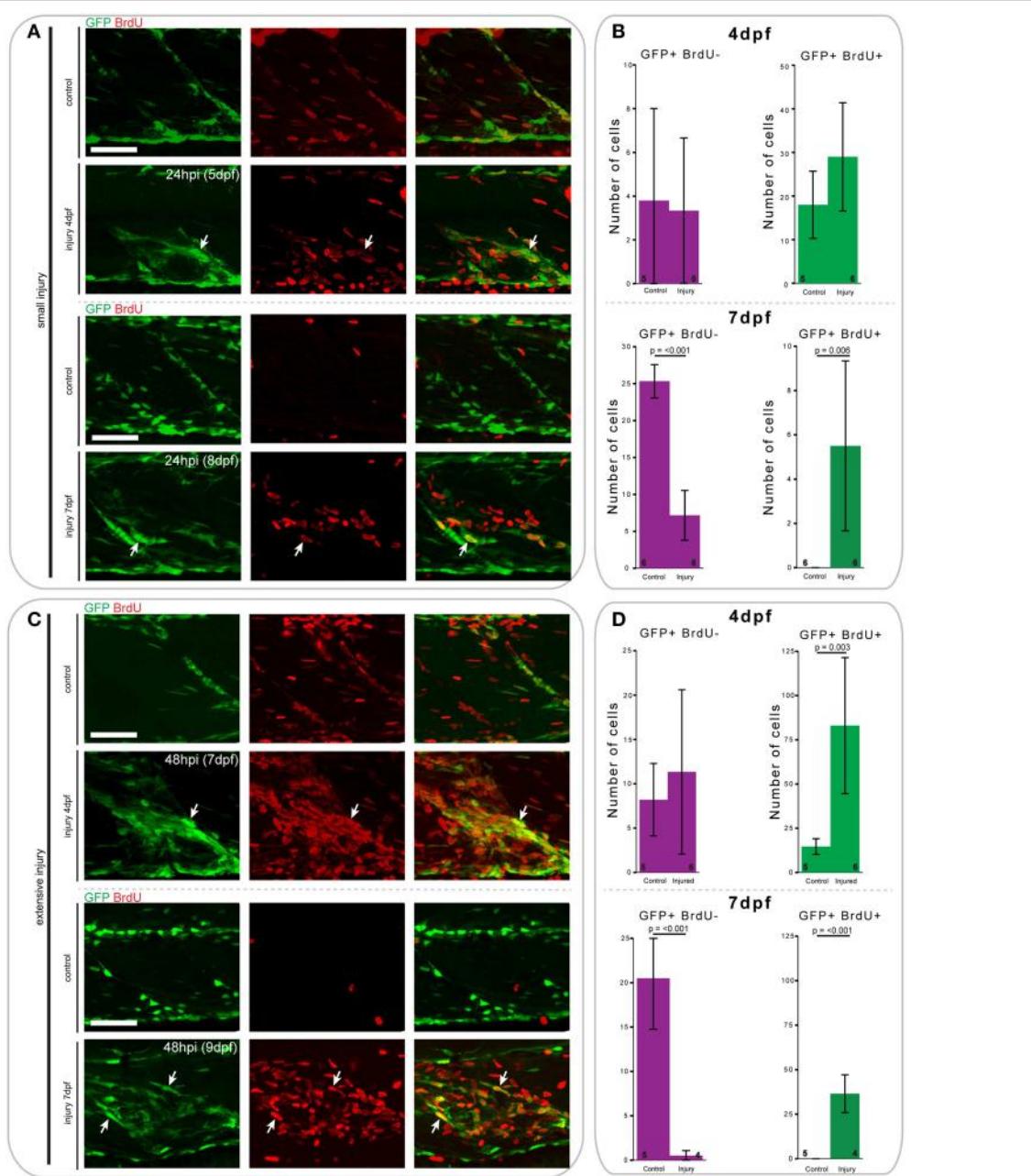


FIGURE 8 | Proliferation patterns of *pax7a*-expressing cells differ between 4 and 7 dpf larvae following small and extensive single myotome injury. **(A,C)** Z-projections of 4 and 7 dpf *pax7a*:eGFP larva immunostained for GFP (green) and BrdU (red) at 24 hpi after small (**A**) or extensive (**C**) single myotome injury. Larvae were incubated in 10 mM BrdU/1% DMSO during the entirety of the recovery period. Arrows indicate cells which

show double staining. Scale bars are 50 μ m. Left is anterior, top is dorsal. **(B,D)** Bar charts indicating the average number of GFP+Brdu- or GFP+Brdu+ cells after small (**B**) or extensive (**D**) single myotome injury compared to un-injured controls. Single cells were counted at 24 hpi (**B**) or 48 hpi (**D**) in larvae injured at 4 or 7 dpf. Numbers of larvae counted are indicated in small font at the bottom of each bar.

models, resulting in minor or extensive damage to muscle fibers. We show these injuries are repaired and involve the expression of MRF genes, as seen in other vertebrates. Such injuries elicit the response of a population of *pax7a*-expressing cells resident in the myotome, which migrate to sites of muscle injury

in a transgenic *pax7a*:eGFP line. The contribution of *pax7a*-expressing (GFP+) cells to regenerating muscle was dependent on the injury extent: they contributed to fiber formation after extensive single myotome injury, whereas a small injury did not lead to participation of *pax7a*-expressing (GFP+) cells in fiber

repair. We also found that developmental stage was important for the speed of these cells and the duration of MRF expression in the regenerative phase. Overall, this highlights the diverse response of a muscle-resident *pax7a*-expressing cell population to injury and suggests that their role during regeneration can be affected by several parameters.

Variations in Injury Size Might Alter muSC Behavior during Regeneration

Little is known about the behavior of muscle progenitor cells during the regeneration of skeletal muscle in zebrafish. In larval muscle, it has been shown that Pax3/Pax7-expressing cells are proliferative and accumulate around the site of injury (Seger et al., 2011). We observe that *pax7a*+ cells migrate toward the wound site through the extension of cytoplasmic processes, though we do not see evidence of proliferation using time-lapsed imaging. This mode of migration may in part be regulated by Rho/Rac GTPases (Murali and Rajalingam, 2014). On the contrary, in an *in vitro* single fiber system, mammalian SCs have been described to migrate through blebbing (Otto et al., 2011). This process also appears to be dependent on Rho kinase (ROCK) function, as application of small molecule inhibitors caused cells to display a slower movement (Collins-Hooper et al., 2012). Although we cannot clearly discriminate between a mesenchymal or amoeboid cell movement, we note that migrating *pax7a*-expressing cells *in vivo* clearly extend cytoplasmic processes.

Initially, we hypothesized that *pax7a*-expressing cells in injured larvae would contribute to tissue regeneration, as seen in mammals. However, we were unable to verify any contribution of GFP+ cells to fiber formation after small injury in *pax7a*:eGFP larvae. An earlier study, in which a different *pax7a*:GFP line was used, likewise did not show a contribution of GFP+ cells to regenerating fibers following cardiotoxin injection (Seger et al., 2011). Despite this, MRFs are expressed in response to small single myotome injury and GFP+ cells proliferate and migrate to the site of injury. Further, the presence of multi-nuclear and closely associated GFP+ cells is strongly indicative that fusion occurs in our small injury model. Perhaps, the number of *pax7a*-expressing cells contributing to repair in this injury model is not extensive, preventing detection of residual GFP protein in newly regenerated fibers. Alternatively, other cell populations may play a more prominent role in the context of small injuries. For instance, zebrafish also possess a paralog of the *pax7a* gene, named *pax7b*. The Pax7 antibody used in this study is unable to distinguish between these two Pax7 isoforms. Examination of fish models with a reporter gene targeted to the *pax7b* locus would be useful to determine the contribution of this isoform to muscle repair in the context of small injuries.

In mouse, Pax7+ cells are crucial for muscle to sustain its regenerative ability (Relaix and Zammit, 2012). Many muscle-resident cell populations, which do not express Pax7, have previously been shown to be able to contribute to muscle regeneration, such as side-population cells (Gussoni et al., 1999; Asakura et al., 2002), muscle-derived stem cells (Qu-Petersen et al., 2002) and CD133+ progenitor cells (Torrente et al., 2004). Even though these particular cell populations have not

been described in zebrafish, it is possible that such a non-*pax7*-expressing cell population acts to repair small injuries to the muscle tissue, whereas *pax7a*-expressing cells only regenerate muscle following larger insult.

In vitro data from a related species of cyprinid, *Devario aequipinnatus* or giant Danio, shows that Pax7 is actually only expressed in newly activated muSCs, whereas Pax3 is present throughout myoblast proliferation (Froehlich et al., 2013). It is thus possible that *pax3*-expressing cells or other myogenic progenitor cells in fish might actually resemble a mammalian muSC population more closely than the cells we observed in our work. Considering our rudimentary understanding of sub-populations of muSCs and their roles in regeneration, especially in humans (Boldrin et al., 2010), studying these in zebrafish might lead to new insights other animal models cannot provide. We have not examined the role of *pax3*-expressing cells during muscle regeneration in zebrafish, though it is well-established that Pax3 acts to specify early muscle progenitor cells in mouse (Schubert et al., 2001). Given that Pax3 and Pax7 are related transcription factors that are both important for specifying muscle progenitor cell populations, it is possible that Pax3 may also be important for regeneration in zebrafish.

Proteins of the muscle extra-cellular matrix, such as the dystrophin-associated protein complex and laminins, are localized at the myosepta in zebrafish (Gupta et al., 2012; Wood and Currie, 2014). In accordance with prior findings (Seger et al., 2011), we note that the *pax7a*-expressing cells in the myotome of larval fish are localized to the myosepta, suggesting it is an environment that maintains this cell population. Given that we observe many *pax7a*-expressing cells with low levels of division by 7 dpf, it is tempting to speculate the myoseptum may act as a niche for these cells. Our extensive single myotome injury paradigm may indeed perturb the myoseptum, which could explain the differential regeneration response we see with respect to injury size. In this context, it is conceivable that signals from the myoseptum dictate the response of *pax7a*-expressing cells to injury.

Variations in Age Might Alter muSC Behavior during Regeneration

The difference in the dynamics of regeneration between larvae injured at 4 and 7 dpf is very consistent. Most interestingly, GFP+ cells in *pax7a*:eGFP larvae migrate toward the injury significantly slower when injured at 7 dpf compared to 4 dpf. *In vitro* studies of SCs isolated from mouse have previously shown that older cells show slower migration along myofibers (Collins-Hooper et al., 2012). The amount of GFP+ cells that proliferate in response to injury is also consistently lower in 7 dpf larvae compared to 4 dpf larvae. Further, we observe prolonged expression of MRFs and fewer regenerative fibers in larvae injured at 7 dpf relative to 4 dpf. These observations suggest that the cell populations contributing to muscle regeneration in older animals are less efficient or slower in their responses. It is possible that *pax7a*-expressing cells at 4 dpf may be more developmentally plastic or poised, thus more prone to respond to stimuli than at later stages. This phenomenon might

well-reflect the entry of *pax7a*-expressing cells into quiescence after the completion of developmental myogenesis. This is also supported by our observation that we did not observe GFP+ cells undergoing proliferation in uninjured 7 dpf *pax7a:eGFP* animals, whereas there is ample proliferation in uninjured 4 dpf control fish.

It has been suggested that stem cell quiescence may be caused by the accumulation of histone marks, which promote the formation of heterochromatin, thus inhibiting gene expression (Grigoryev et al., 2004; Srivastava et al., 2010). Furthermore, the chromatin of muSCs shows an increased deposition of inhibitory histone marks during aging, which may correlate with impaired muSC function in regeneration (Liu et al., 2013). It is possible that similar mechanisms of epigenetic gene inactivation lead to decreased muSC activity between 4 and 7 dpf, explaining the difference in cell speed and regenerative responses.

Alternatively, the changes in cell behavior we observed may reflect an alteration of the environment of muSCs, such as changes to the ECM. Indeed, significant changes occur to the ECM between 3 and 6 dpf larvae (Charvet et al., 2011). Most notably, collagen organization changes from a loose, disorganized meshwork into a dense, regular network of collagen fibrils. Since it is well-known that the cellular environment of SCs can greatly impact their behavior (Montarras et al., 2013), it is conceivable that this maturation of the ECM in the myotome may lead to changes in *pax7a*-expressing cell response to injury and thus affects the speed of tissue regeneration.

Conclusion

Our finding that responses of *pax7a*-expressing cells are dictated by the extent of the muscle injury are intriguing, especially since most injury models in mouse, such as cardiotoxin injection or crush injuries, result in a severe disruption of the tissue, leading to extensive regeneration. The impact of age on the behavior of muSCs in zebrafish and their efficiency in regenerating muscle also provides a new consideration for the study of muSC plasticity.

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Supplementary Material

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fnagi.2015.00161>

Video S1 | Z-projection of a confocal time-lapse stack of an uninjured *pax7a:eGFP* larva at 3 dpf. Stacks were captured every 30 min in 1 μm slices from skin to neural tube. Left is anterior, top is dorsal.

Video S2 | Z-projection of a confocal time-lapse stack of an uninjured *pax7a:eGFP* larva at 5 dpf. Stacks were captured every 30 min in 1 μm slices from skin to neural tube. Left is anterior, top is dorsal.

Video S3 | Z-projection of a confocal time-lapse stack of an uninjured *pax7a:eGFP* larva at 7 dpf. Stacks were captured every 30 min in 1 μm slices from skin to neural tube. Left is anterior, top is dorsal.

Video S4 | Z-projection of a confocal time-lapse stack of *pax7a:eGFP* larva injured at 4 dpf. Capture was started 30 min after injury and stacks were captured every 30 min in 1 μm slices from skin to neural tube. Left is anterior, top is dorsal.

Video S5 | Z-projection of a confocal time-lapse stack of *pax7a:eGFP* larva injured at 7 dpf. Capture was started 30 min after injury and stacks were captured every 30 min in 1 μm slices from skin to neural tube. Left is anterior, top is dorsal.

Figure S1 | (A,B) Confocal slices of the 12th ventral myotome of *pax7a:eGFP* larvae injured at 4 dpf (A) or 7 dpf (B) and stained for phalloidin (f-actin, red), GFP (green), and DAPI (cyan) 24 h after small injury. Arrowheads indicate cells of interest, which appear to be multinuclear. Scale bars are 25 μm. Left is anterior, top is dorsal.

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The emergence of Pax7-expressing muscle stem cells during vertebrate head muscle development

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Pax7 expressing muscle stem cells accompany all skeletal muscles in the body and in healthy individuals, efficiently repair muscle after injury. Currently, the *in vitro* manipulation and culture of these cells is still in its infancy, yet muscle stem cells may be the most promising route toward the therapy of muscle diseases such as muscular dystrophies. It is often overlooked that muscular dystrophies affect head and body skeletal muscle differently. Moreover, these muscles develop differently. Specifically, head muscle and its stem cells develop from the non-somitic head mesoderm which also has cardiac competence. To which extent head muscle stem cells retain properties of the early head mesoderm and might even be able to switch between a skeletal muscle and cardiac fate is not known. This is due to the fact that the timing and mechanisms underlying head muscle stem cell development are still obscure. Consequently, it is not clear at which time point one should compare the properties of head mesodermal cells and head muscle stem cells. To shed light on this, we traced the emergence of head muscle stem cells in the key vertebrate models for myogenesis, chicken, mouse, frog and zebrafish, using *Pax7* as key marker. Our study reveals a common theme of head muscle stem cell development that is quite different from the trunk. Unlike trunk muscle stem cells, head muscle stem cells do not have a previous history of *Pax7* expression, instead *Pax7* expression emerges *de-novo*. The cells develop late, and well after the head mesoderm has committed to myogenesis. We propose that this unique mechanism of muscle stem cell development is a legacy of the evolutionary history of the chordate head mesoderm.

Keywords: head muscle, muscle stem cells, *Pax7*, chicken, mouse, *Xenopus*, zebrafish, vertebrate embryo

Introduction

Adult skeletal muscle stem cells (satellite cells) accompany contractile muscle fibers and efficiently repair muscle after injury (reviewed in Relaix and Zammit, 2012). It is generally thought that one of the factors contributing to this efficient repair is that skeletal muscle stem cells are tissue-specific stem cells, solely committed to myogenesis. However, in a number of diseases including

muscular dystrophies, cancer and HIV/Aids, the ability of muscle stem cells to repair muscle is compromised. Moreover, muscle regeneration declines when we age. This has been ascribed to inflammatory responses, changes to the stem cell niche and changes to the stem cells themselves. Current approaches investigate how these parameters could be targeted to reinstate the full regenerative capacity of muscle.

Overall, skeletal muscle function and repair is much the same in all areas of the body. Therefore, it is often overlooked that muscular dystrophies differentially target muscle groups in the head and in the trunk (reviewed in Emery, 2002). Moreover, head and trunk muscle and their accompanying muscle stem cells have a different developmental history (reviewed in Sambasivan et al., 2011, and see below). This tissue also contributes to the heart, an organ that in amniotes including humans cannot regenerate (reviewed in Garbern and Lee, 2013). Moreover, adult head and trunk muscle stem cells have divergent gene expression, proliferation and differentiation profiles (Sambasivan et al., 2009; Ono et al., 2010; Hebert et al., 2013). Thus, the head mesoderm and the muscle stem cells derived thereof are of great interest to develop both specialized skeletal muscle stem cells and cardiac cells for human therapy.

In the body, skeletal muscles and their accompanying stem cells develop from the segmented paraxial mesoderm, the somites (reviewed in Bryson-Richardson and Currie, 2008; Buckingham and Vincent, 2009; Relaix and Zammit, 2012). Muscles (myotomes) are laid down in waves, and while the first cells differentiate into contractile fibers, more cells are being added on from a dual muscle-dermis-competent precursor pool, stored in a specialized somitic compartment, the dermomyotome. This compartment also provides cells that emigrate into the periphery to provide the limb, hypobranchial/hypopharyngeal/hypoglossal, and in mammals, diaphragm muscles. Importantly, cells in the dermomyotome eventually shed their dermal competence, enter the myotome and become specialized muscle stem cells (Gros et al., 2005; Kassar-Duchossoy et al., 2005; Relaix et al., 2005; Schienda et al., 2006). These cells actively self-renew and provide differentiating cells during fetal and juvenile stages of development, thereby providing the bulk of the adult musculature. Eventually, the stem cells settle underneath the basal lamina of the differentiated muscle fibers and become adult muscle stem cells (satellite cells). In amniotes, these stem cells adopt a quiescent state, only to be activated when injuries occur; in anamniotes, the cells may remain mitotically active and continue to drive muscle growth (Bryson-Richardson and Currie, 2008; Buckingham and Vincent, 2009; Relaix and Zammit, 2012).

In amniotes, somites express the paralogous transcription factors Pax3 and Pax7 as soon as they form; in all jawed vertebrates, these genes continue to be expressed in the dermomyotome, with either Pax3 or both proteins also labeling the migratory muscle precursors (reviewed in Bryson-Richardson and Currie, 2008; Buckingham and Vincent, 2009; Relaix and Zammit, 2012). The genes keep cells in a proliferative state, but are also required to initiate myogenesis, and hence are referred to as premyogenic genes (Collins et al., 2009; Diao et al., 2012; Kawabe et al., 2012). Cells undertaking differentiation then switch on members of the MyoD family of transcription factors,

which are crucial for myogenic differentiation (Weintraub et al., 1989). In the somites, *Myf5* and *MyoD* are expressed first and commit cells to myogenesis. In a feed forward mechanism, they activate *Myogenin* which promotes cell cycle exit and entry into terminal differentiation (Penn et al., 2004). *Mrf4* has an early expression phase in the mouse (Summerbell et al., 2002), but in most models, acts mainly during fetal myogenesis (Hinitz et al., 2009; Della Gaspera et al., 2012, and Dietrich, unpublished observations).

The *Pax3* and *Pax7* genes arose as a result of the second of two rounds of whole genome duplications that occurred in the ancestors of jawed vertebrates 500 million years ago (Ohno et al., 1968; Holland et al., 1994). In jawless vertebrates, the single *pax3/7* gene is also expressed in dermomyotomal muscle precursors (Kusakabe et al., 2011). Likewise, *pax3/7* expression has also been found in the somites and muscle stem cell-like cells of the cephalochordate *Amphioxus* (Holland et al., 1999; Somorjai et al., 2012), indicating an ancient role as premyogenic genes. In jawed vertebrates, both genes were subject to subfunctionalisation: cells retaining muscle stem cells properties rely on the presence of *Pax7* rather than *Pax3*, and in the absence of *Pax7* function, the deposition and maintenance of the skeletal muscle stem cell pool is impaired (Seale et al., 2000; Kassar-Duchossoy et al., 2005; Relaix et al., 2006; Lepper et al., 2009; von Maltzahn et al., 2013). Moreover, in anamniote vertebrates such as the axolotl, in which fully differentiated, functional muscle can contribute to regeneration by returning to a stem cell state, or in experimental models where de-differentiation is induced *in vitro*, this occurs concomitant with a reactivation of *pax7* (Kragl et al., 2009; Pajcini et al., 2010). Thus, the *Pax7* gene is accepted as the universal skeletal muscle stem cell marker in jawed vertebrates.

In the head, the muscles that move the eye ball, move the gill arches and in jawed vertebrates, open and close the mouth, are derived from the non-somitic paraxial head mesoderm (Noden, 1983; Couly et al., 1992; Harel et al., 2009; Sambasivan et al., 2009; reviewed in Sambasivan et al., 2011). This tissue does not form segments, and in contrast to the trunk mesoderm, contributes to both, skeletal muscle and the heart. The early head mesoderm does not express the *Pax3* gene and instead, harbors a complement of markers whose expression pattern is established in a step-wise fashion; eventually, the eye and jaw closure muscle anlagen express *Pitx2*, the most posterior eye muscle and muscle anlagen for the jaw and throat (branchiomeric muscles) express *Tbx1*, and all express *Musculin* (*Msc/MyoR*) (Mootoosamy and Dietrich, 2002; Bothe and Dietrich, 2006; Bothe et al., 2011). These transcription factors have overlapping roles. Notably, similar to *Pax3* and *Pax7* in the trunk, they keep cells in an immature state, control their survival and activate *MyoD* family members; once *Mrf* genes are expressed, myogenic differentiation is thought to occur in a similar fashion as in the body (Kitamura et al., 1999; Lu et al., 2002; Kelly et al., 2004; Diehl et al., 2006; Dong et al., 2006; Zacharias et al., 2011; Moncaut et al., 2012; Hebert et al., 2013; Castellanos et al., 2014).

In the adult, head muscle is equipped with muscle stem cells which express *Pax7*, underlining that *Pax7* is the bona fide muscle stem cell marker (Harel et al., 2009; Sambasivan et al., 2009,

reviewed in Sambasivan et al., 2011). These stem cells however are not immigrants from the somites. Rather, like the muscle they accompany, they are derived from the head mesoderm itself. In tune with this observation, head muscle stem cells continue to express the early head mesodermal markers. This implies that head muscle stem cells may have retained some of the properties of the early head mesoderm, and may therefore be suited to developing specialized muscle stem cells and cardiac cells for therapy.

To explore the developmental and therapeutic potential of head muscle stem cells, we need to understand when and how these cells are being generated. This is currently not known. The aim of this study therefore is to establish when and where head muscle stem cells emerge, using *Pax7* as lead-marker. In order to understand the basic process common to all jawed vertebrates, we investigated the key models for vertebrate myogenesis, chicken, mouse, frog (sarcopterygians), and zebrafish (an actinopterygian). Our work shows that unequivocally, *Pax7* expressing cells arise late in head muscle development, well after the onset of *Myf5* and *MyoD*. Importantly, the cells arise from *MyoD* expressing precursor cells, and we propose that head mesodermal cells have to commit to myogenesis before being able to become a muscle stem cell.

Materials and Methods

Culture and Staging of Embryos

Chicken Embryos

Fertilised chicken eggs (Henry Stewart Ltd, Norfolk) were incubated in a humidified atmosphere at 38.5°C and staged according to Hamburger and Hamilton (1951). Embryos were harvested in 4% PFA.

Mouse Embryos

Wildtype mice were provided by the Animals Resource Centre at the University of Portsmouth. Transgenic mouse driver lines carrying the improved *Cre* gene introduced into the *Pax7* or the *MyoD* locus and reporter lines carrying a Cre-activateable *LacZ*, *GFP*, or *YFP* gene in the *Rosa26* locus are described in Hutcheson et al. (2009), Kanisicak et al. (2009), and Wood et al. (2013) and were provided by the Kardon and Goldhamer laboratories. Mice were mated overnight; the appearance of a vaginal plug the next morning was taken as day 0.5 of development (E0.5). Pregnant females were sacrificed by cervical dislocation and the embryos were fixed in 4% PFA.

Xenopus Embryos

Adult J-strain and cardiac actin:GFP transgenic *Xenopus laevis* frogs were maintained in the European Xenopus Resource Centre (EXRC) at the University of Portsmouth at 18°C in a 14 h light 10 h dark cycle and fed 5 days each week using high protein trout pellets. Embryos were generated as described in Guille (1999), then dejellied in 2% cystein-HCl (pH 8.0), grown at 18–23°C in 0.1 × MBS (Gurdon, 1977), staged according to Nieuwkoop and Faber (1994) and harvested in MEMFA (Harland, 1991).

Zebrafish Embryos

Zebrafish embryos were provided by the INCT de Medicina Molecular, Faculdade de Medicina, Universidade Federal de Minas Gerais. Breeding zebrafish (*Danio rerio*) were maintained at 28°C on a 14 h light/10 h dark cycle. Embryos were obtained by natural spawning, grown in egg water (0.3 g/l Instant Ocean Salt, 1 mg l/l Methylene Blue) at 28°C and staged according to Kimmel et al. (1995). To prevent pigment formation, embryos post-24 hpf were raised in 0.2 mM 1-phenyl-2-thiourea (PTU, Sigma). Embryos were harvested in 4% PFA.

Whole Mount *In Situ* Hybridisation, Immunohistochemistry, Beta Galactosidase Staining, and Sectioning

In chicken and mouse, whole mount *in situ* hybridisation, double *in situ* hybridisation, antibody staining, *in situ* hybridisation followed by antibody staining and vibratome sectioning was carried out as described by Dietrich et al. (1997, 1998, 1999), Mootooosamy and Dietrich (2002), Alvares et al. (2003), and Lours and Dietrich (2005). *In situ* hybridisation and antibody staining in *Xenopus* followed the protocols by Harland (1991) and Baker et al. (1995); for zebrafish the protocols by Thisse and Thisse (2008) were used. Beta galactosidase staining and antibody staining on cryosections was performed according to Hutcheson et al. (2009), a heat-induced epitope retrieval in 1.8 mM Citric Acid, 8.2 mM Sodium Citrate and signal amplification using the Streptavidin system was used for *Pax7*. Probes and antibodies are detailed in the table below (Tables 1A–E).

Photomicroscopy

Whole embryos were cleared in 80% glycerol/PBS or, when fluorescent antibodies had been used, in 2.50 mg/ml 1,4-diazabicyclo[2.2.2]octane (DABCO) in 90% glycerol/ PBS. Vibratome sections were mounted with glycerol, cryosections with Fluoromount (Sigma). Embryos and sections were photographed on a Zeiss Axioskop, using fluorescence or Nomarski optics. Sections in Figure 9 were photographed using a Zeiss LSM710 confocal microscope.

Research Ethics

The work has been approved by the University of Portsmouth Ethical Review Committee (AWERB No14005) and follows the jurisdiction of the Animals (Scientific Procedures) Act. The work involving *Pax7*-*Cre*, *MyoD**iCre*, *Rosa26-lacZ*, and *Rosa26-GFP* mouse lines and the cardiac actin; GFP frog line is covered by personal licenses to G. Kardon, D. Goldhamer, and M. Guille.

Results

Emergence of *Pax7* Expressing Myogenic Cells in Chicken Craniofacial Muscles

Time Course of *Pax7* Expression

Muscle stem cells have the ability to self renew and generate differentiating daughter cells, and this ability is linked to the expression and function of *Pax7* (reviewed in Bryson-Richardson and

TABLE 1A | Chicken ISH probes.

Gene	Source	Fragment size (base pairs)
Pax7	gift from P. Gruss (Goulding et al., 1993)	582
Pitx2	gift from S. Noji (Yoshioka et al., 1998)	800
Alx4	gift from T. Ogura (Takahashi et al., 1998)	1245
MyoR = Msc	own clone (von Scheven et al., 2006)	550
Capsulin	own clone (von Scheven et al., 2006)	600
Tbx1	gift from D. Srivastava (Garg et al., 2001)	380
Myf5	open reading frame, synthesized and cloned into pMK-RQ	785
MyoD	open reading frame, synthesized and cloned into pMK-RQ	909
MyoG	open reading frame, synthesized and cloned into pMK-RQ	694
Mrf4	open reading frame, synthesized and cloned into pMK-RQ	738
Troponin I 1 (Tnni 1)	RT-PCR fragment obtained from E4 cDNA using the primers F2: 5'-AGCAGCTCCAGGAGATCAG-3'; R2 T7: 5'-TAATACGACTCACTATAAGGGAGA-CATGCAGCTGCATGGGCAC-3' The fragment was verified by sequencing.	921
Cdh4 = R-Cadherin	gift from C. Redies (unpublished PRC fragment)	900
Pax3	gift from P. Gruss (Goulding et al., 1993)	660
Paraxis	gift from E. Olson (Šošić et al., 1997)	717
Six1	gift from C. Tabin (Heanue et al., 1999)	700
Eya1	gift from A. Streit (Christophorou et al., 2009)	1000

TABLE 1B | Mouse ISH probes.

Gene	Source	Fragment size (base pairs)
Pax7	gift from P. Gruss (Jostes et al., 1991)	900
Msc = MyoR	gift from R. Kelly (Kelly et al., 2004)	542
Myf5	gift from T. Braun (Braun et al., 1989)	310
MyoD	gift from T. Braun (Braun et al., 1989)	1785
MyoG	gift from T. Braun (Braun et al., 1989)	290

Currie, 2008; Buckingham and Vincent, 2009; Relaix and Zammit, 2012). Adult head muscle stem cells express *Pax7* (Harel et al., 2009; Sambasivan et al., 2009, reviewed in Sambasivan et al., 2011), and hence we used the emergence of *Pax7* expression in the head mesoderm as a sign that head muscle stem cells are being laid down. We first analyzed the onset of *Pax7* expression in the chicken head mesoderm, because chicken embryos are large and easy to obtain, and craniofacial muscle formation is well characterized in this model (reviewed in Noden and Francis-West, 2006). Using whole mount *in situ* hybridisation, we performed a time course for the expression of *Pax7* mRNA from the stage the head mesoderm is being laid down by the primitive streak at HH4 to stage HH24 when craniofacial muscle anlagen are well established (Noden et al., 1999; Camp et al., 2012; **Figures 1A–L**).

TABLE 1C | Xenopus tropicalis (Xt) and Xenopus laevis (Xl) ISH probes.

Gene	Primers/Subcloning	Fragment size (base pairs)
Xt pax7	F: 5'-AAGCAGGCAGGAGCCAATCA-3'; R-T7: 5'-TAATACGACTCACTATAAGGGAGA-ATGGACAGGTCTCAGAAGATG-3'	804
Separate Xl pax7a and 7b probes	RT-PCR Fragments obtained with the above F and R primers and cloned into pGEMT Easy.	804
Xt msc = myor	F: 5'-GGATCTGTGAGTGACACTGAG-3'; RT7: 5'-TAATACGACTCACTATAAGGGAGA-AGGTAGAGAGGTGATGTTCTAG-3'	550
Xt myf5	F: 5'-AGAACAGGTAGAAAATCTACTACA-3'; R T7: 5'-TAATACGACTCACTATAAGGGAGA-AATACAAATGCAGCCAAGTAGA-3'	531
Xt myod	F: 5'-CCTGCRGCTCCAGGAGAAG-3'; R T7: 5'-TAATACGACTCACTATAAGGGAGA-AAGTTCCCTTGGCCTCAGG-3'	587
Xt myog	F: 5'-CAGACCAAAGGTTTATGACAA-3'; R T7: 5'-TAATACGACTCACTATAAGGGAGA-AATGCATATTGTCATGATGG-3'	880
Xt mrf4	F: 5'-GCACAGTTGGATCAGCAGG-3'; R T7: 5'-TAATACGACTCACTATAAGGGAGA-TTCAACACTGTCCATAATTAC-3'	596
Xt desmin	Degenerate primers were used. F: 5'-TCTGCACTCAGTTTAYAGAGAA-3'; R T7: 5'-TAATACGACTCACTATAAGGGAGA-CATATSTAAGMGAATYATGGG-3'; M = A + C; Y = C + T; S = G + C	470

All cDNA fragments were obtained by RT-PCR using the primers below. To generate templates for probe synthesis, either the binding site for the T7 RNA polymerase was introduced with the reverse PCR primer, or fragments were subcloned. The identity of PCR fragments or clones was confirmed by sequencing.

TABLE 1D | Zebrafish ISH probes.

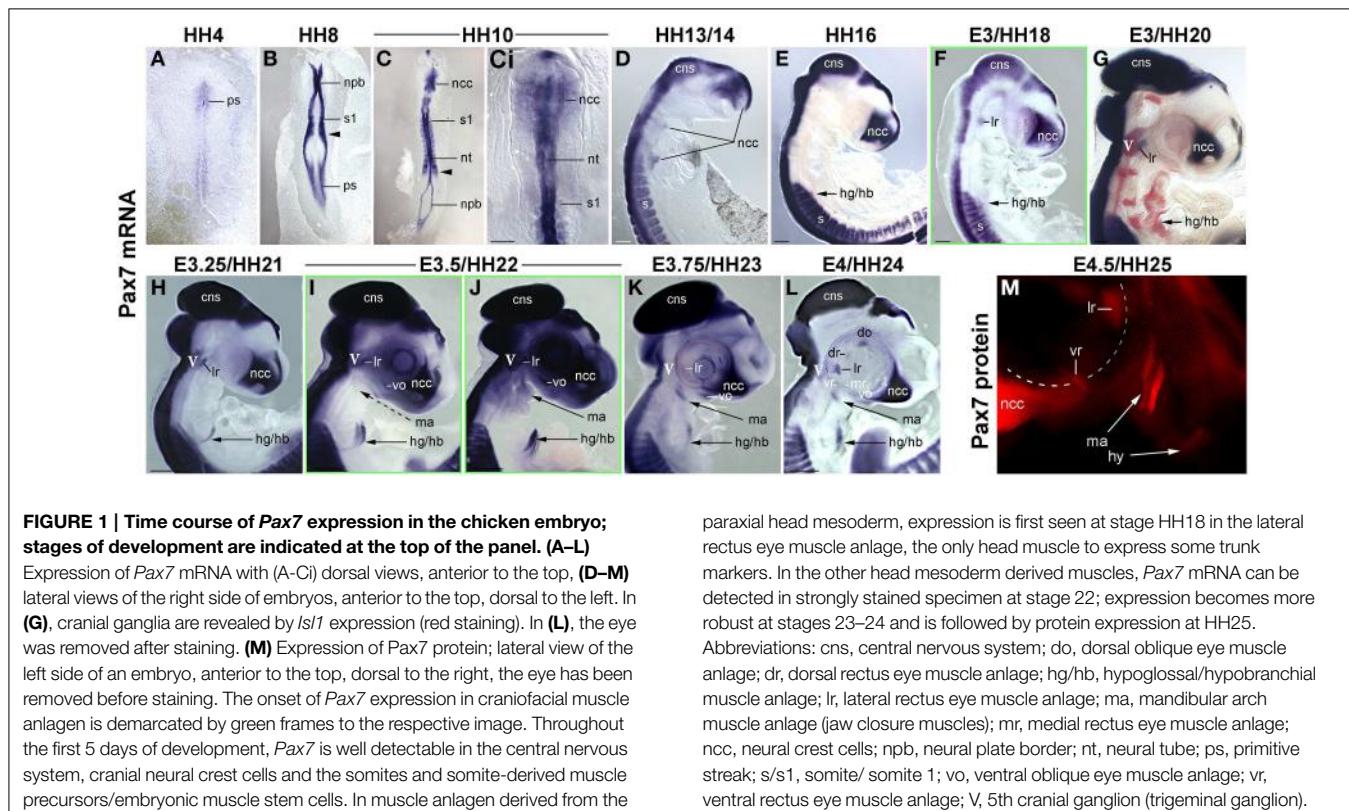
Gene	Source	Fragment size (base pairs)
pax7a isoform 1	gift from A. Fjose (Seo et al., 1998)	2000
myod1	RT-PCR Fragment obtained with F: 5'-TTCTACGACGCCCTGCTT-3'; R: 5'-GGATTCCGCCTTTCTGCT-3'; cloned into pGEMT Easy and sequenced	858

Moreover, we analyzed the onset of *Pax7* protein expression (**Figure 1M** and not shown) and we confirmed the association of *Pax7* expression domains with craniofacial skeletal muscle on serial frontal and cross sections (**Figure 2**).

We found that during early stages of development, *Pax7* expression was associated with the epiblast bordering the primitive streak, the neural plate border/ dorsal neural tube and emerging neural crest cells (**Figures 1A–C**). Expression in the dorsal

TABLE 1E | Antibodies.

	Source	Dilution
PRIMARY ANTIBODIES		
Rabbit IgG anti-GFP	Life technologies	1:1000
mouse IgG2b anti-sarcomeric Myosin (MF20)	Developmental Studies Hybridoma Bank	1:200
Mouse IgG1 anti-embryonic skeletal muscle Myosin (F1.652)	Developmental Studies Hybridoma Bank	1:10
Mouse IgG1 anti-slow skeletal muscle Myosin (NOQ7.5 4D)	Sigma	1:4000
Mouse IgG1 anti-fast skeletal muscle Myosin (My32)	Sigma	1:1000
mouse anti-NFM 160kd (RMO 270)	Invitrogen	1:2000
mouse IgG1 anti-Pax7	Developmental Studies Hybridoma Bank	1:25
SECONDARY ANTIBODIES		
alkaline phosphatase conjugated sheep anti-Digoxigenin, Fab fragments	Roche	1:2000
horse radish peroxidase (HRP) conjugated goat anti-mouse IgG + IgM (H + L)	Jackson Immuno	1:500
Alexa fluor 594 conjugated goat anti-mouse IgG + IgM (H + L)	Jackson Immuno	1:200
Alexa fluor 488 conjugated donkey anti-rabbit IgG (H + L)	Jackson Immuno	1:200
Biotin conjugated goat anti-mouse IgG1; developed with Streptavidin conjugated Alexa Fluor 594	Jackson Immuno	1:200

**FIGURE 1 | Time course of Pax7 expression in the chicken embryo; stages of development are indicated at the top of the panel. (A–L)**

Expression of Pax7 mRNA with (A–C) dorsal views, anterior to the top, (D–M) lateral views of the right side of embryos, anterior to the top, dorsal to the left. In (G), cranial ganglia are revealed by *Isl1* expression (red staining). In (L), the eye was removed after staining. (M) Expression of Pax7 protein; lateral view of the left side of an embryo, anterior to the top, dorsal to the right, the eye has been removed before staining. The onset of Pax7 expression in craniofacial muscle anlagen is demarcated by green frames to the respective image. Throughout the first 5 days of development, Pax7 is well detectable in the central nervous system, cranial neural crest cells and the somites and somite-derived muscle precursors/embryonic muscle stem cells. In muscle anlagen derived from the

paraxial head mesoderm, expression is first seen at stage HH18 in the lateral rectus eye muscle anlage, the only head muscle to express some trunk markers. In the other head mesoderm derived muscles, Pax7 mRNA can be detected in strongly stained specimen at stage 22; expression becomes more robust at stages 23–24 and is followed by protein expression at HH25. Abbreviations: cns, central nervous system; do, dorsal oblique eye muscle anlage; dr, dorsal rectus eye muscle anlage; hg/hb, hypoglossal/hypobranchial muscle anlage; lr, lateral rectus eye muscle anlage; ma, mandibular arch muscle anlage (jaw closure muscles); mr, medial rectus eye muscle anlage; ncc, neural crest cells; npb, neural plate border; nt, neural tube; ps, primitive streak; s/s1, somite/somite 1; vo, ventral oblique eye muscle anlage; vr, ventral rectus eye muscle anlage; V, 5th cranial ganglion (trigeminal ganglion).

neural tube remained high at later stages of development while expression in neural crest cells only persisted in the trigeminal ganglion and the frontonasal neural crest (Figures 1D–M, cns, V, ncc). In the trunk, from HH7 onwards Pax7 was also expressed in the epithelialising somites (Figures 1B,C, arrowhead, and not shown), subsequently becoming confined to the muscle precursor/muscle stem cell lineage (Figures 1C–L). From HH16 onwards, Pax7 was also expressed in migratory muscle precursors that leave the somites to form the hypoglossal/hypopharyngeal (Figures 1E–L, hg/hb) and limb

musculature (not shown). From HH20 onwards, expression was found in the embryonic muscle stem cells that populate the myotome, drive both fetal and perinatal muscle growth and give rise to the trunk adult muscle stem cells (Gros et al., 2005; Relaix et al., 2005; Ahmed et al., 2006; Schienda et al., 2006) and not shown. These findings are in agreement with published data and underline the robustness of our approach.

Expression associated with craniofacial muscles was first seen at HH18 in the developing lateral rectus eye muscle (Figure 1F,

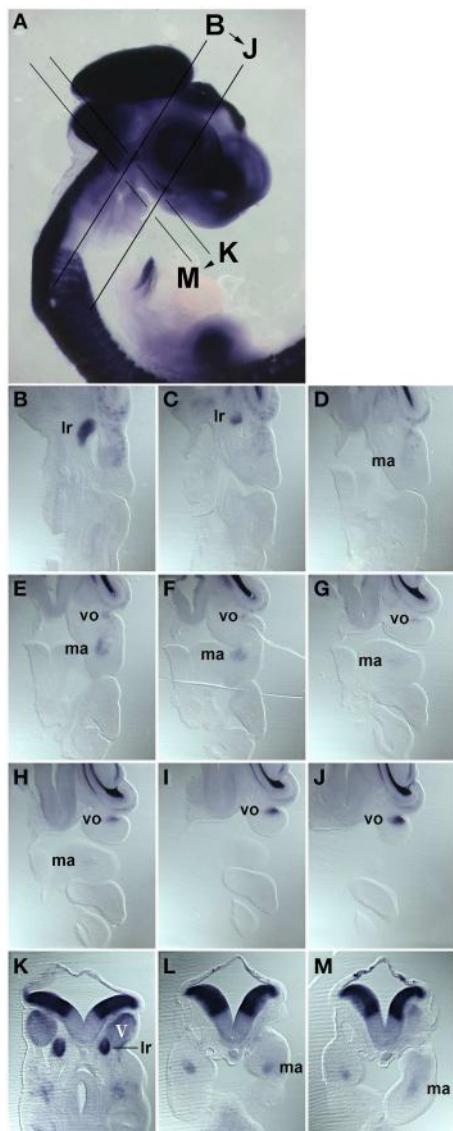


FIGURE 2 | Series of frontal (B–J) and cross (K–M) sections of a HH22 chicken head, stained for the expression of *Pax7* mRNA, the plane and order of sections in indicated in (A). (B–J) anterior to the top, lateral to the right; (K–M) dorsal to the top. Abbreviations as in **Figure 1**. The position of the *Pax7* signals beneath the trigeminal ganglion, beneath the eye and in the core of the mandibular arch confirms that these are expression domains associated with developing head muscles.

lr), located just beneath the also *Pax7* positive trigeminal ganglion (**Figures 1G–J, V**, in G red staining for *Isl1*, **Figure 2B, V**). However, the lateral rectus is somewhat unusual as it is the only craniofacial muscle to express trunk markers such as *Paraxis* and *Lbx1* (Mootoosamy and Dietrich, 2002, and see below). In the anlagen of the other craniofacial muscles, *Pax7* staining did not emerge before day 3.5 of development. Expression was first seen in the ventral oblique eye muscle (**Figures 1I,J, 2, vo**), in strongly stained specimen followed by the anlagen of the jaw closure muscles (1st pharyngeal arch = mandibular

arch muscles; **Figures 1J, 2, ma**). *Pax7* mRNA expression became more robust at E3.75/HH23 and E4/HH24 and eventually began to encompass all muscle anlagen, with the staining in the ventral and medial rectus lagging behind that of the other eye muscle anlagen (**Figures 1K,L**). However, signals were always weak compared to the expression in the frontonasal neural crest cells, the central nervous system and the trunk musculature. Moreover, expression of *Pax7* protein in craniofacial muscle anlagen was delayed compared to the expression of *Pax7* mRNA and could only be detected from HH25 onwards (**Figure 1M** and not shown).

Comparison of *Pax7* Expression with the Expression of Markers for the Early Head Mesoderm, for Myogenic Commitment and for Myogenic Differentiation

In the trunk, *Pax7* expression precedes the expression of any marker for myogenic commitment and differentiation (Jostes et al., 1991). However, the late onset of *Pax7* expression in the head musculature suggested that here, the sequence of marker gene expression and the set up of gene regulatory networks might be quite different. To explore this, we systematically analyzed the spatiotemporal distribution of early head mesoderm markers (*Pitx2*, *Alx4*, *Musculin* = *Msc* = *MyoR*, *Tcf21* = *Capsulin*, *Tbx1*; **Figure 3**), of markers indicating the onset of myogenesis (*Mrf* family members; **Figure 4**), and of markers indicating cohesion and terminal differentiation of muscle anlagen (*Cadherin4* = *Cdh4* = *R-Cadherin*, *Tnni1*, sarcomeric Myosin; **Figure 5**).

Markers for the cranial mesoderm

The early chicken head mesoderm is known to express the transcription factors *Pitx2*, *Alx4*, *Msc*, *Capsulin*, and *Tbx1* (Bothe and Dietrich, 2006; von Scheven et al., 2006; Bothe et al., 2011), and in accord with this work, mRNA expression of *Pitx2* and *Tbx1* was first seen at HH6 in distinct rostro-caudal regions of the head mesoderm (not shown). At HH9 *Alx4* expression emerged within the confines of the *Pitx2* territory, followed by *Msc* expression at HH10. Between HH10 (**Figures 3A,F,K,P,U**) and HH13/14 (**Figures 3B,G,L,Q,V**), *Tbx1* expression spread anteriorly and *Msc* expression spread posteriorly, co-labeling the branchiomeric mesoderm and the anlage of the caudal-most eye muscle, the lateral rectus. Also at HH13/14, *Tcf21* expression commenced in the anlagen of the branchiomeric muscles, but remained weaker than that of its paralog *Msc* throughout (**Figure 3Q**). *Alx4* on the other hand became strongly upregulated in craniofacial neural crest cells, thus, masking any residual mesodermal expression (**Figures 3G–J, ncc**). Owing to these changes, *Pitx2*, *Msc*, and *Tbx1* became the most prominent markers for the myogenic head mesoderm, between HH16 to HH22–24 labeling the anlagen of the eye and mandibular arch muscles (*Pitx2*, **Figures 3C–E**), all eye and branchiomeric muscles (*Msc*; **Figures 3M–O**) or the branchiomeric muscles and the lateral rectus eye muscle (*Tbx1*, **Figures 5W–Y**). Respective expression domains for these markers were wider than those for *Pax7* but included the *Pax7* domains.

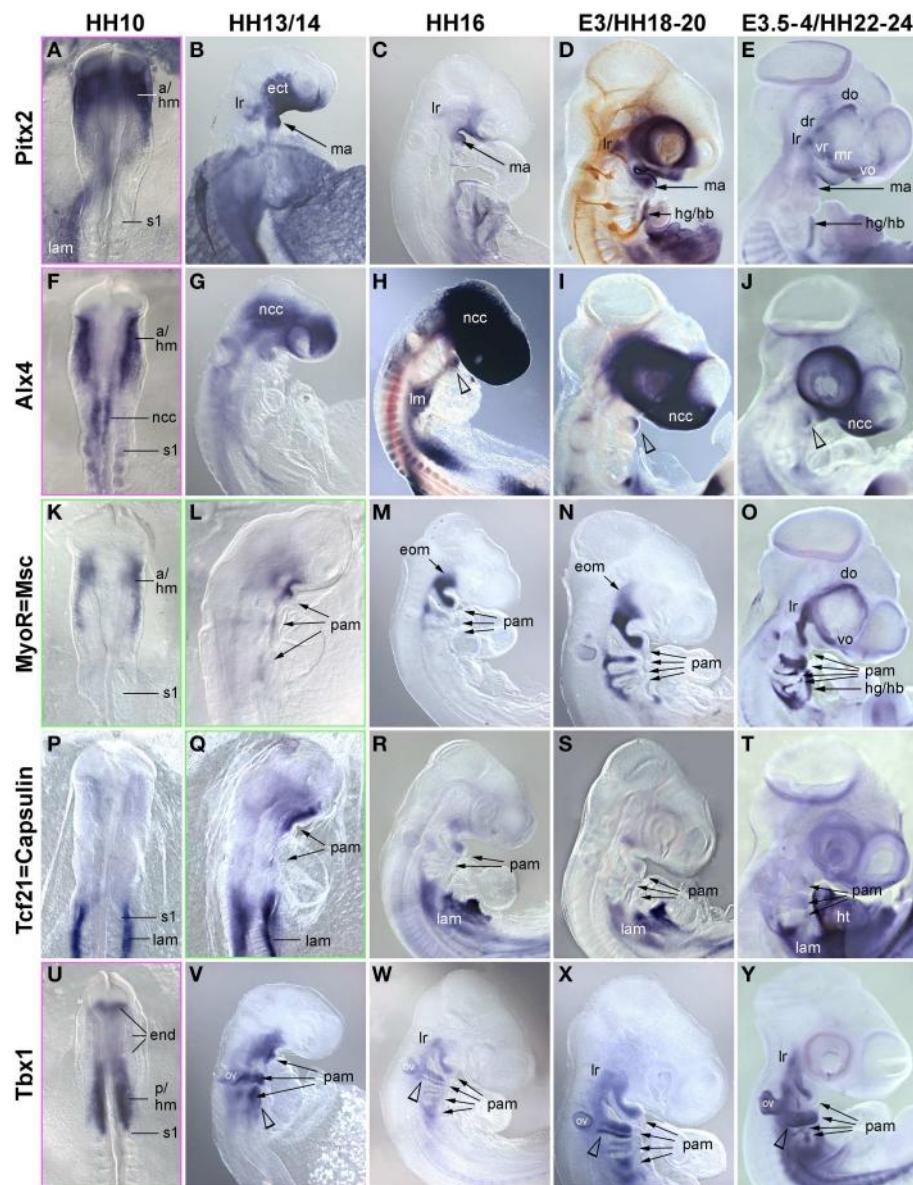


FIGURE 3 | Time course for the mRNA expression of head mesoderm markers in chicken embryos at HH10 (dorsal views) and HH13/14-E4 (lateral views); in (D), cranial nerves are revealed with the RMO270 antibody (brown staining). Gene names are displayed on the left of the panel; developmental stages are indicated at the top. The onset of marker gene expression is demarcated by a green frame, for genes being expressed earlier than HH10, frames are displayed in magenta. Abbreviations as in **Figure 1** and: a/hm, anterior head mesoderm; ect, surface ectoderm; eom, extraocular muscle anlagen; end, endoderm; lam, lateral mesoderm; ht, heart; ov, otic vesicle; pam, pharyngeal arch muscle anlagen; p/hm, posterior

head mesoderm. The open arrowhead in (H,J) points at *Alx4* expression in the mandibular arch ectoderm and in (V-Y) at *Tbx1* expression in the posterior ectoderm of the hyoid (2nd pharyngeal) arch. Note that all head mesoderm markers begin their expression well before *Pax7*. With the exception of *Alx4* which from HH13/14 onwards mainly labels cranial neural crest cells and *Tcf21/Capsulin* which throughout has lower expression levels than its paralog *MsC/MyoR*, all head mesoderm markers continue to strongly label the myogenic head mesoderm. Their expression domains are wider than that of *Pax7*, whose expression domain is nested in the expression domain of the head mesoderm genes (compare **Figures 1, 4**).

Markers for myogenic commitment and the initiation of myogenesis

In the developing chicken somites, commitment to myogenesis and entry into differentiation is demarcated by the sequential expression of the *MyoD* family of *Mrf* genes, with *Myf5* commencing first, followed by *MyoD*, *MyoG* and *Mrf4*

(Berti and Dietrich, unpublished observations). In the avian head mesoderm, expression of the *Mrf* family commenced at HH13/14, when *Myf5* labeled the anlagen of the lateral rectus and the mandibular and hyoid arch muscles (**Figure 4B**, see also Noden et al., 1999). At HH16, expression of *Myf5* was accompanied by that of *MyoD* (**Figures 4C,H**). Between

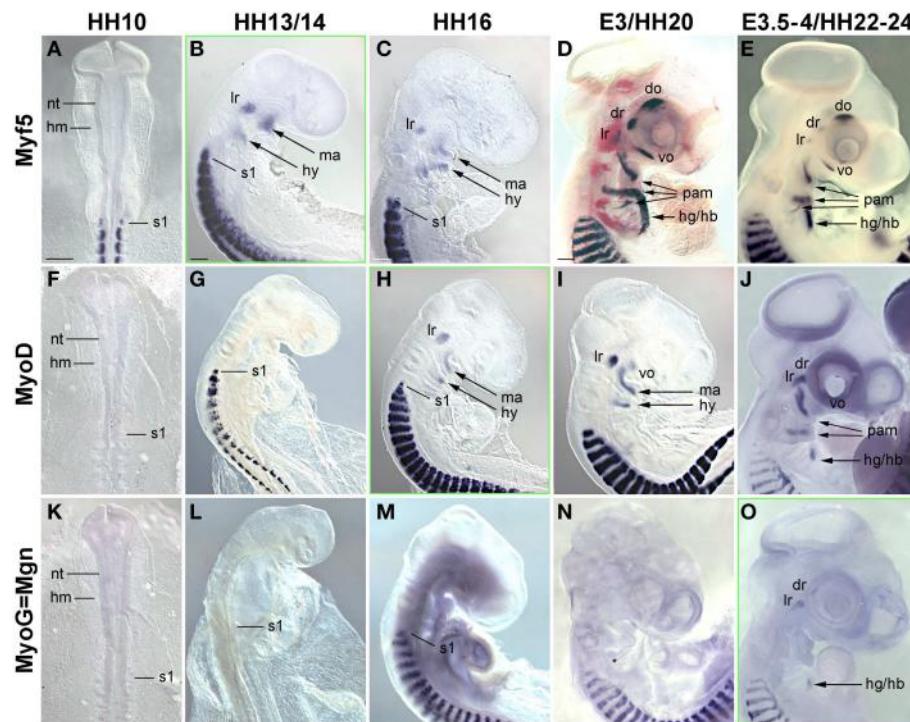


FIGURE 4 | Time course for the mRNA expression of Mrf transcriptions factors in chicken embryos from HH10-E4; orientation of specimen and frames indicating the onset of expression as in Figure 3. Gene names and developmental stages are displayed as in Figure 3. Abbreviations as in Figures 1, 3 and: hy, hyoid arch. *Myf5* and *MyoD* indicate the myogenic commitment of

precursor cells and are expressed in developing craniofacial muscle anlagen from HH13/14 (*Myf5*) and HH16 (*MyoD*) onwards, i.e., significantly before the onset of *Pax7*. Expression of *Myogenin* (*MyoG/Mgn*) indicates the entry of cells into muscle differentiation and commences at E3.5-4, i.e., about the same time as *Pax7* (compare with Figure 1).

HH20-HH24, all craniofacial muscle anlagen began to express these genes, with *MyoD* expression always following that of *Myf5* (Figures 4D,E,I,J). *MyoG* expression was detected at HH22-24 (Figure 4O), yet *Mrf4* was still silent at this stage (not shown). Thus, for all craniofacial muscles, including the peculiar lateral rectus eye muscle, expression of *Myf* and *MyoD* emerged well before that of *Pax7*; and *MyoG* expression began at approximately the same time as *Pax7*. This is different from the trunk where *Pax7* is expressed before any of the *Mrf*s.

Markers for muscle cohesion and terminal differentiation
Cadherin 4 has been shown to act in the communication and differentiation of myogenic cells (Rosenberg et al., 1997) and to be expressed in the chicken lateral rectus eye muscle (Mootoosamy and Dietrich, 2002). Troponins and sarcomeric Myosins are components of the contractile proteins complexes in both cardiac and skeletal muscle, with *Tnni1* specifically acting in the early developing slow-twitch muscle and (during embryogenesis) in the heart (see <http://geisha.arizona.edu/geisha/>). We therefore used these markers as indicators for the cohesion and terminal differentiation of muscle anlagen. Expression of these markers was first seen at HH16, when *Cadherin4* labeled the lateral rectus and mandibular arch muscle anlagen (Figure 5C). *Tnni1* expression commenced at HH20, at HH22-24 encompassing all craniofacial

muscle anlagen (Figures 5I,J). At this stage, sarcomeric Myosins were also expressed, indicating the presence of functional skeletal muscle (Figure 5O and Noden et al., 1999). The onset of *Cdh4* and *Tnni1* expression before or concomitant with that of *Pax7* suggests that in the head, the process of skeletal muscle development is well under way when the *Pax7* cell lineage is being established.

Comparison of *Pax7* Expression with the Expression of Trunk Premyogenic Genes

In previous studies, we had shown that the early head mesoderm does not express the *Pax7* paralog *Pax3* (Mootoosamy and Dietrich, 2002; Bothe and Dietrich, 2006). However, stages at the onset of *Pax7* expression have not been analyzed. Moreover, in the trunk Paraxis, *Six1* and the *Six1* co-activator *Eya1* also act as premゆogenic regulators (Wilson-Rawls et al., 1999; Grifone et al., 2005, 2007; Relaix et al., 2013). To explore if any of these genes might be in the position to serve as intermediaries between the head mesoderm genes, the *Mrf* genes and *Pax7*, we investigated the expression of these trunk premゆogenic genes (Figure 6). Our analysis revealed that *Pax3* expression overlapped with that of *Pax7* in the neural tube, the trigeminal ganglion, the frontonasal neural crest and somites, but remained absent from craniofacial skeletal muscle anlagen (Figures 6A–E). *Paraxis* expression

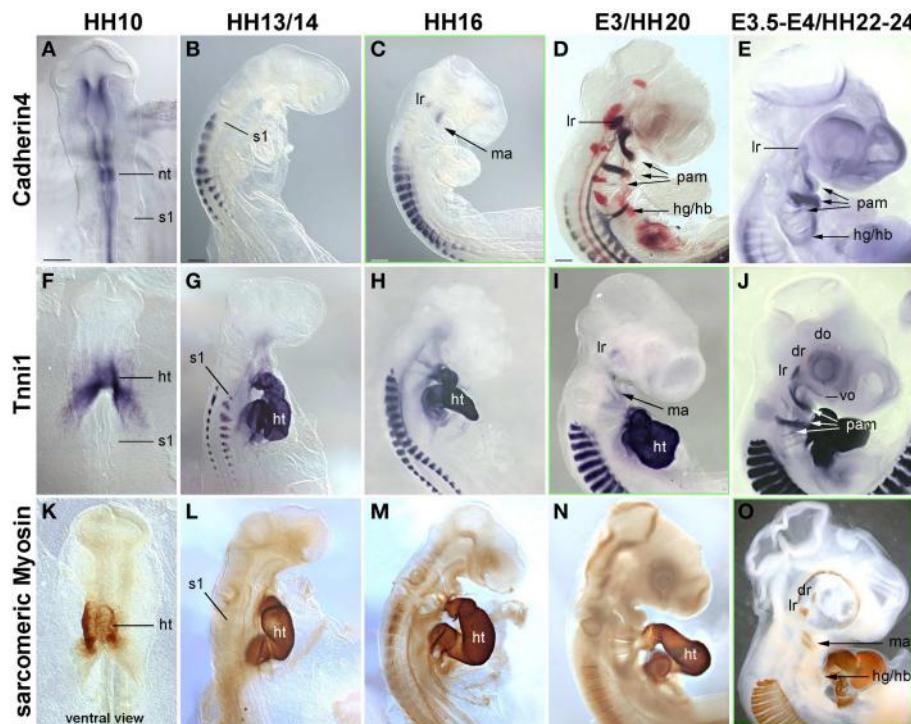


FIGURE 5 | Time course in chicken embryos from HH10-E4 for markers indicating the cohesion of muscle anlagen (Cadherin 4-mRNA expression) and terminal differentiation [Troponin I 1 (Tnni1)-mRNA expression; sarcomeric Myosin–MF20 antibody staining]. Orientation of specimen and frames indicating the onset of

expression as in **Figures 3, 4**; abbreviations as in **Figures 1, 3, 4**. The time course of Cadherin 4 expression resembles that of *MyoD*, and *Tnni1* expression commences in many craniofacial muscle anlagen E3, i.e., both are expressed before or at the onset of *Pax7* expression. Sarcomeric Myosins can be detected at E3.5-4, simultaneous to the onset of *Pax7*.

overlapped with the expression of *Pax3* and *Pax7* in the frontonasal neural crest cells and the somites, but, with the exception of the lateral rectus muscle, was also not expressed in craniofacial muscle anlagen (Mootooosamy and Dietrich, 2002; **Figures 6F–J**). *Six1* showed a widespread expression, at HH5–10 encompassing the preplacodal ectoderm, both the mesoderm and the endoderm underneath the neural plate, and weakly, the developing somites (shown for HH10, **Figure 6K**). From HH13/14 onwards (**Figures 6L–O**), expression was strong in the otic vesicle and nasal pit, the trigeminal placodes, the posterior edge of the 2–4th pharyngeal arches and the pharyngeal pouches, the somites and the emerging migratory muscle precursors. Moreover, low-level, widespread *Six1* expression was found throughout the head mesenchyme. However, expression was also found in the anlagen of branchiomeric muscles, with strongest expression in the hyoid arch. Expression of *Eya1* (**Figures 6P–T**) was similar to that of *Six1*. Yet, while strong and lasting expression was detected in craniofacial neural crest cells, expression levels in craniofacial muscle anlagen declined. This suggests that in contrast to the other trunk pre-myogenic genes, *Six1* and *Eya1* may play an–albeit more subordinate than in trunk–role in head skeletal muscle development and may influence muscle development indirectly via the control of connective tissue development. Yet none of the trunk pre-myogenic markers seems to take over from the head mesoderm to prepare for myogenesis and/or muscle stem cell deployment.

Emergence of Pax7 Expressing Myogenic Cells in Mouse Craniofacial Muscles

Time Course of Murine Pax7 Expression

Our analysis in the chicken suggested that, in contrast to the trunk, *Pax7* expressing cells associated with cranial skeletal muscle emerge late, well after the onset of markers for myogenic commitment and at the time that cells begin to enter terminal differentiation. To explore whether this unexpected timing is true also for other amniotes, we next investigated the mouse, establishing both the onset of mRNA (**Figures 7A–D**) and protein expression (**Figures 7E–G**; see also **Figure 9**). Moreover, we investigated the position of Pax7 protein expressing cells at birth (**Figures 7H–J**) and analyzed the fate of *Pax7* positive cells from embryonic to late fetal stages of development (**Figures 7K–Q**).

As in the chicken, mouse *Pax7* expression was seen in neural crest cells, the central nervous system and in somites from early neurulation stages onwards (Jostes et al., 1991, and not shown). At E9.5, even though the first two pharyngeal arches were well developed, there was no expression in their myogenic mesodermal core (**Figure 7A**, ma, hy; dotted arrows). Expression in the pharyngeal arch muscle anlagen was first seen at E10.5, and became more robust between E11.5–12.5 (**Figures 7B–D**, ma, hy; solid arrows). Also similar to the chicken, expression of Pax7 protein was delayed compared to the expression of mRNA and was first detected at E12.5, with the Pax7 domain overlapping with that of *MyoD* (**Figures 7E–G**). Both at E12.5 and in

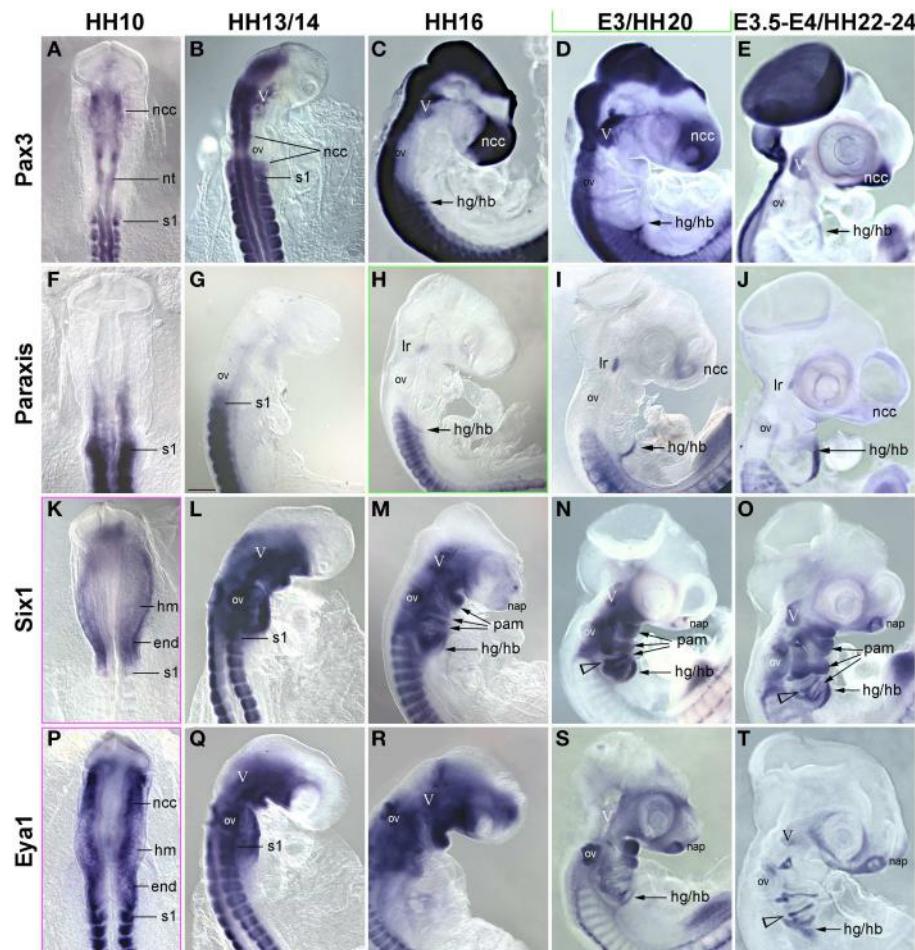


FIGURE 6 | Time course for the mRNA expression of trunk pre-myogenic genes; embryos are displayed and annotated as in Figures 3–5. Abbreviations as before and: na, nasal pit. **(A–E)** *Pax3* labels the central nervous system, the frontonasal neural crest, the trigeminal ganglion, the somites and the somite-derived hypobranchial and limb muscle precursors, but remains absent from genuine craniofacial muscle anlagen. **(F–J)** *Paraxis* expression overlaps with that of *Pax3* and 7 in the

somite-derived muscle precursors and in the frontonasal crest. Similar to *Pax7*, *Paraxis* is also expressed in the lateral rectus eye muscle, but is absent from all other craniofacial muscles. *Six1* **(K–O)** and *Eya1* **(P–T)** are expressed in the head mesoderm before and at HH10. From that stage onwards mesoderm expression becomes somewhat obscured by the overlying expression in neural crest cells. However, *Six1* (but not *Eya1*) remains detectable in craniofacial muscle anlagen.

the newborn, *Pax7* protein was located in the nuclei of cells (note the punctate staining in **Figures 7Gi, Gii, J**). In the newborn, the *Pax7* staining was associated with muscle fibers revealed by antibodies detecting sarcomeric Myosins (compare **Figures 7Ii, J**), in line with studies that showed that at this stage, *Pax7* expressing cells had assumed their mature satellite cell (adult muscle stem cell) position (Harel et al., 2009; Sambasivan et al., 2009).

Using the *Pax7 Cre* driver and the *Rosa26R^{LacZ}* reporter, we traced cells that in their past expressed robust levels of *Pax7* (Hutcheson et al., 2009). This approach allowed to visualize the contribution of *Pax7* expressing neural crest cells to the trigeminal ganglion, the pharyngeal arches and the developing frontonasal skeleton with a delay of 1 day compared to the onset of mRNA expression; likewise, the *Pax7* cell lineage in the central nervous system and in the somites could readily be traced with a delay of 1 day (**Figures 7K–L**, and not shown). *LacZ* positive

cells contributing to the mandibular arch muscle anlagen were just about detectable at E11.5 (**Figure 7M**, ma, arrow), at E12.5, this contribution was more evident (**Figure 7N**, ma, arrow). By E13.5, virtually all developing craniofacial muscles had received a contribution of cells that once had expressed *Pax7* (compare **Figure 7O** and **Figure 9D**, and see also **Figures 9F, G, I**), and at E14.5 (**Figure 9P**) and E18.5 (**Figure 9Q**), the cells had contributed to muscle fibers. This suggests that at fetal and perinatal stages of development *Pax7* positive cells contribute to the growth of head skeletal muscle in a similar fashion as in the trunk, inferring a convergence of developmental pathways.

Comparison of *Pax7* Expression with the Expression of Head Mesoderm and Myogenic Markers

In the chicken, *Pax7* expression in craniofacial muscle anlagen commenced after the onset of head mesoderm markers and after the onset of *Myf5* and *MyoD*. To explore whether this is also true

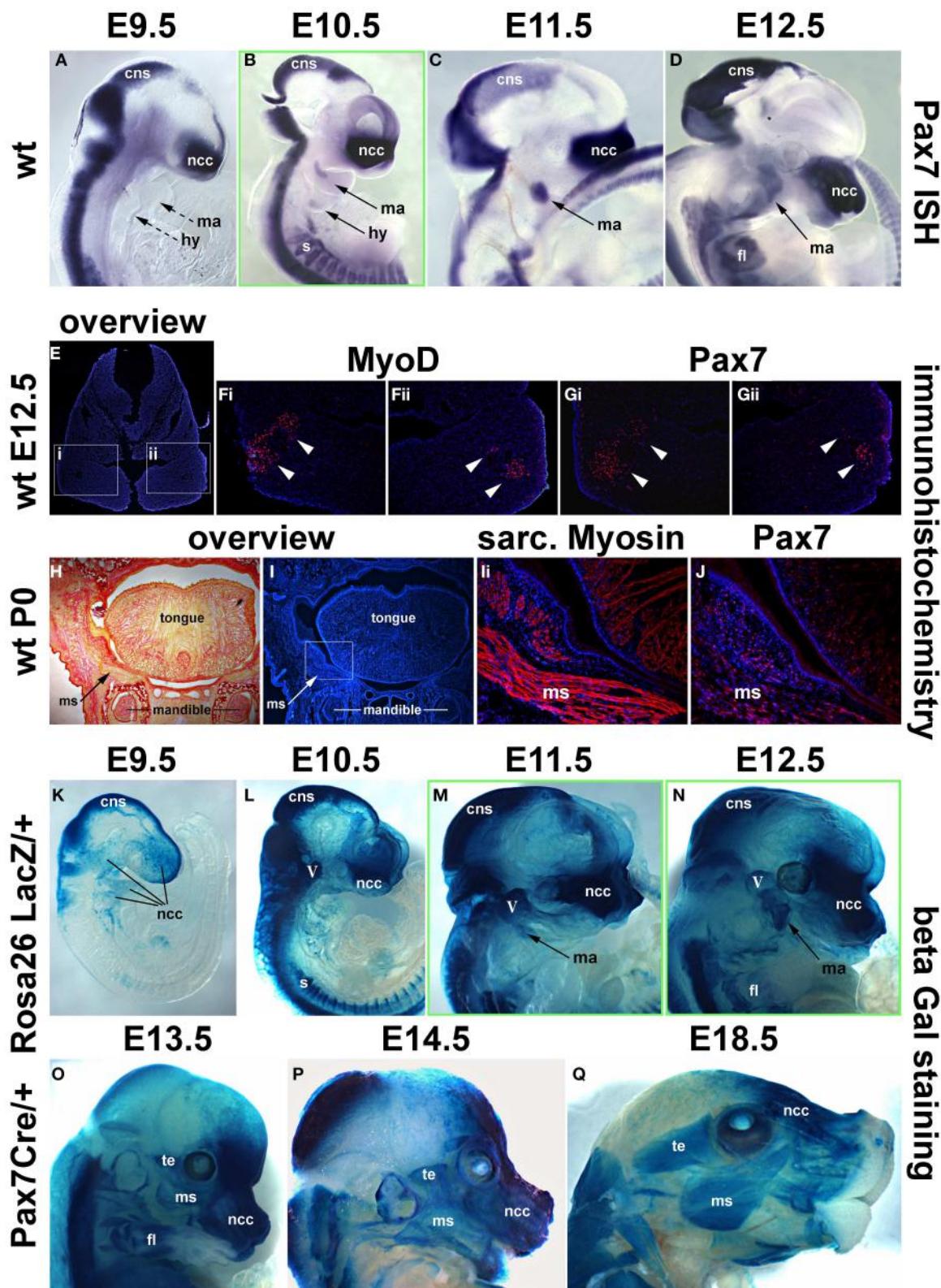


FIGURE 7 | Time course of *Pax7* expression in the mouse. (A–D) *Pax7* mRNA expression from E9.5–E12.5 of development; lateral views of the right side of embryos, anterior to the top. Expression is readily detectable in the

developing central nervous system, emigrating neural crest cells (prolonged expression in the frontonasal neural crest) and the somites. Head muscle
(Continued)

FIGURE 7 | Continued

anlagen show expression first at E10.5. (E) Serial cross sections of the mandibular arch at E12.5, dorsal to the top; (F,G) higher magnifications of the areas indicated by the boxes in (E) and stained for Dapi and MyoD protein (Fi,ii) or Dapi and Pax7 protein (Gi,ii). Note that MyoD and Pax7 domains overlap. (H,I) Serial frontal sections of the mandible at birth (P0), dorsal to the top, lateral to the left. (H) Sirius Red staining showing muscle fibers in yellow and bone and connective tissue in red. (I) Dapi staining of the same region, with (II) showing a magnification of the cheek and the floor of the mouth as indicated in (I). Skeletal muscle fibers are shown in red. (J) Subsequent section stained for Pax7 protein in red. Note the punctate, nuclear staining for Pax7, associated with the

Myosin-positive muscle fibers. (K–Q) Lineage tracing of *Pax7* expressing cells, revealed by beta galactosidase staining; lateral views of the right side of embryos, dorsal to the top. With a delay of 1 day, cells with a history of *Pax7* expression can be detected in the central nervous system, the trigeminal ganglion, the frontonasal neural crest and the somites. In craniofacial muscle anlagen, cells with a history of *Pax7* expression can be detected between E11.5 and E12.5, with a more robust staining appearing at E13.5. Eventually, all craniofacial muscles are stained and the staining is found in muscle fibers, indicating that, similar to the trunk, *Pax7*-positive cells contribute to fetal and perinatal muscle growth. Abbreviations as in **Figures 1, 3, 4** and: fl, forelimb; ISH, *in situ* hybridisation; ms, masseter; te, temporalis muscle; wt, wildtype.

for mammals, we investigated the expression of *Pitx2*, *Tbx1*, *Msc*, *Myf5*, *MyoD*, and *MyoG* at E7.5–E10.5 of development (**Figure 8** and not shown). Expression of *Pitx2*, *Tbx1*, and *Msc* commenced much earlier at E7.5–8 (not shown). At E9.5, *Msc* labeled the myogenic cells that will engage with the eye as well as the core of the first two pharyngeal arches; the same pattern was found at E10.5 (**Figures 8A,B**). Significantly, at both stages, both *Myf5* as well as *MyoD* were well expressed in the anlagen of first and second arch muscles (**Figures 8C–F**), while *MyoG* was not yet active (**Figures 8G,H**). Thus, in both amniote models, the expression of the head mesoderm markers preceded the expression of *Myf5* and *MyoD*, which in turn preceded the expression of *Pax7*.

Pax7 Expression in Regions with a History of MyoD Expression

Since *MyoD* expression in craniofacial muscle anlagen preceded the onset of *Pax7*, we began to explore whether *MyoD* might be upstream of *Pax7*, similar to what has been shown for P19 EC cells misexpressing *MyoD* (Gianakopoulos et al., 2011). For this we turned to the *MyoDiCre* mouse driver line (Kanisicak et al., 2009; Wood et al., 2013). We first established when the *Rosa26 GFP* reporter (R26NG; Yamamoto et al., 2009) may reveal activity of the *MyoDiCre* driver in craniofacial muscle anlagen; we found that this was the case from E10.5 onwards (**Figures 9A–D**). At E12.5 and E13.5, the GFP expression pattern was highly similar to that of *Pax7* mRNA and *Pax7*-driven LacZ (compare **Figures 9C,D, 7D,N,O**). To test whether the *Pax7* mRNA we had detected earlier at E11.5 might colocalise with the GFP read-out of the *MyoD* locus, we simultaneously visualized the *Pax7* mRNA and GFP driven by *MyoDiCre* (**Figures 9E–I**). We found that indeed, cells with a history of *MyoD* expression engulfed the *Pax7* domain located at the maxillary-mandibular junction. To directly test whether *Pax7* expressing cells have a history of *MyoD* expression, we stained for *Pax7* and GFP proteins on cryosections of *MyoDiCre/+; R26NG/+* embryos at E12.5 (not shown) and E13.5 (**Figures 9F–I**). This revealed that not all cells with a history of *MyoD* expression also expressed *Pax7*. However, for head mesoderm-derived muscles, the majority of *Pax7*-positive nuclei were located in cells with *MyoDiCre* driven GFP expression (**Figures 9G–I**). In contrast, in the somite-derived tongue muscle most *Pax7*-positive nuclei were in GFP-negative cells (**Figure 9H**). Taken together, our data support the idea that head mesodermal cells express early *Mrf* and become myogenic before turning on *Pax7*.

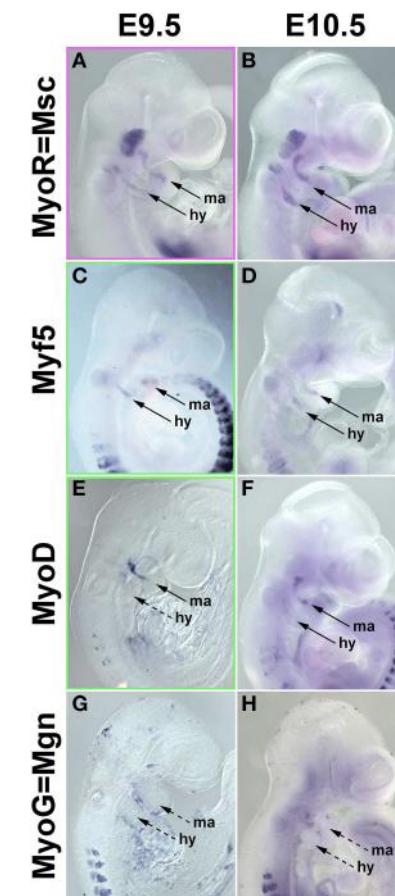


FIGURE 8 | mRNA Expression of mouse head mesoderm markers and markers for myogenic commitment before and at the onset of *Pax7* expression. Lateral views, dorsal to the top. Stages of development are indicated at the top of the panel, gene names on the left. (A,B) *Musculin* expression commences before E9.5 (not shown); at E9.5–10.5, the gene is widely expressed in the myogenic head mesoderm. *Myf5* (C,D) and *MyoD* (E,F) expression commences at E9.5, i.e., before the onset of *Pax7*. (G,H) *Myogenin* expression is not yet detectable at these stages and commences slightly later at E11.5 (not shown).

Emergence of Pax7 Expressing Myogenic Cells in Anamniote Craniofacial Muscles

Our analysis suggested that in amniotes, cells that eventually will populate the head muscle stem cell niche are being deployed

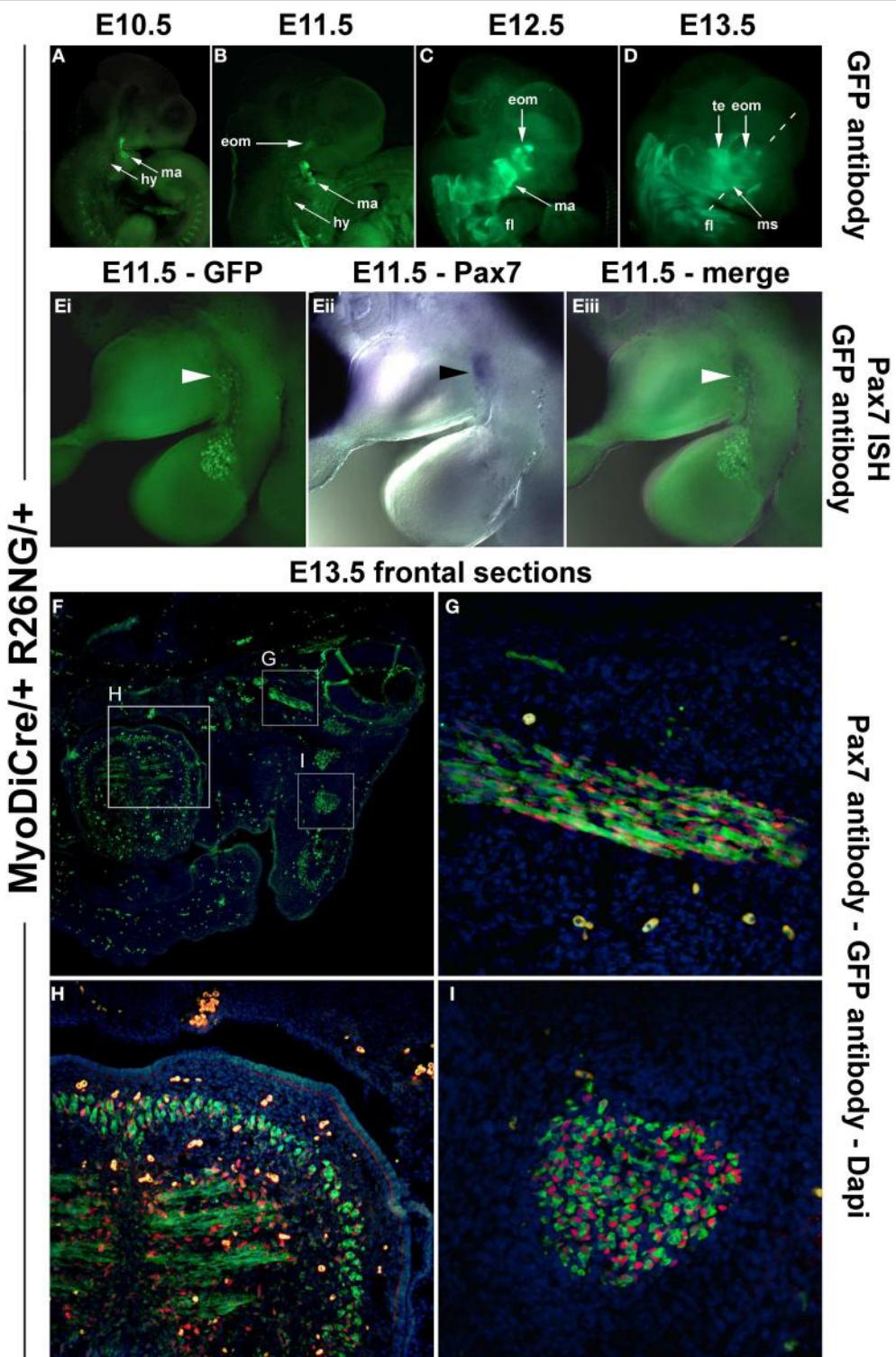


FIGURE 9 | Lineage tracing of *MyoD* expressing cells in *MyoDiCre/+ R26NG* embryos, revealed by anti-GFP antibody (green) staining.

(A–D) Lateral views of the right side of E10.5–E13.5 embryos; the dotted line indicates the sectional plane in (F–I). (Ei–iii) Lateral views of the left side of an E11.5 embryo, stained for *Pax7* mRNA (blue) and GFP protein (green); dorsal to the top. (F) Frontal section of an E13.5 embryo, stained for *Pax7* protein

(red), GFP (green), and Dapi (blue). (G) Detail of the ventral rectus eye muscle, (H) detail of the tongue, (I) detail of the masseter as indicated in (D,F). The widely distributed bright green (F) or yellow cells (G–I) are autofluorescing blood cells. Cells with a history of *MyoD* expression can readily be detected at E10.5 and 11.5, first in the mandibular and hyoid arch,

(Continued)

FIGURE 9 | Continued

then in the developing extraocular muscles. In head-mesoderm-derived muscles, *Pax7* mRNA and subsequent protein expression colocalises with that of MyoD-Cre driven GFP, and *Pax7* containing nuclei reside in

GFP expressing cells. In contrast, in the somite-derived tongue muscle, most *Pax7*-positive nuclei are not located in GFP expressing cells. Abbreviations as in **Figures 1, 4, 5** and: eom, developing extraocular muscles.

after, possibly from, cells committed to skeletal muscle formation. However, in amniotes, overall skeletal myogenesis is delayed compared to anamniotes that rely on functional muscle during larval stages of development. Therefore, we investigate the emergence of *Pax7* positive cells in craniofacial muscles of two anamniote models, the African clawed frog *X. laevis* (a sarcopterygian vertebrate like mouse and chicken) and the teleost fish *D. rerio* (zebrafish, an actinopterygian vertebrate).

Emergence of Pax7 Expressing Myogenic Cells in Craniofacial Muscles of Xenopus laevis

In *X. laevis*, the genome was duplicated upon hybridisation between two ancestral species approximately 65 million years ago, and extant *X. laevis* is considered allotetraploid (Hughes and Hughes, 1993; Evans et al., 2004; reviewed in Evans, 2008). However, when we cloned partial cDNA sequences of the two duplicate *pax7* genes, they had an identity of 87% (data not shown). Moreover, the *pax7a* probe had an identity of 85.5% and the *pax7b* probe of 78.7% with the corresponding sequence of the single *Xenopus tropicalis pax7* gene, this however only shared 55% of nucleotides with its paralog *pax3*. Correspondingly, *in situ* hybridisation of *X. laevis* embryos with the *pax7a* and *b* probes alone, with a mix of both probes or with the *X. tropicalis pax7* probe produced the same expression patterns, and hence only the data for the *X. tropicalis* probe are being shown (**Figure 10**). This analysis revealed expression in the central nervous system, in craniofacial neural crest cells, in the ventral diencephalon and the hypophysis as well as in the somitic dermomyotome, recapitulating the data by Maczkowiak et al. (2010), Daughters et al. (2011), Della Gaspera et al. (2012), Bandin et al. (2013) (**Figures 10A,B** and data not shown). *Pax7* expression in areas of developing head muscle anlagen was detected from stage 39 onwards and became somewhat stronger at stages 40/41 [**Figures 10C–Di**; muscles were identified according to Ziermann and Olsson (2007) and Schmidt et al. (2013)]. However, expression levels remained low compared to other expression domains.

Previous studies have investigated the expression of some mesodermal and myogenic markers in the *Xenopus* embryos (Della Gaspera et al., 2012), but a systematic comparison with *pax7* has not been carried out. We therefore cloned probes for the head mesodermal gene *msc*, for all *mrf* genes and for the muscle structural gene *desmin*. As expected, *msc* labeled the myogenic head mesoderm from early stages onwards (**Figures 11A–Di** and not shown); the exception is the mesoderm of the first arch which however is *pitx2* and *tbx1* positive (Della Gaspera et al., 2012). These markers are followed by the expression of *myf5* and *myod* (**Figures 11E–Hi,I–Li** and not shown). *myf5* and *myod* showed overlapping but non-identical expression patterns, with *myf5* strongly labeling the 1st arch derived intermandibularis muscle anlage (**Figures 11H,Hi, im**) and *myod* the 1st arch derived

levator mandibularis muscle (**Figures 11L,Li, lm**). Yet when *myog* and *desmin* expression commenced in developing head muscles at st36, the markers encompassed all craniofacial muscle anlagen, and they were followed by *mrf4* expression at st39 (**Figures 11M–Pi,Q–Ti,U–Xi**). Thus, as in the two amniote models, frog *pax7* expression in craniofacial muscle anlagen began late, after the commitment of cells to myogenesis and the onset of differentiation.

In order to determine the onset of *pax7* protein expression and to ascertain that expression domains are associated with skeletal muscle, we compared the expression of sarcomeric myosins (MF20 antibody staining, **Figures 12A,C,E,G,I**) and the read-out of the cardiac actin promoter (cardiac actin; GFP frogs; **Figure 12K**) with that of *pax7* protein (**Figures 12B,D,F,H,J,L,M**). To associate expression with anatomical features, a diaminobenzidine staining was performed (**Figures 12A–F**); to better detect signals away from the surface, a fluorescent secondary antibody was used (**Figures 12G–J,L,M**). As a control, we performed an antibody staining at st26, focusing on the somitic expression (**Figures 12A,Ai,B,Bi**); this recapitulated the data by Daughters et al. (2011). Our stainings at st40 revealed *pax7* protein expression in craniofacial muscle anlagen, with expression levels being significantly lower than those of sarcomeric myosins (**Figures 12C–J**).

Emergence of Pax7 Expressing Myogenic Cells in Craniofacial Muscles of the Zebrafish

Chicken, mouse and frog all belong to the lobe-finned/limbed (sarcopterygian) class of osteichthyans, while zebrafish is a teleost that belongs to the ray-finned (actinopterygian) class (Clack, 2002). Thus, zebrafish is the model most distantly related to humans/ mammals. Teleosts have undertaken a 3rd genome duplication 350 million years ago (Postlethwait, 2007), and retained both *pax7* copies (Seo et al., 1998). The coding sequences of these genes are 80.9% identical, and they share 83.6 and 82.6% (*pax7b*) identity with coding sequence of the single *pax7* gene in the spotted gar, a holost fish (data not shown). Yet zebrafish *pax7a* sequences are 63.5/57.3% identical with *pax3a/b* sequences, and *pax7b* sequences are 61.3/56.2% identical with those from *pax3a/b*, respectively. This suggests that *pax7a* and *b* are likely to cross-hybridize with the mRNA of the duplicate gene but not with *pax3a/b* mRNAs. Here, we used a *pax7a* probe as it provides a more robust signal than the *pax7b* probe (Hughes, personal communication). This probe and the *pax7* antibody (see below) had been used earlier (Seo et al., 1998; Hammond et al., 2007) and recapitulated *pax7* expression in the nervous system and somites as displayed in these studies (not shown).

Craniofacial muscle anlagen begin to express *myf5* and *myod* at 24 and 32 h post fertilization (hpf), with *myod* showing a more widespread expression (Lin et al., 2006; Hinitz et al., 2009) and data not shown). *myog* and *mrf4* are readily detectable

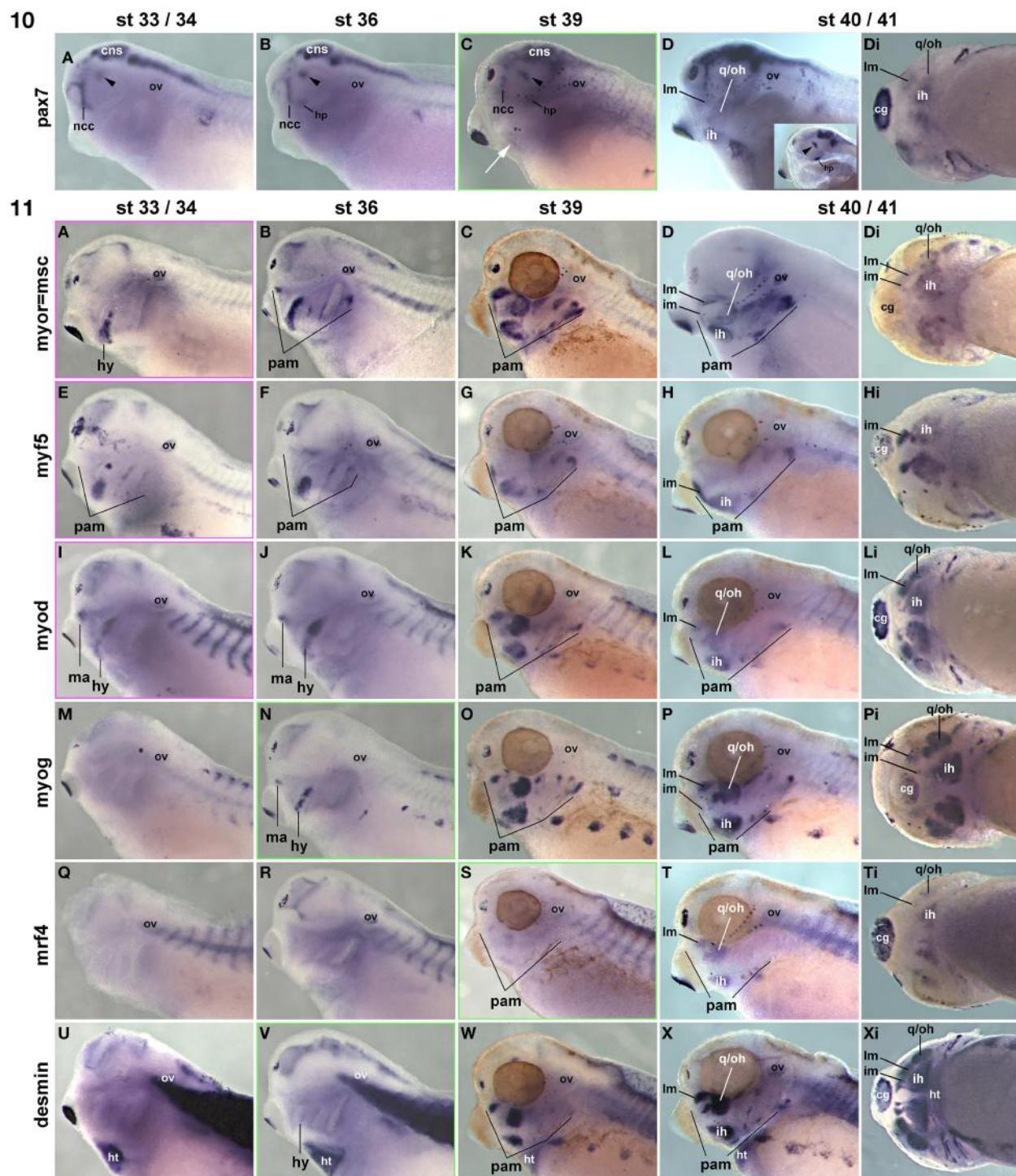


FIGURE 10 | Time course of *pax7* mRNA expression in *Xenopus laevis*. Lateral views, anterior to the left. Embryonic stages are indicated at the top. Inset in (D): pharyngeal arches and head mesenchyme were dissected away from the left side to reveal the brain. Up to stage 36, Pax7 expression is confined to the central nervous system including the ventral diencephalon (arrowhead), the hypophysis (hp), and the frontonasal neural crest cells. Weak expression is also seen in the somites. From stage 39 onwards, weak expression can be detected in craniofacial muscle anlagen. Abbreviations as before and: cg, cement gland; hp, hypophysis; ht, heart; first arch derived muscle: im, m. intermandibularis anlage; lm, m. levatores mandibulae anlage; second arch derived muscle: ih, m. interhyoideus anlage; oh, m. orbitohyoideus anlage; q/h, m. quadrato-hyoangularis anlage; q/oh, common oh and qh precursor.

FIGURE 11 | Time course of head mesoderm and muscle gene expression in *Xenopus laevis*. Same stages, views, and abbreviations as in Figure 11; markers are indicated on the left. Note that *msc*, *myf5*, *myod*, *myog*, and *mrf4* are expressed before, *mrf4* concomitant with the onset of *pax7* expression.

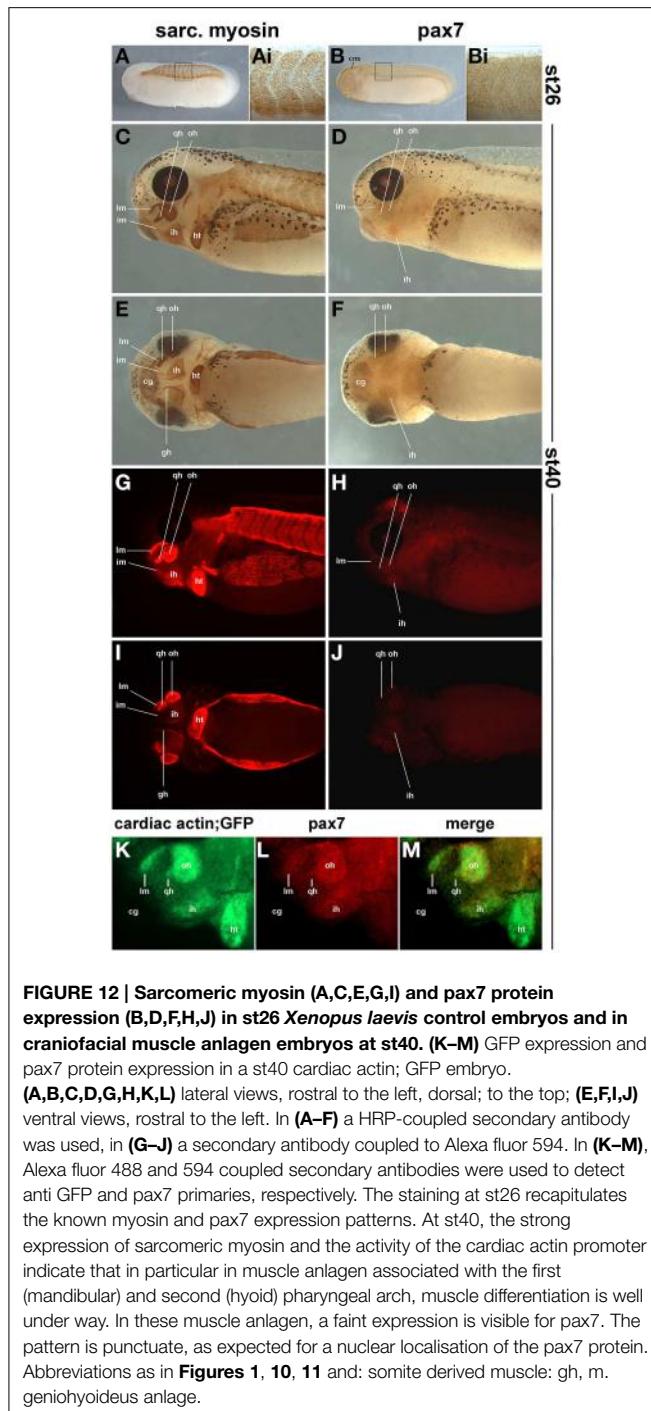


FIGURE 12 | Sarcomeric myosin (A,C,E,G,I) and pax7 protein expression (B,D,F,H,J) in st26 *Xenopus laevis* control embryos and in craniofacial muscle anlagen embryos at st40. (K–M) GFP expression and pax7 protein expression in a st40 cardiac actin; GFP embryo. (A,B,C,D,G,H,K,L) lateral views, rostral to the left, dorsal; to the top; (E,F,I,J) ventral views, rostral to the left. In (A–F) a HRP-coupled secondary antibody was used, in (G–J) a secondary antibody coupled to Alexa fluor 594. In (K–M), Alexa fluor 488 and 594 coupled secondary antibodies were used to detect anti GFP and pax7 primaries, respectively. The staining at st26 recapitulates the known myosin and pax7 expression patterns. At st40, the strong expression of sarcomeric myosin and the activity of the cardiac actin promoter indicate that in particular in muscle anlagen associated with the first (mandibular) and second (hyoid) pharyngeal arch, muscle differentiation is well under way. In these muscle anlagen, a faint expression is visible for pax7. The pattern is punctuate, as expected for a nuclear localisation of the pax7 protein. Abbreviations as in Figures 1, 10, 11 and: somite derived muscle: gh, m. geniohyoideus anlage.

at 48 hpf, and when at 72 hpf the animals rely on their head muscles to ventilate the gills and feed, structural proteins are well established (Schilling and Kimmel, 1997). In contrast, *pax7a* expression in craniofacial muscles was barely detectable at 72 hpf (Figures 13A,B, arrowheads) whereas expression of *myod* mRNA (single copy gene; Figures 13C,D) and of sarcomeric myosins (MF20 antibody staining, Figures 13E,F) was very strong at this stage. Thus, also in teleosts *pax7* expressing future

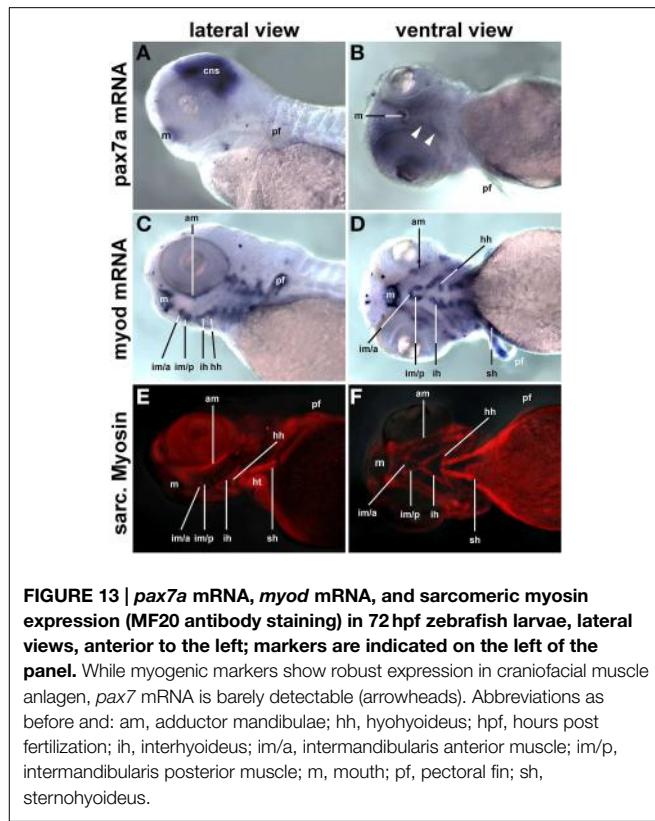


FIGURE 13 | *pax7a* mRNA, *myod* mRNA, and sarcomeric myosin expression (MF20 antibody staining) in 72 hpf zebrafish larvae, lateral views, anterior to the left; markers are indicated on the left of the panel. While myogenic markers show robust expression in craniofacial muscle anlagen, *pax7a* mRNA is barely detectable (arrowheads). Abbreviations as before and: am, adductor mandibulae; hh, hyohyoideus; hpf, hours post fertilization; ih, interhyoideus; im/a, intermandibularis anterior muscle; im/p, intermandibularis posterior muscle; m, mouth; pf, pectoral fin; sh, sternohyoideus.

head muscle stem cells arise late and possibly after the cells committed to myogenesis.

Discussion

The vertebrate head mesoderm is a unique type of mesoderm as it forms both skeletal muscle and cardiac tissue. Specifically, the head mesoderm lining the lateral and ventral aspects of the pharynx retains the ability to contribute to skeletal muscle and the heart for a prolonged period of time, and it contributes the ventral muscles of the pharyngeal arches and the outflow tract of the heart (reviewed in Sambasivan et al., 2011). It is conversely debated whether in amniotes including humans, there are any proliferative cells in the mature heart; it is clear however that in contrast to for example the zebrafish (an anamniote) amniote heart regeneration is currently not possible (reviewed in Garbern and Lee, 2013). Yet, adult head skeletal muscle stem cells, besides expressing the muscle stem cell marker *Pax7*, retain the expression of the early head mesodermal markers (Harel et al., 2009; Sambasivan et al., 2009). These cells, when transplanted into trunk skeletal muscle lose their head-specific expression profile and contribute to trunk muscle regeneration. However, it is appealing to explore whether in the appropriate environment or niche, cells may be able to repair muscle in dystrophies predominantly affecting head muscles, or could be reprogrammed to regenerate the heart. Given that cardiovascular diseases are the predominant cause of death in the Western world (Garbern and Lee, 2013), the latter is of great medical importance.

Prerequisite to exploring the properties and therapeutic potential of head muscle stem cells is an understanding of their developmental biology. However, while some inroads into the unraveling of head skeletal muscle development have been made (reviewed in Sambasivan et al., 2011), timing and mechanisms controlling head muscle stem cell deployment are still largely unknown. Muscle stem cells rely on the expression and function of the *Pax7* gene, and *Pax7* is currently the most reliable marker for muscle stem cells (Seale et al., 2000; Kassar-Duchossoy et al., 2005; Relaix et al., 2006; Lepper et al., 2009; von Maltzahn et al., 2013). In this study, we used a comparative approach in the commonly used vertebrate models for myogenesis, chicken, mouse, *Xenopus* and zebrafish, and established, when and how *Pax7* expressing head muscle stem cells emerge. These models represent both the lobe-finned/ limbed (sarcopterygian) and ray-finned (actinopterygian) class of “bony” (osteichthyan) vertebrates, and any shared characteristics point at evolutionarily conserved, basic mechanisms.

Head Muscle Stem Cells Arise Late in the Development

In vertebrates, the longitudinal body axis is laid down sequentially during gastrulation, proceeding from anterior to posterior (reviewed in Gilbert, 2000). The head therefore is always developmentally advanced, yet head skeletal muscles are known to develop late (Sambasivan et al., 2011). In tune with this delay, we found that *Pax7* expressing head muscle stem cells also develop late. However, this delay is not proportionate: the avian embryo, for example, takes 21 days to develop, the head mesoderm is being laid down within the first day, the final pattern of head mesoderm markers is established within a further day, yet it takes about 1.5 days to *Pax7* mRNA expression and another 12 h for readily detectable protein levels. This discrepancy is even more pronounced in amniotes where *Pax7* levels are low throughout and first detectable around the time of larval hatching. This suggests that the head mesoderm undertakes a series of so far ill-defined steps before head muscle stem cells can be deployed.

Head Muscle Stem Cells Arise after the Onset of *Myf5* and *MyoD* Expression

Using the aid of marker genes, we investigated the processes the head mesoderm is engaged in before the onset of *Pax7*. Notably, in all models examined here, the myogenic head mesoderm expresses *Myf5* and/or *MyoD* (amniotes: co-expression, anamniotes: coexpression in most, but differential expression of *myf5* or *myod* in selected muscle anlagen) before *Pax7*, indicating that the majority of cells have committed to a skeletal muscle fate. Moreover, several markers indicating the onset of myogenic differentiation are also expressed before the onset of *Pax7*. This is particularly evident in amniotes where *myog*, the *mrf* that drives cell cycle exit and entry into terminal differentiation, is expressed before *pax7*; in amniotes, *Pax7* expression begins at a similar time point as *MyoG*. With the exception of *Six1*, trunk pre-myogenic genes are not expressed (e.g., *Pax3*) or not expressed consistently (e.g., *Eya1*). Moreover, head mesoderm genes have been shown to act directly

upstream of *Mrf* (Zacharias et al., 2011; Moncaut et al., 2012; Castellanos et al., 2014). This suggests that in the phase before the onset of *Pax7*, the myogenic head mesoderm proceeds from a precursor state to a state where skeletal muscle formation is initiated, without recruiting the upstream factors controlling trunk myogenesis. This also suggests that in contrast to the trunk, head muscle precursor cells do not go through a phase of *Pax* gene expression before becoming a muscle stem cell.

Head Mesodermal Cells May Require a Defined Muscle Environment to Settle as Muscle Stem Cells

When *Pax7* expression becomes detectable in the head mesoderm, the signal either occupies the same region as the *Mrf* signals or is nested within the *MyoD* expression domain. In turn, these markers overlap with the expression domains of the early head mesodermal genes. This indicates that in contrast to the early somite, the head mesoderm is not compartmentalized, and stem cells and differentiating cells emerge from within the same cell pool. This scenario is akin to the simultaneous renewal of stem cells and production of differentiating cells after -and from- *Pax7* expressing cells that have populated the myotome; the same occurs in the muscle masses of the limbs, and in all muscles during fetal and perinatal stages of development (reviewed in Buckingham and Vincent, 2009). Elegant studies in the mouse showed that both in the head and in the trunk, differentiating muscle displays the membrane-bound ligand Delta which triggers Notch signaling in the neighboring cells. This in turn suppresses *MyoD* expression and maintains the muscle stem cell state of these cells (Mourikis et al., 2012; Czajkowski et al., 2014; reviewed in Mourikis and Tajbakhsh, 2014). However, in the trunk, the initial expression of *Pax7* in the mouse dermomyotome is not controlled by a Notch-Delta lateral inhibition mechanism (Schuster-Gossler et al., 2007; Vasyutina et al., 2007). Similarly, the expression of *Pax7* in the head mesoderm was not Delta-dependent (Czajkowski et al., 2014). Thus, additional parameters have to be considered for the establishment of the head muscle stem cell pool.

Head Mesodermal Cells May Commit to Myogenesis before Becoming Skeletal Muscle Stem Cell

It is commonly held that at least in the amniote somite, all myogenic cells first express *Pax3* and *Pax7*, the *Pax* genes are genetically and molecularly upstream of *MyoD*, and when *MyoD* expression commences, the pre-myogenic genes are downregulated (reviewed in Buckingham and Vincent, 2009). The same observation has been made in satellite cells where, upon asymmetric cell division, the cell set up to activate *MyoD* will switch off *Pax7* and differentiate (Troy et al., 2012). Yet evidence is emerging that the linear progression from a *Pax3/7*+ state to a *MyoD*+ state is not obligatory: Lineage tracing and genetic cell ablations in the mouse have revealed that adult muscle stem cells have a history of *Myf5*, *MyoD* and *Mrf4* expression, indicating

that the expression of Mrfs that control the initial myogenic commitment does not prevent the maintenance of a stem cell state (Kanisicak et al., 2009; Biressi et al., 2013; Sambasivan et al., 2013; Wood et al., 2013). In amniotes, the first cells to form contractile muscle do not express *pax3/7* before undertaking myogenesis (reviewed in Bryson-Richardson and Currie, 2008), and in *Xenopus*, the *pax7* lineage is established in a zone lateral to the somite that also expresses *myoD* (Daughters et al., 2011; Della Gaspera et al., 2012). In the mouse myoblast cell line C2C12, quiescent stem cells arise concomitant with contractile cells when the cells are cultured differentiation promoting medium (Yoshida et al., 1998), and when *MyoD* is misexpressed in P19 embryonic carcinoma cells, the gene activates pre-myogenic rather than myogenic genes, and does so directly (Gianakopoulos et al., 2011). Thus, evidence is accumulating that *MyoD* can act upstream of *Pax7*. Our data showed that in the mouse, cells with current and with a history of *MyoD* expression are situated in the same territory and arise well before the onset of *Pax7*. Importantly, the majority of *Pax7* expressing cells develop from cells that previously expressed *MyoD*, and for the cells that do not display a history of *MyoD* expression, it cannot be excluded that they expressed *Myf5* before. Interestingly, the closest chordate relatives of vertebrates, the ascidians, develop cardiac and pharyngeal muscles from a bi-potential precursor in a similar fashion to vertebrates (Stolfi et al., 2010; Wang et al., 2013). In these animals, the pharyngeal muscle stem cells express the single *mrf* gene before some cells are set aside to become stem cells (Razy-Krajka et al., 2014). Thus, while more detailed lineage tracing will be required to fully elucidate this question; our data suggests that vertebrate head mesodermal cells similarly proceed through a phase of *Mrf* gene expression which sets the stage for the activation of *Pax7* (a model is proposed in Figure 14).

Myogenic Commitment of Head Muscle Stem Cells May Be Obligatory and Reflects the Evolutionary History of the Head Mesoderm

Circulatory pumps equipped with contractile cells—hearts—are widespread in the animal kingdom, and a conserved, core regulatory network involving Nk4/timman-type transcription factors may already have been established in Cnidarians (Shimizu and Fujisawa, 2003). Likewise, skeletal muscle for locomotion, generated with the help of a MyoD-like basic-helix-loop-helix transcription factor, is widespread and may predate the evolution of bilaterians (Muller et al., 2003, but see also Steinmetz et al., 2012). Yet, typically cardiac and skeletal muscle lineages are exclusive. Vertebrates and their closest chordate relatives, the ascidians, have evolved a program that generates cells for the heart as well as skeletal muscle. However, this muscle is not used for locomotion but is associated with the function of the pharynx. The muscularisation of the pharynx has been seen as a key step during vertebrate evolution as it provided the basis for the active ventilation of gills and eventually, the evolution of jaws (Gans and Northcutt, 1983). A central component in this system is the *Tbx1* gene (ascidians: single *Tbx1/10* gene; (Stolfi et al., 2010). In the more distantly related cephalochordate *Branchiostoma*, the *Tbx1/10* gene is expressed in the pharyngeal endoderm and mesoderm as well as the (ventral) somites (Mahadevan et al., 2004), while in the even further distant hemichordate *Saccoglossus kowalevskii* *Tbx1/10* is only found in the pharyngeal endoderm and gill slits (Gillis et al., 2012). This suggests that during evolution of the ascidian-vertebrate ancestor, *Tbx1/10* gene function has been linked both to the cardiac as well as the myogenic regulatory cascades. This implies that in contrast to the somite whose evolutionarily basic function is to generate skeletal muscle for locomotion, bi-potential pharyngeal cells have to commit to a myogenic fate before any muscle and muscle stem cells can be

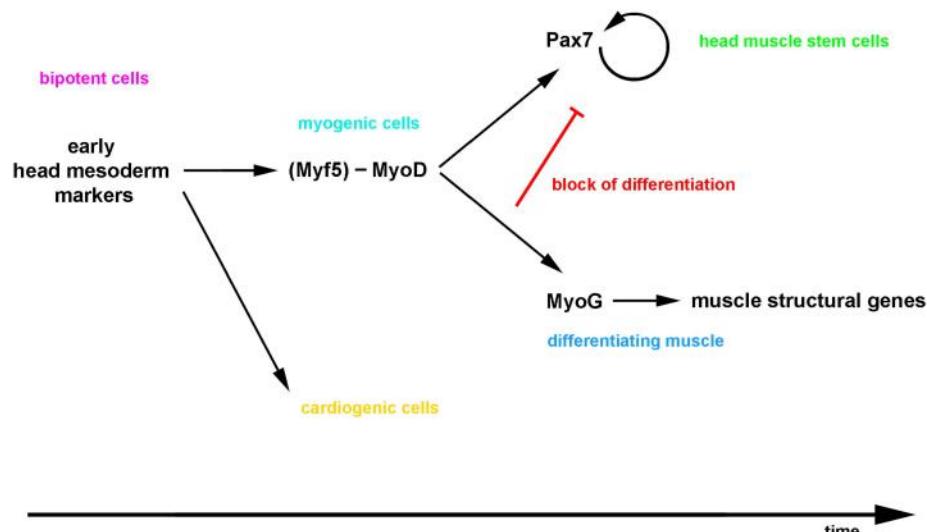


FIGURE 14 | Proposed model for the emergence of craniofacial muscle stem cells: the bi-potential head mesodermal cells commit to myogenesis before adapting a muscle stem cell state, and then a

lateral inhibition mechanism initiated by the differentiating cells controls the simultaneous production of functional muscle and the maintenance of the stem cell pool.

laid down. It is thus conceivable that, in the head, mesodermal cells have to express *Myf5*, *MyoD*, or *Mrf4* before they can be set aside as a muscle stem cell.

Outlook

Our work provides the basis for the—testable—hypothesis that head mesodermal cells have committed to myogenesis, and once this is achieved, cells can faithfully execute standard myogenic programs. In line with this, we have observed that *Pax7*-expressing head muscle stem cells provide the bulk of the head fetal muscles similar to muscle stem cells in the trunk (this study). Moreover, while the early head mesoderm is unable to provide muscle in a somitic environment (Mootoosamy and Dietrich, 2002), head muscle stem cells can shed their head mesodermal marker gene expression and regenerate trunk muscle (Sambasivan et al., 2009). Having established the emergence of head muscle stem cells, we can now explore the underlying molecular mechanisms and test, whether and in which environment head muscle stem cells can be redirected toward an earlier, bi-potential or a cardiogenic state.

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Conditional Cripto overexpression in satellite cells promotes myogenic commitment and enhances early regeneration

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Skeletal muscle regeneration mainly depends on satellite cells, a population of resident muscle stem cells. Despite extensive studies, knowledge of the molecular mechanisms underlying the early events associated with satellite cell activation and myogenic commitment in muscle regeneration remains still incomplete. Cripto is a novel regulator of postnatal skeletal muscle regeneration and a promising target for future therapy. Indeed, Cripto is expressed both in myogenic and inflammatory cells in skeletal muscle after acute injury and it is required in the satellite cell compartment to achieve effective muscle regeneration. A critical requirement to further explore the *in vivo* cellular contribution of Cripto in regulating skeletal muscle regeneration is the possibility to overexpress Cripto in its endogenous configuration and in a cell and time-specific manner. Here we report the generation and the functional characterization of a novel mouse model for conditional expression of Cripto, i.e., the *Tg:DsRed^{loxP/loxP}Cripto-eGFP* mice. Moreover, by using a satellite cell specific Cre-driver line we investigated the biological effect of Cripto overexpression *in vivo*, and provided evidence that overexpression of Cripto in the adult satellite cell compartment promotes myogenic commitment and differentiation, and enhances early regeneration in a mouse model of acute injury.

Keywords: Cripto, transgenic mice, satellite cells, muscle regeneration, myogenic commitment

Introduction

The responses of skeletal muscle tissue following acute or chronic damages are highly complex and coordinated processes, involving many different cell populations that interact each other to promote muscle regeneration, inflammation and angiogenesis, until full regeneration of the tissue and its functional recovery. This process is tightly controlled by signals released by the damaged fibers, which lead to the activation of the quiescent satellite cells, i.e., the myogenic stem cell population that is among the major players responsible for the regeneration of skeletal muscle (Collins et al., 2005). Following an injury, the satellite cells are activated and proliferate as myogenic progenitors that migrate to the damaged site, differentiate and fuse each other to form new myofibers (Hawke and Garry, 2001; Chargé and Rudnicki, 2004). This sequential process is correlated with the finely regulated expression of the Myogenic Regulatory Factors (MRFs). Indeed, quiescent satellite cells express Pax7 and, upon activation, upregulate

the myogenic transcription factor MyoD. Upon commitment to differentiation these transient amplifying cells ($\text{Pax}7^+/\text{MyoD}^+$) downregulate Pax7 and upregulate differentiation genes, as Myogenin and MRF4, while a subset of these cells downregulate MyoD retaining Pax7 expression to replenish the pool of quiescent satellite cells (Zammit et al., 2004; Olgun et al., 2007; Tajbakhsh, 2009). Beside satellite cells, regeneration process is also orchestrated by the crosstalk between heterogeneous cell populations that are recruited/activated after damage, such as the inflammatory cells that are always associated with tissue regeneration, supporting myogenic progression by interacting with satellite cells (Kharraz et al., 2013; Saclier et al., 2013). Besides extensive studies, knowledge of the mechanisms underlying this highly orchestrated process and how the different cell populations establish their fate is still far to be fully elucidated, and much less is known on the extrinsic regulation of this process (Bentzinger et al., 2010). We have recently demonstrated that Cripto, a key regulator of early vertebrate embryogenesis (Shen and Schier, 2000; Minchiotti et al., 2001), is a new player in this process (Guardiola et al., 2012). Cripto is a GPI-anchored protein (Minchiotti et al., 2001) and a developmental factor required for correct orientation of the anterior-posterior axis in the vertebrate embryos (Chu et al., 2005; Minchiotti, 2005). Despite its well-characterized role in embryo development and embryonic stem cell differentiation (Minchiotti, 2005), the role of Cripto in adult life has been poorly investigated also due to the fact that *Cripto* knockout mice are embryonic lethal (Ding et al., 1998; Xu et al., 1999), and that its expression is almost absent in adult physiological conditions. Indeed, Cripto expression is undetectable in skeletal muscles under baseline conditions. However, it becomes rapidly and transiently re-expressed after acute injury, both in myogenic and inflammatory cells, and it is required in the myogenic compartment to achieve an efficient regeneration (Guardiola et al., 2012). Interestingly, a soluble form of the protein (sCripto) is able to rescue the effect of genetic inactivation of *Cripto*, thus recapitulating the function of endogenous membrane form of the protein (Guardiola et al., 2012). Yet, the cellular contribution of Cripto in skeletal muscle repair remains to be further clarified, and its knowledge is limited also by the absence of mouse models for conditional and tissue-specific Cripto expression. Here we report the generation and characterization of novel transgenic mice for conditional expression of *Cripto* in its endogenous configuration, which allowed us to study the biological effect of satellite cell-specific *Cripto* overexpression on muscle regeneration and myogenic cell fate determination.

Results

Generation of Conditional Cripto Gain of Function Transgenic Mice

To get insight into the cellular contribution of Cripto in skeletal muscle regeneration, and to finely modulate Cripto expression *in vivo*, we generated a novel transgenic mouse line for conditional *Cripto* expression based on the *Cre-loxP* strategy. To generate the pDsRed $^{loxP/loxP}$ *Cripto* targeting

vector, a *Cripto*-IRES-eGFP cassette was inserted downstream of the *DsRed* gene sequence followed by three termination sequences, and flanked by two *LoxP* sites (see Materials and Methods for details; **Figure 1A**). The effectiveness of the pDsRed $^{loxP/loxP}$ *Cripto* vector was first evaluated *in vitro*. To this end, HEK-293T cells were transfected with the pCMV-Cre, expressing the Cre recombinase, and the pDsRed $^{loxP/loxP}$ *Cripto* plasmids, either alone or in combination, and Cripto protein expression was evaluated. We first verified that eGFP expression was induced in cells cotransfected with pDsRed $^{loxP/loxP}$ *Cripto* and pCMV-Cre (Figure S1A). Accordingly, Cripto protein was specifically induced (**Figure 1B**) and, as expected, it localized at the cell membrane (Minchiotti et al., 2000) of eGFP expressing cells (Figure S1B). Following the *in vitro* validation of the targeting vector, transgenic mice were generated by pronuclear injection, and the presence of the transgene in the offspring was assessed by PCR genotyping of tail biopsies (**Figures 1C,D**). One out of three transgenic mice obtained gave germline transmission and carried two copies of the transgene that segregated independently in the offspring (**Figure 1E**). Two founder lines were thus established and bred to FVB/N *wild type* mice to generate the *Tg:DsRed* $^{loxP/loxP}$ *Cripto-eGFP*^(A) and *Tg:DsRed* $^{loxP/loxP}$ *Cripto-eGFP*^(B) colonies (from now onwards named *Tg:Cripto*^(A) and *Tg:Cripto*^(B), respectively).

Functional Characterization of the Tg:Cripto Transgenic Lines

Different studies have shown that significative differences exist in the expression level of the same transgene between individual founder siblings, due to different integration loci, and the influence of the genomic sequences flanking the integration site (Palmeter et al., 1982; Overbeek et al., 1986). To characterize *Tg:Cripto*^(A) and *Tg:Cripto*^(B) transgenic lines, we first assessed DsRed expression in freshly isolated muscles by direct fluorescence and found a stronger DsRed signal in *Tg:Cripto*^(A) compared to *Tg:Cripto*^(B) muscles (**Figure 1F**). We thus evaluated whether Cripto was expressed upon *Cre*-mediated recombination *in vivo*. To this end, TA muscles of both transgenic lines were injected with Adeno Associated Virus 9 (Inagaki et al., 2006) encoding Cre recombinase (AAV-Cre) or empty vector (AAV-Cntl) as control. Ten days after infection, TA muscles were explanted and genotyped by PCR. As expected, a 838-bp band characterizing the recombining allele was specifically detected in the AAV-Cre injected muscles (**Figure 1G**), and Cripto protein was induced at different levels as determined by ELISA-based assay (2.36 ± 0.06 ng/mg in *Tg:Cripto*^(A) vs. 0.43 ± 0.09 ng/mg in *Tg:Cripto*^(B); $**P = 0.005$) (**Figure 1H**).

All together these data demonstrate that Cripto expression is regulated upon *Cre*-mediated recombination and is induced at different levels in the two transgenic lines.

Time -Dependent Effect Of Cripto Overexpression in Adult Satellite Cells on Skeletal Muscle Regeneration

We have recently shown that adenovirus-mediated soluble Cripto (sCripto) overexpression accelerates muscle

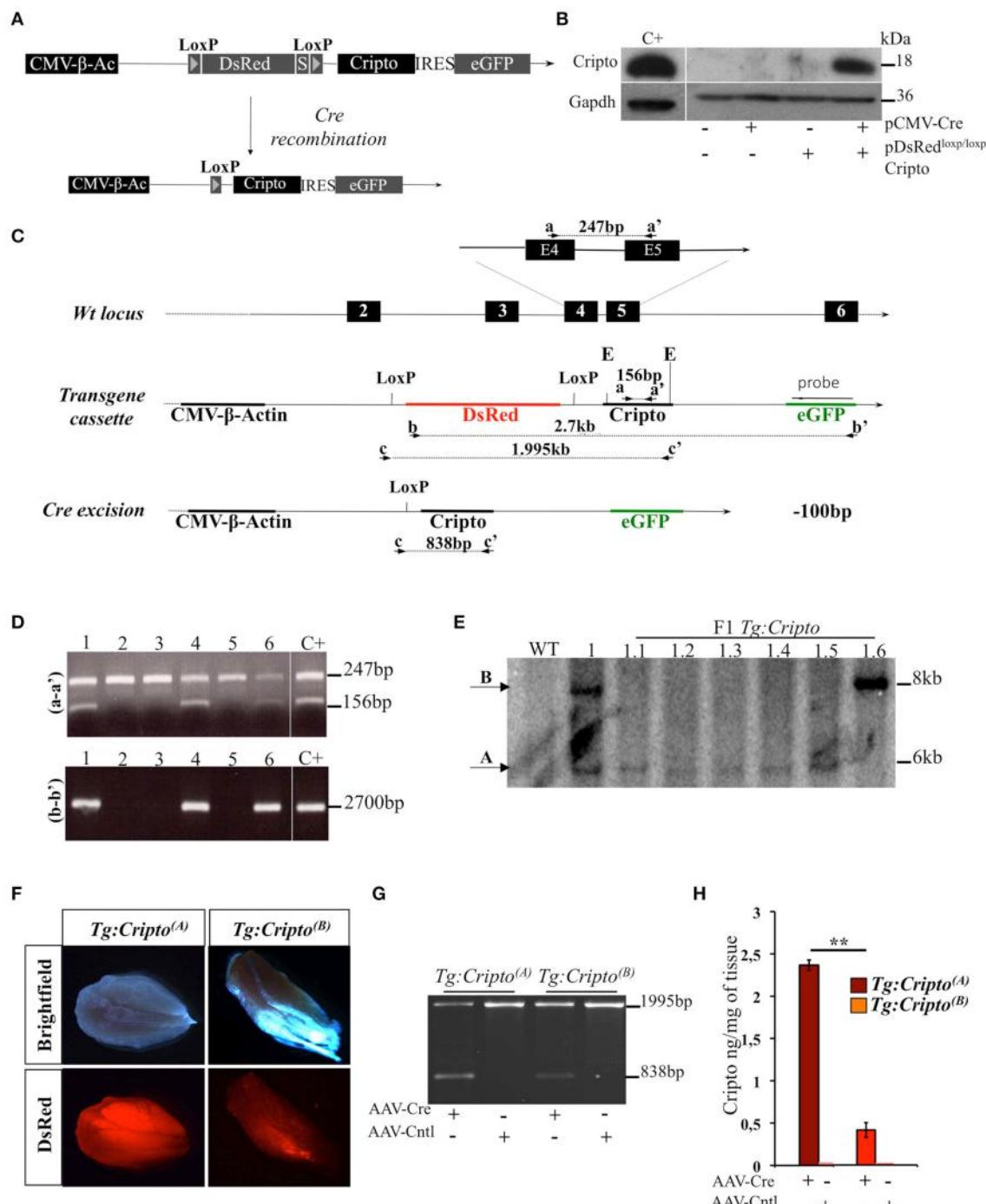


FIGURE 1 | (A) Schematic representation of the pDsRed^{loxP/loxP}Cripto targeting vector. The nuclear DsRed gene flanked by two loxP sites and followed by three termination sequences (S) is transcribed by a constitutive CMV-β-Actin (CMV-β-Ac) promoter. Upon cre- mediated recombination, the DsRed sequence is excised and the Cripto-IRES-eGFP cassette is transcribed from the CMV-β-actin promoter. **(B)** Western blot analysis using anti-Cripto antibody on total protein extracts of HEK-293T cells transfected with pDsRed^{loxP/loxP}Cripto vector either alone or in combination with pCMV-Cre vector. Gapdh was used as a loading control. Protein extracts from undifferentiated mouse embryonic stem cells (ESCs) were used as

positive control (C+). **(C)** Schematic representation of the genotyping strategy. Location of PCR primers (black arrowheads) and the probe (black bar) used for genotyping are indicated. Numbered boxes represent wild-type exons (black). E, EcoRI. **(D)** Representative PCR screening on tail genomic DNA from mice generated after oocyte injection. Primers a-a' (top panel) amplified a 247-bp fragment of the wild-type Cripto locus and a 156-bp fragment of the Cripto transgene. Primers b-b' (bottom panel) amplified a 2700-bp fragment spanning the DsRed-IRES-Cripto cassette. **(E)** Genotyping by Southern blot analysis. Genomic DNA from wild type (WT) and F1 Tg:Cripto mice (1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6) was analyzed. Probes B and A detect bands at approximately 8 kb and 6 kb, respectively. **(F)** Fluorescence microscopy images of Tg:Cripto(A) and Tg:Cripto(B) mice. Top row: Brightfield images. Bottom row: DsRed fluorescence images. Labels indicate the genotypes. **(G)** RT-PCR analysis of Cripto mRNA levels in Tg:Cripto(A) and Tg:Cripto(B) mice. Lanes are grouped by genotype (Tg:Cripto(A) and Tg:Cripto(B)) and individual mice. AAV-Cre and AAV-Cntl treatments are indicated. Molecular weight markers (1995 bp and 838 bp) are shown on the right. **(H)** Quantitative RT-PCR analysis of Cripto mRNA levels. The graph shows Cripto ng/mg of tissue for Tg:Cripto(A) and Tg:Cripto(B) mice under different AAV-Cre and AAV-Cntl treatments. ** indicates statistical significance.

(Continued)

FIGURE 1 | Continued

(WT), founder (1) and F1 offspring mice (from 1.1 to 1.6) was digested with EcoRI and hybridized with eGFP probe shown in **(C)**. The sizes of hybridized fragments are indicated in kilobases. **(F)** Representative pictures of direct fluorescence of freshly isolated skeletal muscle from *Tg:Cripto^(A)* and *Tg:Cripto^(B)* mice, showing different levels of DsRed expression. **(G)** PCR screening of biopsy from *Tg:Cripto^(A)* and

Tg:Cripto^(B) TA muscles infected with either AAV-Cre or AAV-Control (AAV-Cntl). Primers c-c' **(C)** amplified a 1995-bp fragment of the transgenic allele and a 838-bp fragment of the recombining allele. **(H)** ELISA assay of Cripto protein levels expressed as ng/mg of tissue in *Tg:Cripto^(A)* and *Tg:Cripto^(B)* muscles infected with AAV-Cre and AAV-Cntl (2.36 ± 0.06 ng/mg in *Tg:Cripto^(A)* vs. 0.43 ± 0.09 ng/mg in *Tg:Cripto^(B)*; $^{**}P \leq 0.005$).

regeneration induced by acute injury (Guardiola et al., 2012). Nevertheless, viral mediated Cripto expression does not allow us to discriminate between the inflammatory and satellite cell contribution of Cripto overexpression. To overcome this limitation, we crossed *Tg:Cripto^(A)* and *Tg:Cripto^(B)* mice with the tamoxifen-inducible *Tg:Pax7CreER^{T2}* mice (Mourikis et al., 2012) and obtained the *Tg:Pax7CreER^{T2}::DsRed^{loxP/loxP}Cripto-eGFP^(A)* and *Tg:Pax7CreER^{T2}::DsRed^{loxP/loxP}Cripto-eGFP^(B)* transgenic lines (from now onwards named *Tg:Pax7CT2::Cripto^(A)* and *Tg:Pax7CT2::Cripto^(B)*) (**Figure 2A**). We first assessed the effects of satellite cell-specific Cripto overexpression on skeletal muscle regeneration. To this end, *Tg:Pax7CT2::Cripto^(A)* and *Tg:Pax7CT2::Cripto^(B)* adult mice and their control littermates were treated with tamoxifen once a day for 5 days; at day 4, muscle regeneration was triggered in TA muscles by local injection of cardiotoxin (CTX; **Figure 2A**). Genetic recombination was first confirmed by PCR analysis on TA muscle genomic DNA (**Figure 2B**, Figure S2A), and Cripto protein levels were quantified by ELISA assay on total protein extracts at different time points after injury. Increased Cripto protein levels were detected in both *Tg:Pax7CT2::Cripto^(A)* and *Tg:Pax7CT2::Cripto^(B)* mice compared to control, with *Tg:Pax7CT2::Cripto^(A)* showing the highest levels of Cripto upon Cre-mediated recombination (**Figure 2C**, Figure S2B). To assess if Cripto overexpression might influence muscle regeneration, we first evaluated the expression of the embryonic Myosin Heavy Chain (eMyHC), which is a marker of newly regenerated fibers, at day 8 after injury. Both immunofluorescence and Western blot analysis clearly showed increased eMyHC protein levels in *Tg:Pax7CT2::Cripto^(A)* mice compared to their control littermates (**Figures 2D,E**). In line with these findings, expression of both neonatal Myosin Heavy Chain (nMyHC) and the early muscle differentiation marker Myogenin (Myog) similarly increased in the overexpressing mice (**Figures 2F,G**). Furthermore, expression of Myostatin (Mstn), which is a negative regulator of muscle growth (Thomas et al., 2000), was significantly reduced in *Tg:Pax7CT2::Cripto^(A)* mice compared to control (**Figure 2H**). Of note, similar results were obtained from the analysis of the other transgenic line (*Tg:Pax7CT2::Cripto^(B)*; Figures S2C–F). Morphological analysis of H&E-stained TA muscle sections at day 8 showed that the number of myofibers containing more than one nucleus ($n > 1$) significantly increased in Cripto overexpressing mice compared to control (0.12 ± 0.02 for *Tg:Pax7CT2::Cripto^(A)* vs. 0.06 ± 0.01 for *Tg:Cripto^(A)* of fibers; $^{*}P \leq 0.05$; **Figures 3A,B**). Interestingly, while there was no significant difference in Cross Sectional Area (CSA) between the two groups at day 8 (**Figures 3A,C**), later on

(i.e., at day 15) both CSA distribution and the relative average values significantly increased in the *Tg:Pax7CT2::Cripto^(A)* mice compared to control (**Figures 3A,D,E**). To assess if the positive effect of Cripto overexpression on muscle regeneration was either transient or persistent, we extended the analysis to a later time point (i.e., at day 30). Morphometric analysis of the CSA showed no difference in the distribution of muscle fibers in the two groups at day 30 (**Figures 3A,F**); thus providing evidence of a time-dependent effect of satellite cell -Cripto overexpression on skeletal muscle regeneration. Most remarkably, similar experiments carried on the *Tg:Pax7CT2::Cripto^(B)* mice gave comparable results (Figures S3A–F), thus ruling out the possibility of a position-effect of the transgene.

Conditional Overexpression of Cripto in Satellite Cells Enhances Myogenic Differentiation

To determine how Cripto overexpression in the satellite cells impact on skeletal muscle regeneration, we first evaluated its effect on early stages of myoblast differentiation *in vitro*. To address this issue, we determined the fusion index (i.e., the percentage of the MF20⁺ cells with $n \geq 2$ nuclei) of the postnatal myogenic progenitor cells (MPCs) isolated from hindlimb muscles of new-born *Tg:Pax7CT2::Cripto^(A)* mice and their *Tg:Cripto^(A)* control littermates at postnatal (P) day 7. Cre-mediated recombination was induced by tamoxifen injection (**Figure 4A**) and assessed by PCR genotyping of muscle tissues (**Figure 4B**). We then verified that Cripto protein was induced in *Tg:Pax7CT2::Cripto^(A)* by ELISA assay on postnatal MPCs ($70.8 \pm 14 * 10^{-2}$ pg/μg for *Tg:Pax7CT2::Cripto^(A)* vs. $32.1 \pm 2.6 * 10^{-2}$ pg/μg for *Tg:Cripto^(A)*; $^{*}P \leq 0.05$; **Figure 4C**). Cells were maintained in culture for 24 h in proliferation medium and then shifted to differentiation medium (DM) for 24 and 72 h (**Figure 4D**). Quantitative analysis showed that the percentage of MF20⁺ cells with $n \geq 2$ nuclei progressively increased in cultures from *Tg:Pax7CT2::Cripto^(A)* mice compared to control, at both time points ($29.03 \pm 1.79\%$ for *Tg:Pax7CT2::Cripto^(A)* vs. $18.4 \pm 2.75\%$ for *Tg:Cripto^(A)* at 24 h; $35.85 \pm 2.24\%$ for *Tg:Pax7CT2::Cripto^(A)* vs. $26.87 \pm 1.08\%$ for *Tg:Cripto^(A)* at 72 h; $^{*}P \leq 0.05$; **Figure 4E**), thus suggesting that Cripto overexpression enhanced *in vitro* myoblasts to myotubes differentiation. In line with these findings, the percentage of Pax⁻/MyoD⁺ cells significantly increased in *Tg:Pax7CT2::Cripto^(A)* mice compared to control ($9.92 \pm 1.88\%$ Pax⁻/MyoD⁺ for *Tg:Pax7CT2::Cripto^(A)* vs. $3 \pm 1.62\%$ Pax⁻/MyoD⁺ for *Tg:Cripto^(A)*; $^{*}P \leq 0.05$; Figures S4A,B). Interestingly, these results are in line with data previously reported on isolated myofibers in culture in which addition of sCripto increases the number of Pax7⁻/MyoD⁺ myogenic

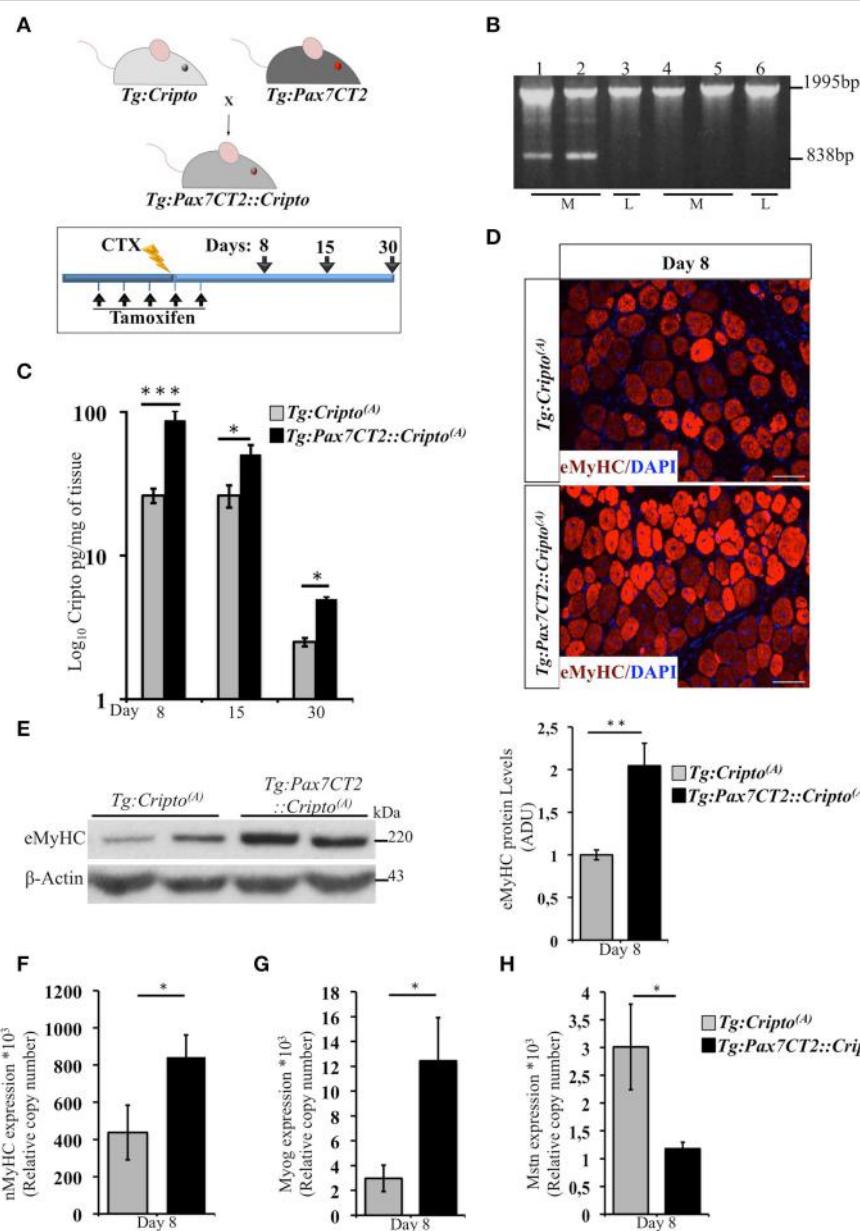


FIGURE 2 | (A) Schematic representation of mouse breeding (upper panel) and *Cripto* conditional gain of function strategy in adult satellite cells (lower panel). Tamoxifen were injected i.p., in adult (5 weeks old)

Tg:Pax7CT2::Cripto^(A) mice and *Tg:Cripto^(A)* control littermates once a day for 5 days. At day 4, regeneration was triggered by CTX injection in TA muscle of both groups, and analysis performed at the indicated time points (days 8, 15, and 30). The consecutive bars indicate the days. **(B)** PCR screening on muscle genomic DNA showing tamoxifen-induced recombination only in the muscle of *Tg:Pax7CT2::Cripto^(A)*. Primers c-c' (See Figure 1C) amplified a 1995-bp fragment of the transgenic allele and a 838-bp fragment of the recombinant allele. The recombinant allele is detected in the muscle (M; lanes 1, 2) but not in the liver (L; lane 3) of *Tg:Pax7CT2::Cripto^(A)* mice, and is not detected in the muscle (M; lanes 4, 5) and in the liver (L; lane 6) of *Tg:Cripto^(A)* control littermates.

(C) ELISA assay of Cripto protein levels in TA muscles at different time points after CTX injection expressed as pg/mg of muscle tissue at the indicated time points. Values are mean \pm SEM of $n = 5$ mice/group. * $P \leq 0.05$; ** $P = 0.0005$.

CTX injection expressed as pg/mg of muscle tissue at the indicated time points. Values are mean \pm SEM of $n = 5$ mice/group. * $P \leq 0.05$; ** $P = 0.0005$. **(D)** Representative pictures of embryonic Myosin Heavy Chain (eMyHC) Immunofluorescence on TA sections from *Tg:Pax7CT2::Cripto^(A)* mice and *Tg:Cripto^(A)* control littermates at day 8. Scale bars = 100 μ m. **(E)** Representative Western blot using anti-eMyHC antibody on total protein extracts of TA muscles from *Tg:Pax7CT2::Cripto^(A)* mice and their *Tg:Cripto^(A)* control littermates at day 8 after CTX. β -Actin was used as a loading control. The densitometric analysis is expressed as Arbitrary Densitometric Unit (ADU) expressing the eMyHC/ β -Actin ratio. Values are mean \pm SEM of $n = 3$ mice/group. ** $P = 0.005$. **(F–H)** qRT-PCR analysis of neonatal Myosin Heavy Chain (nMyHC) (**F**) Myogenin (Myog) (**G**) and Myostatin (Mstn) (**H**) on TA muscle total RNAs from *Tg:Pax7CT2::Cripto^(A)* mice and the *Tg:Cripto^(A)* control littermates at day 8 after CTX injection. Values are mean \pm SEM, 5 mice/group. * $P \leq 0.05$.

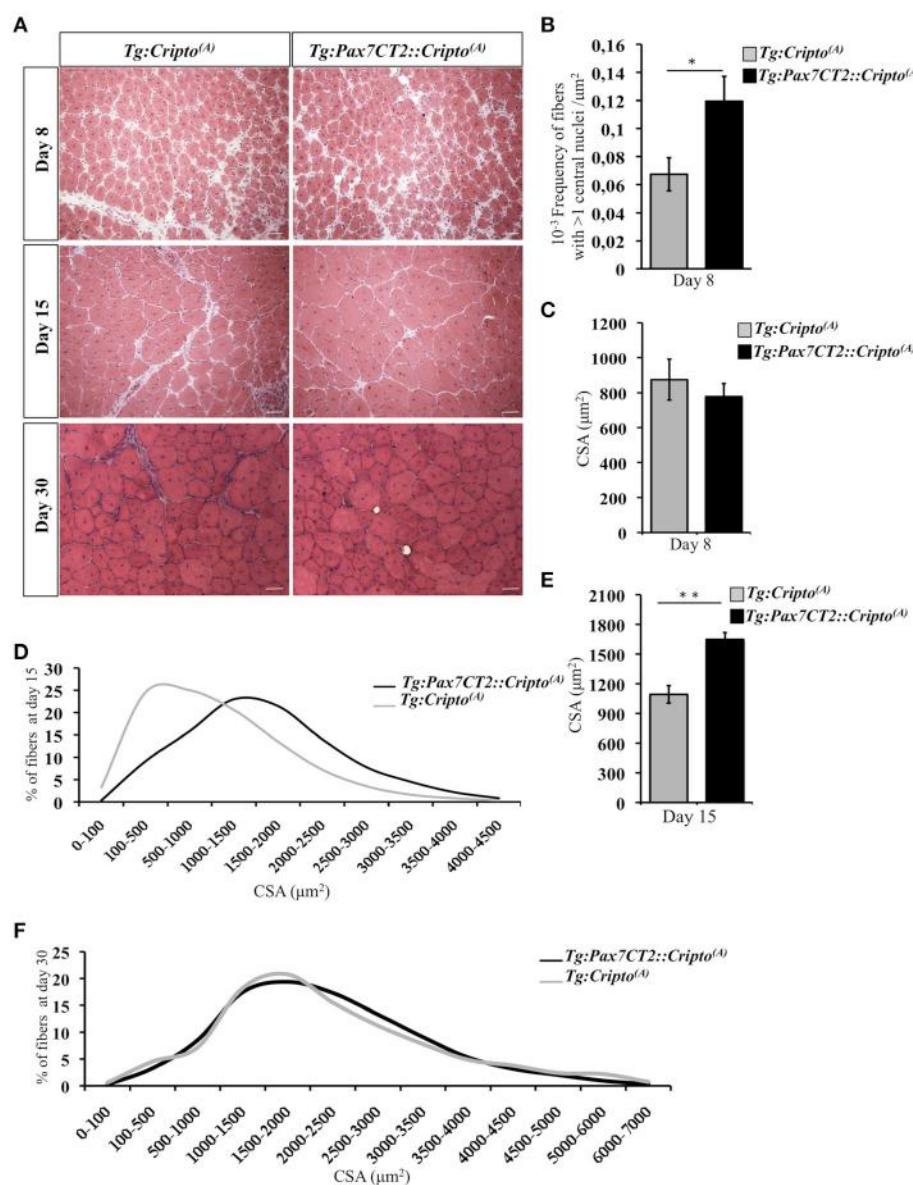


FIGURE 3 | (A) Representative H&E staining of TA muscle sections from *Tg:Pax7CT2::Cripto^(A)* and *Tg:Cripto^(A)* TA muscles at the indicated time points. Scale bars = 50 μm . **(B)** Morphological analysis on muscle sections at day 8 after injury showing the number of myofibers containing more than one central nuclei/area in each group. Values are mean \pm SEM, 5 mice/group *P \leq 0.05. **(C)** Average of centrally nucleated myofibers size values in TA muscle sections at day 8 after

injury. Values are mean \pm SEM, 5 mice/group. **(D)** Myofiber Cross Sectional Area distribution at 15 days after CTX injection; 5 mice/group. **(E)** Average of centrally nucleated myofibers size values in TA muscle sections at day 15 after injury of *Tg:Pax7CT2::Cripto^(A)* mice and *Tg:Cripto^(A)* control littermates. Values are mean \pm SEM, 5 mice/group. **P \leq 0.005. **(F)** Myofiber Cross Sectional Area distribution at 30 days after CTX injection; 4 mice/group.

cell population, promoting satellite cell progression into the myogenic lineage (Guardiola et al., 2012). We thus went on and extended the analysis *in vivo* to assess the effects of Cripto overexpression on the distribution of the satellite cell population during muscle regeneration. To this end, TA muscle sections of *Tg:Pax7CT2::Cripto^(A)* mice and their control littermates at day 8 after injury (see Figure 2A) were double stained with anti-Pax7 and anti-MyoD antibodies (Figure 4F) and the frequency of (i) quiescent satellite cells (Pax7⁺/MyoD⁻),

(ii) transient amplifying cells (Pax7⁺/MyoD⁺), and (iii) cells committed to myogenic differentiation (Pax7⁻/MyoD⁺) was evaluated. Remarkably, the frequency of Pax7⁻/MyoD⁺ cells significantly increased in *Tg:Pax7CT2::Cripto^(A)* mice compared to control (0.32 \pm 0.03 for *Tg:Pax7CT2::Cripto^(A)* vs. 0.16 \pm 0.04 for control of Pax7⁻/MyoD⁺ cells; *P \leq 0.05; Figures 4F,G), at the expense of Pax7⁺/MyoD⁺, which conversely decreased in *Tg:Pax7CT2::Cripto^(A)* mice (0.13 \pm 0.04 for *Tg:Pax7CT2::Cripto^(A)* vs. 0.3 \pm 0.05 for control

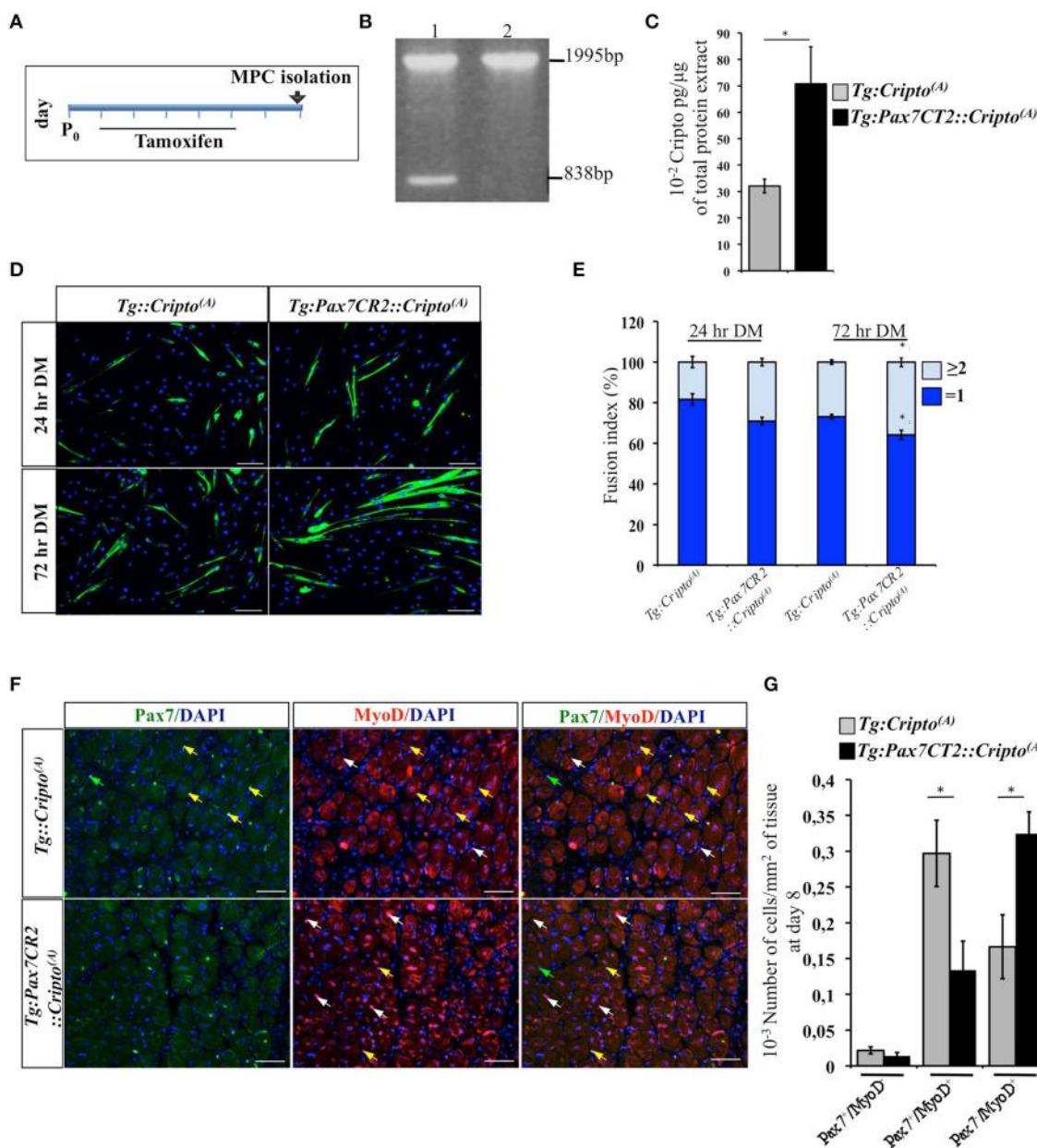


FIGURE 4 | (A) Schematic representation of experimental strategy for myogenic progenitor cells (MPCs) isolation. Tamoxifen was injected once a day for 5 days (from P1 to P5) in new-born *Tg:Pax7CT2::Cripto^(A)* mice and their control littermates, and MPCs were isolated at P7. **(B)** PCR analysis on muscle genomic DNA showing tamoxifen-induced recombination in *Tg:Pax7CT2::Cripto^(A)* mice. Primers c-c' (Figure 1C) amplified a 1995-bp fragment of the transgenic allele and a 838-bp fragment of the recombinant allele. **(C)** ELISA assay of Cripto protein levels in primary myoblasts from *Tg:Pax7CT2::Cripto^(A)* and control mice expressed as pg/μg of total protein extracts. Values are mean ± SEM of $n = 3$. *P ≤ 0.05. **(D)** Representative pictures of *Tg:Pax7CT2::Cripto^(A)* and *Tg:Cripto^(A)* myotubes after 24 and 72 h in differentiation medium (DM) and stained with MF20 antibody. Scale bars = 100 μm. **(E)** Fusion index was calculated as the percentage of MF20⁺ cells with $n = 2$ nuclei at 24 and 72 h in DM. The analysis was performed on 20 independent and random chosen microscope fields. Values are mean ± SEM of 3 independent experiments. *P = 0.05. **(F)** Representative pictures of double immunofluorescence with antibodies anti-Pax7 (green) and anti-MyoD (red) in TA muscles at day 8 after injury. Nuclei were counterstained with DAPI. Arrowheads indicate Pax7⁺/MyoD⁻ (green), Pax7⁺/MyoD⁺ (yellow) and Pax7⁻/MyoD⁺ (white) cells. Scale bars = 100 μm. **(G)** Effect of satellite cells-Cripto overexpression on Pax7[±]/MyoD[±] cell distribution *in vivo*. The number Pax7[±]/MyoD[±]/area from *Tg:Pax7CT2::Cripto^(A)* mice and the *Tg:Cripto^(A)* control littermates was calculated in TA muscles and reported in the chart. Values are mean ± SEM, 5 mice/group. *P ≤ 0.05.

of Pax7⁺/MyoD⁺ cells; *P ≤ 0.05; Figures 4E,G); thus providing *in vivo* evidence that adult satellite cell specific Cripto overexpression accelerated myogenic lineage progression.

bars = 100 μm. (E) Fusion index was calculated as the percentage of MF20⁺ cells with $n = 2$ nuclei at 24 and 72 h in DM. The analysis was performed on 20 independent and random chosen microscope fields. Values are mean ± SEM of 3 independent experiments. *P = 0.05. (F) Representative pictures of double immunofluorescence with antibodies anti-Pax7 (green) and anti-MyoD (red) in TA muscles at day 8 after injury. Nuclei were counterstained with DAPI. Arrowheads indicate Pax7⁺/MyoD⁻ (green), Pax7⁺/MyoD⁺ (yellow) and Pax7⁻/MyoD⁺ (white) cells. Scale bars = 100 μm. (G) Effect of satellite cells-Cripto overexpression on Pax7[±]/MyoD[±] cell distribution *in vivo*. The number Pax7[±]/MyoD[±]/area from *Tg:Pax7CT2::Cripto^(A)* mice and the *Tg:Cripto^(A)* control littermates was calculated in TA muscles and reported in the chart. Values are mean ± SEM, 5 mice/group. *P ≤ 0.05.

Taken together our results provide the first *in vivo* evidence that Cripto overexpression in myogenic compartment accelerates the early stages of

regeneration process promoting satellite cell myogenic commitment.

Discussion

Growing evidence link adult tissue repair to reactivation of developmental gene program; nevertheless, our knowledge on the role of key regulators of early vertebrate embryogenesis in tissue regeneration is still limited also by the lack of adequate *in vivo* genetic tools. Indeed, transcription levels and timing of expression of key developmental genes are finely tuned, and experimental strategies to generate constitutive gain-of-function and loss-of-function mouse mutants often result in embryonic lethality. In this scenario, we have focused our attention on the developmental gene Cripto, which is a key regulator of vertebrate embryogenesis. Here we report the generation and functional characterization of a conditional *Cripto* gain-of-function mouse model *Tg:DsRed^{loxP/loxP}Cripto-eGFP* as a complementary strategy to investigate the effects of Cripto in postnatal and adult life, and potentially in all cell types. Specifically, by crossing *Tg:DsRed^{loxP/loxP}Cripto-eGFP* mice with a *Tg:Pax7CreER^{T2}* mouse line, we have investigated the effect of satellite cell- Cripto overexpression on skeletal muscle regeneration and overcome the limitation of the viral-mediated approach (Guardiola et al., 2012). Consistent with our previous data on isolated myofibers in culture, we provide *in vivo* evidence that satellite cell overexpression of membrane Cripto promotes myogenic commitment and differentiation. However, unlike the viral-mediated overexpression of soluble Cripto (sCripto; Guardiola et al., 2012), the positive effect of satellite cell Cripto overexpression on skeletal muscle regeneration is time-dependent, being restricted to the early phases of regeneration. It is unlikely that the discrepancy in the two approaches is due to protein configuration, i.e., membrane vs. soluble Cripto; indeed, sCripto is able to rescue muscle regeneration defects caused by the genetic ablation of *Cripto* in the satellite cells, indicating that it can recapitulate the role of the endogenous protein (Guardiola et al., 2012). Yet, it is important to consider that Cripto is expressed both in myogenic and inflammatory cells during regeneration. Thus, the different timing of regeneration in the two models might be explained by the fact that while the genetic overexpression is restricted to the satellite cells, the viral-mediated sCripto overexpression has a broader effect and likely affects both the myogenic and the inflammatory cells. Further studies will be necessary to test this hypothesis by deeply investigating the specific contribution of Cripto in inflammatory cells, as well as the autocrine and/or paracrine effects of the endogenous protein.

The positive effect of Cripto overexpression on skeletal muscle regeneration indicated by the increased CSA at day 15 is the expected consequence of its earliest effect on the satellite cell compartment. First, the number of centrally located nuclei is higher in Cripto overexpressing myofibers at day 8 after injury; thus suggesting accelerated differentiation. Accordingly, the myoblast fusion index is increased in postnatal MPCs isolated from Cripto overexpressing mice. Finally, an in depth analysis of *Pax7[±]/MyoD[±]* cell distribution shows that the number

of *Pax7⁻/MyoD⁺* cells committed to differentiation increases both *in vivo* and *in vitro* upon Cripto overexpression. We thus conclude that not only the addition of sCripto protein but also its overexpression in a more physiological context, promotes/accelerates satellite cells commitment to differentiation both *in vitro* and *in vivo*. Taken together our data provide evidence that a timely overexpression of Cripto in the satellite cells promotes myogenic commitment and fusion/differentiation and transiently accelerates regeneration after acute injury. It will be interesting to investigate in future studies the effect of sustained Cripto overexpression on satellite cell self renewal and regeneration after repeated muscle injury.

Finally, in line with the idea that early regeneration is enhanced upon Cripto overexpression, we found that the expression of Myostatin, a member of the TGF β superfamily which inhibits myoblasts differentiation and acts as a negative regulator of skeletal muscle growth (Amthor et al., 2002; Langley et al., 2002) is downregulated in Cripto overexpressing mice. Interestingly, this Cripto/Myostatin inverse correlation is consistent with previous findings on isolated myofibers in culture showing that sCripto antagonizes Myostatin leading to increased number of myoblast population committed to differentiation (*Pax7⁻/MyoD⁺*) (Guardiola et al., 2012).

In conclusion, all together these findings broaden our knowledge on the role of Cripto in skeletal muscle regeneration, and add novel insights into its activity in the satellite cell compartment. We believe that these novel mouse models, which also offer the unique opportunity to study the effect of Cripto in the regulation of satellite cell quiescence, activation and self-renewal *in vivo*, will be instrumental to get fundamental insights into the role of Cripto in skeletal muscle homeostasis and regeneration, as an important step toward the therapeutic use of this molecule.

Materials and Methods

Mice and Genotyping

To generate the pDsRed^{loxP/loxP}Cripto targeting vector, a mouse *Cripto* cDNA (750 bp) (Minichiotti et al., 2001) -IRES-eGFP cassette was cloned downstream of the *Discosoma* sp. red fluorescent protein (DsRed) gene into the pGapDsRed^{loxP/loxP} vector, containing the CMV enhancer/chicken beta-actin promoter (CMV/ β -Actin) and two loxP sites flanking the DsRed gene upstream of triple STOP-polyA sequences (kindly provided by Dr. Silvia Brunelli). Briefly, a Xho-AflII 2.2 kb DNA fragment spanning the Cripto-IRES-eGFP cassette was excised from the pCripto-IRES-eGFP plasmid (kindly provided by Dr. Giovanna L. Liguori), blunt ended and cloned into the backbone of the pGapDsRed^{loxP/loxP} vector previously linearized with EcoRI and filled in with Klenow Fragment of DNA polymerase I (New England Biolabs). Proper orientation of the inserted Cripto-IRES-eGFP DNA fragment was verified by restriction digestion and DNA sequencing. To generate the transgenic mice, a SalI DNA fragment spanning the entire CMV/ β -Actin/DsRed^{loxP/loxP}CriptoIRES-eGFP transcription unit released from the pDsRed^{loxP/loxP}Cripto targeting vector was gel purified and injected into fertilized

oocytes of FVB/N mice, in collaboration with the Core Facility of Conditional Mutagenesis at San Raffaele Hospital, Milan, Italy. The resulting mice were genotyped by PCR-based screening using primers (a-a') spanning the *Cripto* gene exon4-exon5 junction for the amplification of both *Cripto* wt (247 bp) and transgenic (156 bp) alleles. The integrity of the transgene was further evaluated by PCR using primers (b-b') annealing on the DsRed and eGFP sequences (2700 bp). Three out of sixty mice analyzed (5%) were positive for the transgene and only one of them (mouse 1) gave germline transmission of the transgene. Two copies of the transgene, which segregate independently, were identified in the genome of the founder mouse by Southern blot analysis. After germline transmission, heterozygous *Tg:DsRed^{loxP/loxP}Cripto-eGFP^(A)* and *Tg:DsRed^{loxP/loxP}Cripto-eGFP^(B)* mice colonies were maintained by crossing with wild-type FVB mice and named *Tg:Cripto^(A)* and *Tg:Cripto^(B)*, respectively.

For Southern Blot analysis, 20 µg of genomic DNA was prepared from tail biopsies, digested with EcoRI and blotted on Immobilon-Ny+ (Millipore). The ³²P-labeled probe was a PCR amplified DNA fragment spanning the eGFP insert (546 bp) from the pDsRed^{loxP/loxP}Cripto vector. The heterozygous *Tg:Pax7CreER^{T2}::DsRed^{loxP/loxP}Cripto-eGFP^{(A)/(B)}* mice were generated by crossing *Tg:Pax7CreER^{T2}* (Mourikis et al., 2012) with *Tg:Cripto^{(A)/(B)}* animals; the offsprings of these crosses were genotyped by double PCR analysis to identify the *Tg:Cripto* (213 bp) and the *Tg:Pax7CreER^{T2}* (600 bp) alleles (DsRedCripto for/rev and Pax7Cre for/rev primers). The DNA was prepared from tail biopsy samples of breeding animals. To induce genetic recombination, *Tg:Pax7CreER^{T2}::Cripto* and *Tg:Cripto* adult mice (5 weeks old) were injected intraperitoneally (i.p.) with tamoxifen (60 µg/g of body weight per day, Sigma-Aldrich) once a day for 5 days. The genetic recombination was verified by PCR analysis (primers c-c') to distinguish the transgenic (1995 bp) from the recombinant (838 bp) allele. The DNA was obtained from tibialis anterior (TA) muscle biopsy samples. The primer sequences for PCR screening, genotyping and genetic recombination analysis are reported in Table S1.

Cell Culture and Transfection

HEK-293T cells were grown in Dulbecco Modified Eagle Medium (DMEM, Gibco) supplemented with 10% Fetal Bovine Serum (FBS, Euroclone) and 1% penicillin-streptomycin (EuroClone), and plated on six-well plates at a density of 2×10^5 cells/cm². Twenty-four hours after plating, cells were transfected with 4 µg of DNA (pDsRed^{loxP/loxP}Cripto ± pCMV-Cre recombinase expression vector), using Lipofectamin Transfection Reagent (Life Technologies) following manufacturer's instructions. Forty-eight hours after transfection, cells were fixed for immunofluorescence analysis or visualized by fluorescence microscopy (DMI600; Leica), and images were acquired on an Orca R2 camera (Hamamatsu).

Muscle Injections, Preparation, and Analysis

Tibialis Anterior (TA) muscles of adult *Tg:Cripto^{(A)/(B)}* transgenic mice were injected with either *Cre recombinase*-encoding Adeno Associated Virus (AAV-Cre) or the empty

vector (AAV-Cntl) at 1×10^{12} genome copies/ml in 10 µl. Muscles were collected after 10 days and total protein extracts were prepared for further analysis. The AAV vectors were prepared by the AAV Vector Unit at ICGEB Trieste (<http://icgeb.org/avu-core-facility.html>) by packaging AAV2 vector genome backbone into AAV capsid serotype 9, as previously described (Arsic et al., 2003). To induce muscle damage, 10 µl of cardiotoxin (CTX, 70 µM in PBS, Sigma-Aldrich) were injected in the TA muscles of adult (5 weeks old) *Tg:Pax7CreER^{T2}::Cripto* and *Tg:Cripto* mice. For morphometric analysis, muscles were harvested at the indicated time points after damage, and frozen in isopentane-cooled liquid nitrogen for cryosection. Myofiber Cross Sectional Area (CSA) was analyzed on haematoxylin/eosin (H&E) stained muscle sections using ImageJ software (freely available software developed at the National Institutes of Health, Bethesda, Maryland, USA). The number of myofibers having more than one nucleus on total area was evaluated, as previously described (Mittal et al., 2010).

All experiments were conducted in strict accordance with the institutional guidelines for animal research and approved by the Department of Public Health, Animal Health, Nutrition and Food Safety of the Italian Ministry of Health in accordance with the law on animal experimentation.

Western Blot

Western blot analysis was performed as previously described (Parisi et al., 2003) on total protein extracts prepared from either cells or TA muscles samples. TA muscles were homogenized with TissueLyserII (Qiagen) following manufacturer's instructions. These primary antibodies were used: Cripto (1:500, R&D System), eMyHC (1:100, Developmental Studies Hybridoma Bank), Gapdh (1:40.000, Abcam) and β-Actin (1:5000, Sigma-Aldrich).

Isolation of Mouse Satellite Cells and in Vitro Differentiation

Tg:Pax7CreER^{T2}::Cripto and *Tg:Cripto* new-born mice were injected i.p. with tamoxifen (10 µM, 5 µl) every day, from day 1 to day 5 after birth (P1–P5). Preparation of MPCs from new-born mice (P7) was performed as previously described (Rando and Blau, 1994). Briefly the hindlimb and forelimb were removed and separated from the bones. Muscles were mechanically minced with surgical scissors and dissociated by enzymatic digestion [600 U/ml Collagenase Type IV (Gibco) and 1.8 U/ml Dispase II (Roche) in DMEM (Gibco)] at 37°C for 2 h in the shaker. Digested muscles were spun at 500 rpm for 5' and satellite cells were spun down from supernatant (1500 rpm for 10'). Cells were plated on gelatin-coated dishes in proliferation medium [1% Pen/Strep (EuroClone), 1% Glutamin (Euroclone), 20% FBS (Euroclone), 2% UltraSerum (LifeScience) in DMEM (Gibco),] and myoblast population was enriched by several pre-plating steps (Richler and Yaffe, 1970). For Pax7/MyoD cell counting, MPCs were cytospinned at a density of 50.000 cells/spot after 24 h in proliferating medium. For myogenic differentiation, myoblasts were plated in 48 wells at a density of 2×10^4 cells/cm² in proliferation medium. After twenty-four hours, proliferation medium was replaced with differentiation medium for 24–72 h

[DMEM (Gibco) containing 2% (v/v) horse serum (Gibco) and 1% pen/strep (EuroClone)] and the fusion index was determined as the ratio of the number of nuclei in myotubes (MF20⁺ cells with $n \geq 2$ nuclei) on the total nuclei (MF20⁺ cells).

Immunofluorescence Analysis

Cells were fixed for 10' with 4% (wt/vol) paraformaldehyde (PFA) in Phosphate Buffer Saline (PBS) and blocked with 1% bovin serum albumin (BSA) in PBS. Primary antibodies used were: Cripto (Parisi et al., 2003), MF20 (1:50, Developmental Studies Hybridoma Bank), Pax7 (1:10, Developmental Studies Hybridoma Bank) and MyoD (1:50, SantaCruz). Appropriate fluorophore-conjugated secondary antibodies, Alexa Fluor 594 or 488 (1:600, Molecular Probes), biotin-conjugated goat anti-mouse (Jackson), and Cy3- (Jackson) or 488- (Molecular Probes) conjugated streptavidin were used for the visualization using DMI600 or DM600 (Leica) fluorescence microscopes.

Muscles were freshly frozen and cut in cryostat sections. Slides were fixed in 4% (wt/vol) PFA and processed as previously described (Nicolas et al., 2005). Briefly, muscle sections were permeabilized in ice-cold methanol for 6' at -20°C, and boiled 15 min in 10 mM sodium citrate pH 6. Unbinding sites were blocked with 4% IgG-free BSA (Jackson) in PBS for 2–3 h at RT followed by a second blocking step with AffiniPure Fab Fragment goat anti-mouse IgG (Jackson). Primary antibodies were as follows: eMyHC (1:50, F1.652-s, Developmental Studies Hybridoma Bank), Pax7 (1:10, Developmental Studies Hybridoma Bank) and MyoD (1:50, SantaCruz). Appropriate fluorophore-conjugated secondary antibodies, Alexa Fluor 488 or 594 (1:600, Molecular Probes), biotin-conjugated goat anti-mouse (Jackson) and Cy3- (Jackson) or 488- (Molecular Probes) conjugated streptavidin were used for visualization. Nuclei were counterstained with DAPI (1:100 in PBS, VinciBiochem) and FluoSave Reagent (Calbiochem) was used for mounting. Labeling was visualized by epifluorescent illumination using a DM600 microscope (Leica), and images were acquired on a DFC360-FX camera (Leica).

ELISA-based Assay

ELISA-based assay was performed as previously described (Guardiola et al., 2012). Briefly ninety-six well plates were coated with 0.5 µg/ml of sheep anti-mouse Cripto Ab (R&D System, AF1538) in PBS overnight (O.N.) at 4°C. Unbinding sites were blocked with PBS-BSA 1% for 2 h at RT. After washing, protein extracts (300 µg) were added and plates were incubated O.N. at 4°C. The plates were incubated with 1 µg/ml of mouse Cripto biotinylated Ab (R&D System, BAF 1538) in PBET (0.1% BSA, 5 mM EDTA, 0.004% Tween-20 in PBS 1X pH 7,5) for 1 h at 37°C, and then for 1 h at RT. Finally, the plates were incubated for 1 h at RT with avidin/streptavidin complex conjugated with horse-radish peroxidase (Vectastain elite ABC kit, Vector Laboratories). The signals were visualized with *o*-phenylenediamineperoxidase substrate (OPD, Sigma-Aldrich)

and the relative absorbance was read at 490 nm on a Benchmark microplate reader (Bio-Rad Laboratories).

RNA Preparation and qRT-PCR

TA muscles were homogenized with TissueLyserII (Qiagen) in TriReagent (Life Technologies) and total RNA was isolated according to the manufacturer's instructions. One microgram of total RNA was utilized for cDNA synthesis using SuperScript II Reverse Transcriptase and random hexamers (Qiagen). qRT-PCR was performed using SYBR Green PCR master mix (EuroClone). Primers are listed in Table S2.

Statistical Analysis

All values are expressed as mean ± Standard Error of the Mean (SEM). To determine significance between two groups, comparisons were made using unpaired Student *t*-tests. **P* ≤ 0.05, ***P* ≤ 0.005, and ****P* ≤ 0.0005 were considered statistically significant.

Author Contributions

CP, design of the work, data acquisition and analysis, data interpretation. SI, data acquisition and analysis, data interpretation. GA, LZ, FI, data acquisition and analysis. OG, conception and design of the work, data analysis, data interpretation and draft of the article. GM, conception and design of the work, data interpretation, writing and revising of the article. All the authors have read and approved the final manuscript.

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Supplementary Material

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fcell.2015.00031/abstract>

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Chromatin signaling in muscle stem cells: interpreting the regenerative microenvironment

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Muscle regeneration in the adult occurs in response to damage at expenses of a population of adult stem cells, the satellite cells. Upon injury, either physical or genetic, signals released within the satellite cell niche lead to the commitment, expansion and differentiation of the pool of muscle progenitors to repair damaged muscle. To achieve this goal satellite cells undergo a dramatic transcriptional reprogramming to coordinately activate and repress specific subset of genes. Although the epigenetics of muscle regeneration has been extensively discussed, less emphasis has been put on how extra-cellular cues are translated into the specific chromatin reorganization necessary for progression through the myogenic program. In this review we will focus on how satellite cells sense the regenerative microenvironment in physiological and pathological circumstances, paying particular attention to the mechanism through which the external stimuli are transduced to the nucleus to modulate chromatin structure and gene expression. We will discuss the pathways involved and how alterations in this chromatin signaling may contribute to satellite cells dysfunction during aging and disease.

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Muscle Regeneration

Adult muscle is a very stable tissue, with very few fibers being replaced during the normal life of the organism. However, it has a remarkable capacity to regenerate in response to tissue damage. Upon injury, either physical or genetic, changes in the injured microenvironment (i.e., necrosis of damaged fibers, recruitment of the inflammatory infiltrate and release of cytokines and growth factors) lead to the activation, expansion and differentiation of a population of muscle-resident stem cells called satellite cells (Chargé and Rudnicki, 2004). Satellite cells were originally named after their anatomical position beneath the basal lamina of muscle fibers and are characterized by the expression of the transcription factor Pax7 (Mauro, 1961; Cornelison and Wold, 1997; Seale et al., 2000; Chargé and Rudnicki, 2004). Upon activation, satellite cells start to proliferate and up-regulate the early Muscle Regulatory Factors (MRFs) Myf5 and MyoD. After several rounds of cell divisions cells down-regulate Pax7 and induce the expression of the late MRFs (Myogenin and MRF4) and of the cell cycle inhibitor p21. The differentiation program then culminates with the expression of structural and contractile proteins and the fusion of differentiating muscle cells to repair the damaged fibers. Not all activated satellite cells complete the differentiation program and after asymmetric division a small subset of Pax7-positive/MyoD-negative progenitors exit the cell cycle and re-enter quiescence to replenish the satellite cell pool (Chargé and Rudnicki, 2004; Wang et al., 2014).

To achieve their goal satellite cells need to undergo a complex remodeling of chromatin that temporally activates and represses discrete transcriptional programs. Such remodeling is determined by changes in the composition of the satellite cell niche that are transmitted to the nucleus through several cytoplasmic cascades. Here we will discuss how such cascades signal to the chromatin of satellite cells to define the transcriptional response that allows them to proceed through the myogenic program.

At the molecular level, the signaling pathways and transcription factors orchestrating muscle regeneration have been extensively studied. Briefly, myogenesis is controlled by the sequential action of lineage determination markers (i.e., Pax3/Pax7) and early and late MRFs, that act together with Mef2 and Six proteins to recruit chromatin modifying complexes and regulate muscle gene expression. Reprogramming of satellite cells nucleus entails the coordinated activation and repression of discrete subset of genes to progress through the myogenic program. For instance, committed myoblasts keep myogenic identity but repress late muscle genes while continuing to proliferate. Finally, cells switch-off lineage-determination genes and genes necessary for cell cycle progression and activate late muscle-differentiation markers (reviewed in Palacios and Puri, 2006; Guasconi and Puri, 2009; Segalés et al., 2014). Recently, the introduction of high throughput genome-wide studies has been fundamental to provide a deeper insight into the transcriptional and epigenetic programs that modulates myogenesis. By combining ChIP-seq to gene expression analysis, the group of Tapscott showed that MyoD binds to thousands of non-canonical sites both in myoblasts and myotubes, which coincides with local histone hyperacetylation but not necessarily with gene activation. Upon induction of differentiation MyoD-binding to a preferred E-box motif (CAGGTG) located within an accessible chromatin context leads to muscle-specific gene expression (Cao et al., 2010; Fong et al., 2012). Therefore, both genetic (the specific E-box sequence) and epigenetic (local chromatin structure) determinants are fundamental for the activation of the correct differentiation program. Recently, Asp et al. provided an exhaustive analysis of the differentiation-associated epigenetic changes in muscle cells (Asp et al., 2011) and identified novel muscle-specific enhancers associated to MyoD binding (Blum et al., 2012; Blum and Dynlacht, 2013). To add complexity to this scenario, the lab of Sartorelli showed that some muscle-specific enhancers are actually transcribed in what have been called enhancer RNAs (eRNAs), which function as novel epigenetic regulators of gene expression (Mousavi et al., 2013). It is therefore becoming increasingly clear that modulating the epigenome is an essential mechanism that allows muscle cells to proceed through the differentiation program.

Before getting in depth into the mechanisms by which signaling molecules act to modulate the epigenome of satellite cells, it is important to highlight some of the key aspect of epigenetics and its role in regulating gene expression during cellular differentiation.

Epigenetics: From the DNA Sequence to Differential Gene Expression

Historically introduced by Conrad Waddington in 1942, the term epigenetics was coined to explain why specialized cells in a multi-cellular organism exert different functions despite having the same genetic material. This is accomplished by the addition of epigenetic modifications, covalent modifications of the DNA and associated proteins that determine the activation and repression of discrete transcriptional programs during development, cellular differentiation and disease (reviewed in Bernstein et al., 2007; Bergman and Cedar, 2013).

The first layer of epigenetic regulation is the covalent modification of DNA. Mammalian genomes are globally methylated (Eckhardt et al., 2006; Bergman and Cedar, 2013). In eukaryotes, the majority of DNA methylation occurs on cytosines that precede a guanine nucleotide (CpGs). CpG dinucleotides are not evenly distributed through the genome. Ninety eight percent of the dinucleotides are located in regions of low-CpG density, are usually methylated through the genome and may be demethylated in a tissue-specific fashion (Bergman and Cedar, 2013). The remaining two percent of CpGs cluster in regions termed CpG islands (Bird et al., 1985; Gardiner-Garden and Frommer, 1987), stretches of 500–1000 base pairs of DNA that have a higher CpG density than the rest of the genome and are usually kept free of methylation in all tissues (Bergman and Cedar, 2013). Unmethylated CpG islands are associated to DNA sequences more accessible to transcription factor binding, resulting in enhancement of gene expression (Tazi and Bird, 1990; Ramirez-Carrozzi et al., 2009; Choi, 2010). On the contrary, when CpG islands are methylated gene expression under their control is silenced. This is achieved through the binding of Methyl-CpG-binding proteins (MBD; Nan et al., 1993; Hendrich and Bird, 1998; Hendrich et al., 2001), which recognize 5-methyl cytosine (5mC) and recruit repressor complexes leading to changes in chromatin structure (Nan et al., 1998; Ng et al., 1999; Wade, 2005). Aberrations in the DNA methylation pattern are a common feature of many cancers and have been also observed in aging tissues (Feinberg and Tycko, 2004; Bergman and Cedar, 2013).

DNA methylation is catalyzed by DNA methyltransferases (Dnmts). Of them, Dnmt1 is responsible for maintaining methylation after DNA replication whereas Dnmt3a and Dnmt3b catalyze *de novo* DNA methylation (Goll and Bestor, 2005). It has been recently demonstrated that DNA methylation is reversible thanks to the action of the family of ten-eleven translocation methylcytosine dioxygenases (Tet). Tet proteins modify 5mC to generate 5-hydroxymethyl-cytosine (5hmC; Kohli and Zhang, 2013; Piccolo and Fisher, 2014). 5hmC can be then subjected to subsequent chemical modifications producing different intermediates that are recognized and cleaved off by TDG (thymine DNA glycosylase) and replaced with an unmethylated cytosine (He et al., 2011). The conversion of 5mC to 5hmC impairs the binding of the repressive methyl-binding protein such as MeCP2 and plays an important role in regulating gene expression (Valinluck et al., 2004).

The DNA methylation pattern influences and is also influenced by histone modifications, the second layer of epigenetic modifications. In eukaryotic cells the DNA is packed together with histones and other proteins to fit into the limited space of the nucleus, in a structure called chromatin. The basic unit of the chromatin is the nucleosome that is made up of 147 base pairs of DNA wrapped around an octamer of histone proteins called “core”, which contains two copies of each histone, (H2A, H2B, H3, H4) (Luger et al., 1997). Each histone has an N-terminal tail that protrudes from the nucleosome core and is target of different types of covalent modifications such as acetylation, methylation, o-glycosylation and ubiquitylation. Post-translational modifications of histones tails (and bodies) regulate chromatin structure and accessibility (Bannister and Kouzarides, 2011; Tessarz and Kouzarides, 2014). Acetylation of several lysine residues is usually associated with gene activation and is catalyzed by histone acetyltransferases (HATs) such as p300 and PCAF, while it is erased by histone deacetylases (HDACs; Kouzarides, 1999). Differently from histone acetylation, methylation occurs on both lysines and arginines and can either activate and repress gene expression (Ng et al., 2009). Amongst the histone methyltransferases (HMTs), particular interest has been put on the enzymes responsible for H3K27 and H3K9 di- and tri-methylation as these modifications are considered key regulators of gene repression. The first lysine methyltransferase identified was Suv39h1 (KMT1A) that targets H3K9. H3K9 me2/3 mark is recognized by HP1 proteins, which recruit Suv39h1 to the chromatin to spread the repressive mark (Lachner et al., 2001; Bannister and Kouzarides, 2011). Ezh2, the catalytic subunit of the Polycomb Repressive Complex 2 (PRC2), tri-methylates K27 on H3 to keep genes in a repressed state (Margueron and Reinberg, 2011; Riising et al., 2014). H3K27me3 creates a docking site for PRC1, which ubiquitylates H2A, leading to Dnmt recruitment and to a more compact chromatin structure (Sparmann and van Lohuizen, 2006). However, this view has been challenged recently and two studies have shown PRC1-mediated H2AK119ub is sufficient to trigger PRC2 recruitment (Blackledge et al., 2014; Cooper et al., 2014). Histone methylation is reversible and histone demethylases for most of the modified residues have been described (Klose and Zhang, 2007; Ng et al., 2009).

Based on the different combinations of modifications, chromatin can either activate (i.e., H3K4me3, H3K9Ac) or repress (H3K9me2/3, H3K27me3) gene expression. Further, in some cases it can prime associated genes for future regulation (Schneider et al., 2004; Azuara et al., 2006; Bannister and Kouzarides, 2011; Margueron and Reinberg, 2011). For example, in undifferentiated embryonic stem (ES) cells, developmental regulatory genes contain bivalent domains that are characterized by the co-existence of opposing modification in terms of activation (H3K4me3) and repression (H3K27me3). This allows keeping “marked” genes poised in a silenced state for being rapidly activated or repressed upon differentiation process (Azuara et al., 2006; Voigt et al., 2013).

DNA and histone modifications are therefore essential components of an epigenetic program that ultimately modulates chromatin accessibility to transcription factors and chromatin

remodelers and needs to be tightly regulated. For instance, it has been previously shown that hyperacetylated chromatin is preferentially remodeled by chromatin remodeling complexes, whereas hypoacetylation leads to a more compact structure. Chromatin remodeling is accomplished by different families of proteins that use the energy derived from ATP to disrupt or modify nucleosome positioning (Saha et al., 2006). The best-studied chromatin remodelers are the SWI/SNF and ISWI complexes (Deuring et al., 2000; Martens and Winston, 2002). Both families contain a conserved ATPase subunit and a set of additional, conserved core members. SWI/SNF ATPases Brg1 and Brm contain one or multiple bromo-domains that bind acetylated histone tails (Winston and Allis, 1999; Kasten et al., 2004) while ISWI has two domains (SANT and SLIDE) that allow the recognition of the histone tails and the DNA linker (Grüne et al., 2003). In addition, SWI/SNF and ISWI complexes contain structural subunits that contribute to differential gene-specific targeting.

In summary, the high dynamicity and plasticity of the chromatin, which can go from permissive to refractory structures and *vice-versa*, is an important pre-requisite for the proper control of gene transcription. Here we will discuss how chromatin function is modulated during muscle regeneration in response to external cues to activate and repress discrete subset of genes.

When Signaling Cascades get into the Nucleus

Satellite cells receive a myriad of signals from the regenerative microenvironment that guide the cells through the multi-step process leading to activation of a quiescent stem cells pool, expansion of the activated muscle progenitors and induction of the terminal differentiation program to induce regeneration of damaged muscles. Necrotic cues released from the damaged fibers, cytokines secreted by the inflammatory infiltrate, growth factors, free radicals, soluble proteins and changes in the extra-cellular matrix composition alter the satellite cell niche and need to be interpreted (Guasconi and Puri, 2009).

The precise composition of the extra-cellular environment is therefore fundamental to determine the gene expression profile that will guide the step-wise progression from quiescent satellite cells into multinucleated myofibers. Such information is transmitted to the cell nucleus through several cytoplasmic cascades that ultimately target components of the myogenic transcriptosome and chromatin modifying enzymes to regulate gene expression. Here we will review our current knowledge on how extra-cellular signals are transmitted to the chromatin of muscle cells to coordinately regulate the gene expression program needed for muscle regeneration. We will discuss the pathways and key signaling molecules involved as well as the downstream effectors and the changes in chromatin structure and function necessary to activate or repress discrete gene expression programs. The exquisite coordination of such programs may be subject to alterations by both cell-extrinsic changes (changes in the microenvironment) and cell-autonomous modifications (i.e., changes in the metabolome or

in the epigenome). Understanding how this chromatin signaling modulates cell fate will assist us in the rational design of pharmacological therapies to improve satellite cell function in physiological (i.e., aging) or pathological (i.e., muscular diseases) conditions.

Calcium-Dependent Chromatin Signaling

The importance of chromatin signaling in the control of muscle gene expression was first highlighted by work from Olson's laboratory. In their pioneer work McKinsey et al. showed how calcium calmodulin kinase (CamK) activity is necessary to release class II HDACs from Mef2 transcription factors at the onset of muscle differentiation (McKinsey et al., 2000). Mef2 belong to the MCM1-Agamous-Deficiens-Serum response factor (MADS) box domain family of transcriptional regulators that cooperate with the MRFs (Myf5, MyoD, Myogenin and Mrf4) to activate muscle gene transcription (Puri and Sartorelli, 2000; Potthoff and Olson, 2007). In proliferating myoblasts Mef2 binds to and recruits class II HDACs (HDAC4 and 5) to the chromatin of muscle genes, acting as a transcriptional repressor (Lu et al., 2000). Upon induction of differentiation, activation of CamK in response, at least in part, to Insulin Growth Factor I (IGF-1), leads to phosphorylation of HDAC5 on two serine residues, S259 and S498. Phosphorylation of these residues induces the binding of the chaperon protein 14-3-3 with consequent activation of the nuclear export signal and export to the cytoplasm (McKinsey et al., 2001). Further, Ca^{2+} -sensitive HDAC4, which, in spite of its name lacks of known endogenous deacetylase activity, has been shown to regulate satellite cell function through promoting activation of Pax7-positive cells, although the leading mechanism remains largely unknown (Choi et al., 2014b).

High intracellular Ca^{2+} concentration also increases the activity of calcineurin serine/threonine phosphatase, which in turns dephosphorylates NFAT (Nuclear Factor of Activated T cells) transcription factors (Friday et al., 2000). Dephosphorylation unmasks a nuclear localization signal and induces NFATs nuclear translocation and gene activation (Hogan et al., 2003). Several NFAT isoforms are present in muscle cells (Hoey et al., 1995; Parsons et al., 2003; Calabria et al., 2009) and knock out studies have suggested different temporal expression patterns and function of the different isoforms (Horsley et al., 2001; Kegley et al., 2001; Horsley and Pavlath, 2002). Further, *in vitro* studies have dissected the contribution of calcineurin/NFATs in satellite cells and activated myoblasts, showing that NFATs regulate the myogenic program at least at two different steps: first, NFATC isoforms regulates *Myf5* expression in reserve cells (Friday and Pavlath, 2001), then NFATC3 cooperates with MyoD to regulate *Myogenin* gene expression in early differentiating myoblasts (Armand et al., 2008). Finally, calcineurin is also involved in fiber-type specification at the later steps of differentiation in an NFATC3 independent process (Delling et al., 2000).

Growth Factor Activated Signaling Pathways

While CamK activity is necessary to release class II HDACs from Mef2, binding of class I HDAC to MyoD in proliferating, undifferentiated myoblasts is controlled by cell cycle

dependent changes in the phosphorylation status of pRb. Dephosphorylation of Rb upon differentiation sequesters class I HDACs and allows MyoD-dependent transcription (Mal et al., 2001; Puri et al., 2001). Further, we previously showed that another family of protein kinases, namely Akt1/2, is required to recruit HATs such as p300 and CBP to the chromatin of muscle genes (Serra et al., 2007). Akt1, which is activated by IGF-1 in muscle cells, phosphorylates the C-terminal of p300 on two serines, S1734 and S1834. Phosphorylated p300 is recruited to the chromatin to ultimately lead to the hyperacetylation of histones H3 and H4 at target genes. Moreover, Akt1/2 signaling is involved in the disengagement of chromatin repressors such as Polycomb proteins upon differentiation of muscle cells (Serra et al., 2007). This is consistent with the finding that Akt1 directly phosphorylates Ezh2, the catalytic subunit of PRC2 on S21 in cancer cells, an event that modulates binding to histone H3 (Cha et al., 2005). p300 and PCAF are important not only for histone acetylation but also for the post-translational modification of tissue specific factors such as MyoD and Mef2 (Sartorelli et al., 1999; Dilworth et al., 2004; Ma et al., 2005; Serra et al., 2007). Acetylation of MyoD on residues K99, K102 and K104 is essential for recruitment to a discrete subset of genes at defined stages of the muscle program (Di Padova et al., 2007) and for proper myogenesis both *in vitro* and *in vivo* (Sartorelli et al., 1999; Duquet et al., 2006; Di Padova et al., 2007). Acetylation of Mef2, in turn, increases DNA binding and transcriptional activity (Ma et al., 2005). MyoD and Mef2 transcription factors can be also modulated by lysine methylation. In particular, it was shown that G9a methyltransferase not only increases H3K9me2 at MyoD-target genes to repress muscle gene expression (Ling et al., 2012b; Wang et al., 2013), but also directly interacts with MyoD and Mef2 to methylate them in K104 and K267 respectively, reducing their transcriptional activity (Ling et al., 2012a; Choi et al., 2014a).

In addition to IGF-1 several other growth factors have been shown to signal to the chromatin in regenerating muscles. Amongst them, Hepatocyte Growth Factor (HGF) and members of the Transforming Growth Factor β (TGF β) superfamily are fundamental in the regulation of satellite cells function.

TGF β s are potent repressors of myogenesis (Burks and Cohn, 2011). Of them, Myostatin (GDF8) is a well-known negative regulator of muscle differentiation that signals through binding to Activin type II receptors. Binding to type II receptors induces heterodimerization with type I receptors (Alk4/5) (Rebbapragada et al., 2003). Activated receptor phosphorylates Smad2/3, inducing binding to Smad4 and nuclear translocation (Moustakas, 2002). In muscle cells it has been shown that phosphorylated Smad3 binds to and interferes with MyoD and Mef2 transcriptional activity (Langley et al., 2002; Liu et al., 2004). On the contrary, Smad7, an antagonist of the canonical TGF β /Smad signaling, promotes myogenesis through MyoD binding and activation (Zhu et al., 2004; Kollia et al., 2006). Myostatin function can be blocked by the action of Follistatin, which can be induced in muscle cells by treatment with HDAC inhibitors (HDACis; Iezzi et al., 2004). Follistatin is involved in myoblast fusion *in vitro* and contributes to the beneficial

effect of HDACi treatment *in vivo* in a mouse model of muscle regeneration (Iezzi et al., 2004; Minetti et al., 2006). Further, it was recently shown that the EGF-CFC family of proteins (Shen and Schier, 2000) also antagonizes Myostatin function during muscle regeneration. In particular, Cripto, the founder member of the family, is re-expressed in activated satellite cells and positively regulates myogenesis, interfering with Myostatin-mediated Smad2 phosphorylation (Guardiola et al., 2012).

HGF is a heparin binding protein located in the extra-cellular matrix of muscle fibers. Upon injury or mechanical stretch, HGF is released from its anchor in muscle fibers by serum proteases and binds to the surface receptor c-Met, present in satellite cells (Tatsumi et al., 1998, 2002; Miyazawa, 2010). HGF has been shown to regulate satellite cell proliferation and function (Tatsumi et al., 1998; Yamada et al., 2010). Further it was recently shown that high concentrations of HGF are necessary for the re-entry of satellite cells into quiescence through a mechanism involving Myostatin up-regulation (Yamada et al., 2010). Both HGF and the inflammatory cytoquine IL-6 induce the activity of the oxidative stress sensor Nrf2 in myoblasts. Nrf2 is involved in satellite cell proliferation and repression of the myogenic program through direct down-regulation of *Myogenin* gene (Al-Sawaf et al., 2014). On the other hand, signaling through HGF/c-Met activates the mammalian target of rapamycin (mTOR) pathway in satellite cells via the PI3K/Akt cascade. mTOR is a main sensor of the metabolic status of the cell, modulating the cell response to variations in nutrient availability. Although extensive work has been done on the upstream regulators of the pathway, the downstream effectors are less known (for a detailed review on mTOR signaling, see Laplante and Sabatini, 2012). The best-characterized responses are the regulation of protein synthesis through phosphorylation of translational regulators such as eIF4E binding protein or S6 kinase and the regulation of lipid metabolism (Laplante and Sabatini, 2012). Although little endogenous mTOR is found in the nucleus, the pathway has been associated with the modulation of gene expression in skeletal muscle through the transcription factors PGC1 α and YY1 (Cunningham et al., 2007; Blattler et al., 2012). A recent study showed mTOR activation upon limb-muscle injury switches quiescent satellite cells in the contra-lateral limb into what it has been called a quiescent “alert” state or *Galert*. Such state allows satellite cells to quicker activate in response to injury. Consistent with a role of the mTOR pathway in regulating the metabolic status of the cell, amongst the transcriptional changes associated to the “alert” response there is an enrichment in transcripts associated to metabolism and mitochondrial activity. The alert state is reversible and the morphological and transcriptional changes turn to those of normal quiescent satellite cells when mTOR signaling ceases. It will be interesting to dissect the molecular pathways and epigenetic changes associated to this reversible quiescence (Rodgers et al., 2014).

Inflammatory Signals

The inflammatory infiltrate recruited to the site of lesion is the main source of secreted cytokines such as interleukina-1, interleukina-6 or Tumor Necrosis Factor (TNF) that activate

muscle resident cells (Stoick-Cooper et al., 2007). In satellite cells, several signaling cascades respond to cellular stressors by converting inflammatory cues into the epigenetic information that controls gene expression (Lluís et al., 2006; Lassar, 2009). In some circumstances a single signal transducer may control multiple steps of gene regulation. One of the best studied examples of multi-layered control of gene expression by a signaling cascade is provided by the family of Mitogen Activated Protein Kinases (MAPK) α , β , γ and δ (Cuenda and Cohen, 1999; Zetser et al., 1999; Wu et al., 2000). p38 is activated in response to either inflammatory cytokines such a TNF or amphotericin/HMGB1, cell-to-cell contact and growth factors such as TGF β in satellite cells (reviewed in Guasconi and Puri, 2009). Previous studies have shown how p38 kinases α and β contribute to the assembly of the myogenic transcriptosome on the chromatin of muscle loci, leading to the consequent activation of gene expression. p38 α and β promote Mef2 transcriptional activation (Zetser et al., 1999), MyoD-E47 heterodimerization (Lluís et al., 2005) and recruitment of SWI/SNF chromatin remodeling complex (Simone et al., 2004; Serra et al., 2007; Forcales et al., 2012) and Ash2L-containing mixed-lineage leukemia (MLL) methyltransferase complex (Rampalli et al., 2007) to the chromatin of muscle genes. By contrast, activation of p38 γ in satellite cells represses MyoD transcriptional activity by direct phosphorylation, which leads to the association with the H3K9 methyltransferase Suv39h1 (KMT1A) (Gillespie et al., 2009). Further, we have recently shown that p38 α signaling to PRC2 represses the determination gene *Pax7* in satellite cells undergoing terminal differentiation, an event that is necessary for cell cycle exit (Palacios et al., 2010; Mozzetta et al., 2011). Activation of p38 α/β downstream kinase Msk1 has been shown to regulate a chromatin switch between Ezh2-containing and Ezh1-containing PRC2 complexes at the onset of differentiation via phosphorylation of S28 on histone H3 (Stojic et al., 2011). Interestingly, Ezh1-containing complexes have been associated to polII recruitment and transcriptional activation in differentiating myoblasts, challenging the common view of PRC2 complexes as chromatin repressors (Mousavi et al., 2012).

Thus, p38 signaling can either activate or repress gene expression in satellite cells, depending on the activation status of specific p38 isoforms, or chromatin recruitment with specific modifying complexes. Furthermore, evidence supports the notion that chromatin-associated p38 kinases can control gene transcription by directly targeting components of the transcription machinery (Chow and Davis, 2006; Pokholok et al., 2006; de Nadal and Posas, 2010), suggesting a general role of p38 signaling in the control of genome redistribution of chromatin-modifying complexes in response to extrinsic signals.

Underlying a further layer of complexity, the same cytokine may modulate different intra-cellular cascades that ultimately converge to the chromatin of muscle precursors. For instance, in addition to inducing p38 α/β activation, TNF also regulates NF- κ B activation in muscle cells. Work from the Guttridge lab showed that the TNF/NF- κ B pathway repressed *Notch* expression both in satellite cells and C2C12 myoblasts through a mechanism involving Ezh2 and Dnmt3b (Acharyya et al., 2010).

Amongst the signaling pathways activated in response to inflammatory cues in muscle, the Jun Activated Kinase (JAK)/STAT pathway responds to cytokines such as IL-6 or leukemia initiating factor (LIF). The pathway is activated upon binding of IL-6 to the IL-6R-gp130 receptor which leads to JAK activation and phosphorylation of Stat proteins on tyrosines residues. Phosphorylated Stats are able to heterodimerize and translocate into the nucleus (Stark and Darnell, 2012; Muñoz-Cánores et al., 2013). In muscle satellite cells, nuclear Stat3 binds the regulatory regions of target genes, including *MyoD*, to regulate their expression. Consistent with a role in regulating myogenic progression (Sun et al., 2007; Wang et al., 2008), genetic depletion or pharmacological inactivation of JAK2 and/or Stat3 expanded a population of Pax7-positive, *MyoD*-negative cells and improved muscle regeneration (Price et al., 2014; Tierney et al., 2014).

Developmental Programs Re-Activated in Adult Stem Cells

Several signaling pathways essential for embryonic myogenesis have been shown to be re-activated during regeneration in the adult and to play a fundamental role in the activation, commitment and differentiation of satellite cells. Here we will discuss the contribution of Notch, Wnt, Sonic Hedgehog (Shh) and Bone Morphogenetic Proteins (BMPs) pathways to adult muscle regeneration.

The Notch signaling pathway plays a fundamental role in the establishment of cell fate decisions in several tissues. In muscle it has been shown to be essential both during embryogenesis and in the adult. Activation of the Notch signaling starts with the expression of Notch ligand delta-like (Dll) in differentiating cells. Dll binds and activates the membrane receptor Notch in satellite cells, leading to the cleavage of the cytoplasmic portion of the protein and nuclear translocation of the intracellular domain (NICD; Schroeter et al., 1998). NICD is a potent transcriptional activator that binds the regulatory regions of target genes together with Rbpj, the main downstream effector of the pathway (Kopan and Ilagan, 2009). It is currently widely accepted that the Notch pathway, which is activated upon injury in quiescent satellite cells, is required for maintaining the homeostasis of the satellite cells compartment and is involved in regulating proliferation and self-renewal, keeping myoblast in an undifferentiated state (Conboy and Rando, 2002; Wen et al., 2012). By using conditional knock out mice two different labs showed that Rbpj-depleted satellite cells prematurely differentiate while they fail to undergo replication, leading to premature exhaustion of the satellite cell compartment (Bjornson et al., 2012; Mourikis et al., 2012a,b). Genome-wide analysis demonstrated dynamic Rbpj chromatin binding in response to Notch activation in muscle cells, together with NICD and the acetyltransferase p300. A detailed analysis of the chromatin signature at Rbpj-bound regions, showed that different chromatin modifications are present at constitutive vs. inducible genes and confirmed the major role of Rbpj as a transcriptional activator (Castel et al., 2013). Notch signaling is switched off through ubiquitin-mediated degradation of the

protein, which is directed by the cellular adaptor Numb. Numb factors comprise a family of four cytoplasmic proteins that mark Notch for proteome-mediated degradation through the E3 ubiquitin ligase Itch (McGill and McGlade, 2003). Numb has been implied in the replenishing of the satellite cell compartment by mediating asymmetric division of activated satellite cells (Shinin et al., 2006). Further, Numb-depletion has been shown to impair proliferation and differentiation of satellite cells through up-regulation of the cell cycle inhibitor p21 and Myostatin (George et al., 2013).

Work from Rando's lab showed that a temporal switch between Notch and Wnt signaling is necessary for adult myogenesis (Brack et al., 2008). Wnt proteins comprise a family of 19 glycoproteins that play a fundamental role during embryonic myogenesis and adult homeostasis (for a detailed review on the contribution of Wnt signaling to myogenesis, see von Maltzahn et al., 2012). Secreted Wnts bind to Frizzled (Fzd) receptors, located in the plasma membrane of target cells (Sethi and Vidal-Puig, 2010). When bound to the receptor, Wnt elicits a variety of cellular responses through the activation of several intra-cellular cascades. The best known of such cascades, the canonical Wnt pathway, starts the activation of heterotrimeric G proteins and Dishevelled (Dsh) and the recruitment of Axin to the Fzd co-receptor Low Density Protein receptor (LPR)-related protein. This recruitment inactivates the β catenin degradation complex, consisting of Axin, APC and glycogen synthase kinase 3 beta (GSK-3 β). Before Wnt stimulation β catenin levels are regulated through GSK-3 β mediated phosphorylation, which targets the protein to proteasome-mediated degradation. Upon binding of Wnt ligands, stabilized β catenin accumulates and is able to translocate into the nucleus, where it binds members of the TCF and LEF families of transcription factors. Nuclear β catenin acts as a transcriptional co-activator (Katoh, 2007; Nusse, 2008). The canonical Wnt pathway may co-exist with other β catenin-independent signaling, such as the activation of phospholipase C (PLC), CamkII or protein kinase C (PKC) (all of which lead to an increase in the intracellular Ca^{2+} levels) the phosphatidyl-inositol 3 kinase (PI3K)/Akt/mTOR axis, the Protein Kinase A (PKA)/CREB pathway (Chen et al., 2005) and the planar-cell polarity (PCP) pathway (Kühl, 2004; Le Grand et al., 2009; von Maltzahn et al., 2011). The latest is involved in the remodeling of the cytoskeleton, acting through Fzd, Vang1, Dsh and Prickle (Montcouquiol et al., 2003; Vladar et al., 2009). Although it has not been formally proved, it was suggested the PCP pathway could modulate Carm1 (Prmt4)-dependent methylation of Pax7 in activated satellite cells (Kawabe et al., 2012). Carm1 is an arginine methyltransferase important for Pax7-mediated activation of *Myf5* in asymmetrically dividing cells through recruitment of Mll1/2 methyltransferase (McKinnell et al., 2008; Kawabe et al., 2012). It also regulates muscle-specific microRNAs, SWI/SNF-mediated chromatin remodeling and Mef2 transcriptional activity (Chen et al., 2002; Dacwag et al., 2009; Mallappa et al., 2011).

The role of the Wnt proteins in adult muscle regeneration is complex as illustrated by the amount of family members

whose expression is modulated during regeneration (Polesskaya et al., 2003; Brack et al., 2008). In regenerating muscle Wnts are released by the myofibers (Polesskaya et al., 2003) whereas the Fzd receptor is activated only in satellite cells (Brack et al., 2008). Numerous studies have helped to clarify the multi-step control of myogenesis through sequential activation of different Wnt ligands and pathways. First, Wnt7-activation of the PCP pathway stimulates symmetric expansion of satellite cells (Le Grand et al., 2009). Then, activation of the canonical Wnt/βcatenin pathway was proposed to be required for the differentiation of progenitor cells (Polesskaya et al., 2003; Rochat et al., 2004; van der Velden et al., 2006; Brack et al., 2008). Consistently, genetic depletion of Bcl9, the mammalian homologue of the Wnt co-regulator in drosophila *legless*, in Pax7-positive cells abrogates nuclear localization of βcatenin and impairs injury-mediated muscle regeneration (Brack et al., 2009). However, a recent study has challenged this idea, showing that although the Wnt pathway is activated during regeneration, this activation is transient and it is the subsequent Wnt inactivation, rather than activation, that is necessary for proper regeneration (Murphy et al., 2014). Finally, it has been shown that, upon injury, Wnt signaling is also able to induce *Pax7* expression in a subpopulation of muscle-resident CD45+ stem cells through a canonical βcatenin pathway (Polesskaya et al., 2003). However, the contribution of this population of CD45+ cells to normal adult myogenesis is controversial and it is probably limited to pathological conditions such as muscular dystrophies, acting as a compensatory mechanism to the continuous waves of degeneration ad regeneration that lead to the exhaustion of the satellite cells compartment.

BMPs are members of the TGFβ super-family. As with the canonical TGFβ pathway binding of BMPs to type I and II receptors induces phosphorylation of Smad 1, 5 and 8. Phosphorylated Smad1/5/8 interact with Smad4 to form an heterodimer that translocates to the nucleus and binds the chromatin of target genes (Canalis et al., 2003). Upon muscle injury, type IA BMP receptor is activated in satellite cells, and Smad proteins are phosphorylated. Activation of the pathway induces satellite cells proliferation while blocking differentiation (Ono et al., 2011). At the onset of differentiation, the increase of BMP antagonists such as Noggins or Chordin antagonizes BMP signaling to allow satellite cells differentiation (Friedrichs et al., 2011; Ono et al., 2011). Further, it was previously shown that Notch signaling is necessary for BMP-induced block of differentiation, highlighting the functional interplay amongst the two pathways (Dahlqvist et al., 2003).

Hedgehog (hh) is an evolutionary conserved pathway essential for tissue morphogenesis during development. The pathway is activated upon interaction of the extra-cellular ligands Shh, Ihh and Dhh with the transmembrane receptor Patched1 (Ptch1), which leads to the activation of smoothened (Smo) and translocation to the nucleus of Gli transcription factors (Jiang and Hui, 2008). Of the developmental programs that are reactivated upon muscle injury the role of Shh as a regulator of stem cell function in adult muscle has been by far the less studied. It was recently shown that the primary cilia activate Shh at the initial stages of muscle differentiation and is essential

for proper differentiation (Fu et al., 2014). Shh activation induces proliferation of *ex vivo* cultured mouse satellite cells while inhibiting terminal differentiation. In addition, upon induction of differentiation, Shh regulates caspase-3 activation and apoptosis (Koleva et al., 2005). On the contrary, a different study using primary cultures of chicken myoblasts points out to a role of the signaling pathway both in myoblasts proliferation and differentiation through the MAPK/ERK and Akt1 pathways (Elia et al., 2007). Recently it was shown Shh is reactivated during muscle injury *in vivo* and pharmacological inactivation of the pathway reduces the number of myogenic progenitors at the site of lesion and impairs regeneration (Straface et al., 2009). Despite the fact little is known on how Shh modulates chromatin structure in adult muscle cells, it was previously shown that Gli2, together with Zic1 and Pax3, activates *Myf5* epaxial enhancer during somitogenesis (Himeda et al., 2013). Consistent with a role in regulating master regulatory factors, works on developing limbs have mapped genome-wide Gli3 binding by ChIP-seq, showing Shh-responsive genes are associated to gene categories such as development and morphogenesis and are enriched in transcriptional regulators (Vokes et al., 2008; Shi et al., 2014). Further, a recent study has shown that Shh induces an epigenetic switch consisting on disengagement of PRC2 and recruitment of the H3K27me3 demethylase Jmjd3 at target loci in responsive fibroblasts, leading to gene activation (Shi et al., 2014). If similar mechanisms are active also in satellite cells still needs to be elucidated.

Redox Status

Upon injury, inflammatory cytokines can alter the physiological redox status of skeletal muscle stem cells, a fundamental prerequisite for muscle regeneration. Important players in redox status determination are Nitric Oxide (NO), Reactive Oxygen Species (ROS) and the NAD⁺/NADH ratio.

NO is a free radical synthesized from the amino acid L-arginine by three different isoforms of NO synthase (NOS; Nathan and Xie, 1994). In skeletal muscle NO is produced constitutively by neuronal type NO isoform (nNOS; Nakane et al., 1993; Silvagno et al., 1996) that controls its physiological production to hinder a potential toxicity. NO has been described as an epigenetic molecule (Colussi et al., 2008; Nott et al., 2008) capable of inducing global epigenetic modification in Duchenne muscular dystrophy (DMD; Colussi et al., 2009). In DMD, the absence of dystrophin causes delocalization of the dystrophin-associated complex (DAPC) from the cytoskeleton of muscle fibers membrane, leading to structural destabilization of the sarcolemma (Matsumura et al., 1994; Ervasti and Sonnemann, 2008). DAPC displacement determines the dissociation of its interactor, the sarcolemma neuronal NO synthase (nNOS) from the same site, causing an alteration in NO production (Brennan et al., 1995). In healthy muscle, NO deposits S-nitrosylation on HDAC2, which is released from the chromatin, an event that activates a specific pattern of gene expression, including the activation of several microRNAs. Conversely, in dystrophic muscles a decrease of nNOS activity reduces HDAC2 S-nitrosylation, increasing chromatin binding and determining the repression of target genes and microRNAs. Amongst these,

miR-1 repression up-regulates G6PD levels, which sensitizes muscle cells to physiological production of free radicals. This contributes to high oxidative stress levels observed in DMD (Cacchiarelli et al., 2010). When dystrophin is rescued by exon skipping, the recovery of correct nNOS localization on the membrane induces chromatin disengagement of HDCA2 and re-expression of its target genes, contributing to late muscle differentiation (Cazzella et al., 2012).

Differently from NOS, ROS production occurs at different locations in muscle fiber including the sarcoplasmic reticulum, transverse tubules, sarcolemma and the cytosol but the main sites are the mitochondria (Barja, 1999). Changes in redox status of muscle fibers modify kinases and phosphatases activities causing alterations in gene expression (Chiarugi and Cirri, 2003; Torres and Forman, 2003). For example p38 MAPK and JNK are activated not only in response to inflammatory cytoquines but also in response to ROS production (Cuschieri and Maier, 2005) Increases in ROS concentration induces a strong depletion of the glutathione (GSH) pool that leads to NF- κ B activation and reduction of *MyoD* expression, impairing myogenesis (Guttridge et al., 1999; Ardite et al., 2004). NF- κ B is known for its negative regulation of skeletal muscle differentiation (Buck and Chojkier, 1996; Langen et al., 2001) even though in response to ROS alteration is also able to promote the activity of inducible NOS whose role in muscle differentiation is controversial (Kaliman et al., 1999; Piao et al., 2005). Another protein, p66Shc, an isoform of Src homology 2 domain containing transforming protein 1 (Shc), is phosphorylated in response to elevated ROS levels, negatively contributing to myogenesis. p66Shc KO mice show higher regenerative capacity and differentiation of skeletal muscle stem cells compared to wt mice. Probably, active p66Shc produces superoxide anions, which deplete available NO by forming peroxynitrite, which is not generated in KO mice (Zaccagnini et al., 2007). Moreover at high ROS concentration, the ratio of NAD⁺/NADH is shifted in favor of NAD⁺, which promotes the activity of a family of NAD⁺-dependent HDACs, the sirtuins. Sirt1 (the mammalian homologue of yeast Sirtuin2 (Sir2))—mediates MyoD deacetylation and inhibition of transcription (Fulco et al., 2003). Sirt1 senses variations in NAD⁺/NADH ratio during skeletal muscle cell differentiation (MacDonald and Marshall, 2000). In undifferentiated cells, where this ratio is high, MyoD is kept inactive by Sirt1 mediated hypoacetylation whereas when such ratio decreases, skeletal muscle cells start to differentiate. Recently Abdel-Khalek et al. ascribed to Sirt3, a mitochondrial NAD⁺ dependent deacetylase, a role in the regulation of myoblast differentiation (Abdel Khalek et al., 2014). Differently from Sirt1 that is highly expressed in proliferating myoblasts, Sirt3 expression starts to increase when C2C12 cells arrive at confluence and its levels are kept elevated during differentiation. Interestingly, Sirt3-depleted cells show a block of differentiation, high levels of ROS, a decrease in manganese superoxide dismutase (MnSOD) activity and an inhibition of Sirt1 expression (Abdel Khalek et al., 2014). It would be interesting to clarify the mechanism by which Sirt3 regulates *Myogenin* and *MyoD* expression and why Sirt1 is not up-regulated upon Sirt3 depletion.

All together, these data demonstrate that the maintenance of cellular redox homeostasis, as a result of a crosstalk among different free radicals, represents another layer for regulating muscle differentiation through chromatin signaling.

Mechano-Transduction

Due to their particular anatomical position, muscle stem cells are strongly exposed to physical and mechanical cues such as contraction and/or changes in the extra-cellular matrix stiffness (Dupont et al., 2011; Gilbert et al., 2011). Cells convert these mechano-stimuli into biochemical and nuclear signals through the Yap and Taz mediators (Dupont et al., 2011) that usually control cell growth and differentiation. Yap and Taz are the nuclear transducers of the Hippo pathway (Pan, 2010).

The Hippo pathway is a signal transduction pathway involved in development, cell function, regeneration and organ size in many tissues and is altered in several human diseases including muscular dystrophy and cancer (Tremblay and Camargo, 2012; Yu and Guan, 2013; Tremblay et al., 2014; Wackerhage et al., 2014). The central Hippo cascade comprises upstream elements such as the STE20-like protein kinases 1 and 2 (Mst1 and Mst2) and the large tumor suppressor kinases 1 and 2 (Lats1 and Lats2). Phosphorylation and subsequent activation of Lats1/2 by Mst1/2 induces Yap/Taz phosphorylation (Huang et al., 2005) Phosphorylation of the best-characterized phospho-sites on Yap (S127) and on its analogous Taz (S89) determines their retention and inactivation in the cytosol by 14-3-3 proteins (Basu et al., 2003; Wackerhage et al., 2014). In response to different stimuli such as G-protein coupled Receptor (GPCR) signaling (Yu et al., 2012), mechano-stimuli (Dupont et al., 2011) and apico-basal polarity (Huang et al., 2005) Yap and Taz are dephosphorylated and translocate to the nucleus where they are recruited to the chromatin through binding to several transcription factors (Hong and Guan, 2012).

In muscle cells Taz and Yap associate with TEAD transcription factors (TEA domain), which bind to MCAT elements (muscle C, A and T; 5'-CATTCC-3') located in the promoter or enhancer regions of key genes that regulate commitment (*MyoD*, *Myf5*, *Mrf4*), proliferation (*Cyclin D1*) and differentiation (*Myogenin*) of satellite cells. Both Yap and Taz are expressed in skeletal muscle (Jeong et al., 2010; Watt et al., 2010). The group of Wackerhage was the first to assess the expression of Yap in skeletal muscle cells. In particular they have shown that in myoblasts, unphosphorylated Yap is localized in the nucleus where positively controls the expression of proliferation genes. Conversely, as C2C12 undergo differentiation, Yap (S127) phosphorylation increases and the protein translocates from the nucleus to the cytosol where it is kept inactive (Watt et al., 2010). Moreover Yap stimulates proliferation of activated satellite cells and prevents their differentiation by controlling expression of genes associated with cell cycle, ribosome biogenesis and modulation of myogenic differentiation (Judson et al., 2012). In contrast to what observed for Yap, ectopic expression of Taz promotes myogenic differentiation through a cooperative interaction with MyoD that increases its binding to DNA (Jeong et al., 2010). Taz has been shown to interact also with Pax3 during development (Murakami et al., 2006). Jeong et al.

demonstrated Taz levels increase after muscle injury in mice suggesting it is also involved in muscle regeneration (Jeong et al., 2010). Under differentiation conditions, translocation of Taz to the nucleus is required for enhancing the expression of genes such as *Myogenin*, *Mhc* and *Mck*. This process can be stimulated by selenoproteinW which induces Taz nuclear translocation by interrupting its binding with 14-3-3 protein and consequently increasing myogenic differentiation (Jeon et al., 2014). Recently, compounds that promote myogenesis through Taz activation in C2C12 cells have been characterized and shown to induce an increase in *Taz* expression, interaction with MyoD and MyoD recruitment to the chromatin during the initial phase of the muscle differentiation program (Park et al., 2014). Collectively these data point out to Yap and Taz as novel components of the muscle transcriptosome and suggest they could be potential therapeutic targets in muscular dystrophies. Their opposite effects on proliferation and differentiation suggest that fine-tuned modulation of Taz and Yap activity might be a good strategy for increasing muscle regeneration and amelioration of diseased phenotypes.

Altogether, the data discussed here suggest that signaling to the chromatin in response to the regenerative microenvironment plays a crucial role during muscle regeneration. They are summarized in **Figure 1**.

Age-Dependent Decline of Satellite Cell Function

Aging is associated to a loss of the homeostatic and regenerative potential in all tissues and organs. In muscle, it is reflected on a loss of muscle mass and strength (sarcopenia) and to an impaired regeneration potential associated to a dysfunction of the satellite cell compartment (Brack and Rando, 2007).

Both cell-intrinsic changes and changes within the regenerative microenvironment are associated with the stem cell loss of function that occur with age. For instance, replicative damage, proteomic changes (i.e., accumulation of damaged macromolecules) and changes in the epigenome and the proteome have been shown to affect stem cell function in old tissues (Liu and Rando, 2011; Garcia-Prat et al., 2013; Sinha et al., 2014). Amongst the cell-intrinsic changes, modification of the epigenetic profile, including changes in the DNA methylation patterns and post-translational histone modifications are particularly interesting, as they are potentially reversible. Recent work has shown a progressive increase in DNA methylation in aging muscle (Day et al., 2013; Jin et al., 2014; Ong and Holbrook, 2014; Zykovich et al., 2014). Interestingly, key studies in tumor and aged cells indicate that *de novo* DNA methylation in the adult is usually restrained to a subset of CpG islands, most of which are PRC2 target regions (Schlesinger et al., 2007; Bergman and Cedar, 2013). Consistent with this, satellite cells isolated from old mice show altered levels and distribution of H3K27me3 (Liu et al., 2013). The functional interplay between PRC2 and the DNA methylation machinery in aged satellite cells likely contributes to the impaired regeneration potential of old muscles and could represent novel pharmacological target to improve muscle function in aged individuals.

On the other hand, elegant parabiotic studies showed that changes in the regenerative environment of old muscles regulate satellite cell function (Conboy et al., 2005; Brack et al., 2007; Sinha et al., 2014). For instance, attenuated Notch signaling leads to reduced satellite cells proliferation and impaired regeneration in aged muscle. Muscle regeneration can be recovered by ectopic activation of Notch or by exposing old animals to a young environment (Conboy et al., 2005). On the contrary, a progressive increase in Wnt signaling with age alters muscle stem cell fate (Brack et al., 2007; Doi et al., 2014), inducing a myo-to-fibrogenic conversion of muscle progenitors that leads to increased fibrosis *in vivo* (Brack et al., 2007). As in the case of impaired Notch signaling, the fibro-adipogenic conversion observed in old muscles is reversible and can be reduced by exposing mice to conditioned serum from young animals. Conversely, *in vivo* activation of the pathway in young mice by injection of Wnt3A increases fibrotic deposition (Brack et al., 2007). Finally, it was recently shown that the TGF β member Growth Differentiation Factor 11 (GDF11) also reverses the age-related changes in satellite cells dysfunction (Sinha et al., 2014).

The contribution of an old inflammatory infiltrate to the impaired regenerative response of aged muscles can be further predicted by the elevated levels of cytokines such as IL-1, IL-6 and TNF observed in old muscles (Phillips and Leeuwenburgh, 2005; Dirks and Leeuwenburgh, 2006). In a recent study Trendelenburg et al. showed that elevated levels of IL-1 and TNF block differentiation of human myoblasts through activation of a signaling pathway involving TGF β activated kinase (TAK1)/p38/NF- κ B and leading to increased expression of ActivinA (Trendelenburg et al., 2012). Increased levels of TNF, together with alterations in the pathway of FGFR1, also lead to aberrant activation of the p38 α/β cascade in satellite cells derived from old muscles. Two recent studies have highlighted the importance of p38 α/β in maintaining satellite cells function and homeostasis during their lifespan, showing that increased p38 α/β activity leads to a cell-autonomous defect in satellite cells self-renewal and activates a senescence program. This p38 α/β -dependent decline in satellite cells function with age cannot be overcome by exposure to a young microenvironment but instead is partially reversed when satellite cells are treated with the p38 α/β inhibitor SB203580 (Bernet et al., 2014; Cosgrove et al., 2014). Although in these experiments p38 α/β blockade was performed *ex vivo* prior to satellite cell transplantation, pharmacological manipulation of the p38 α/β signaling has been achieved also *in vivo* through the use of antibodies against TNF receptor (Palacios et al., 2010). Acute treatment with neutralizing TNF antibodies expands a population of Pax7+, differentiation-competent satellite cells in young animals (Palacios et al., 2010), whereas long-term treatment with the same antibodies have been shown to have a beneficial effect on muscle regeneration (Radley et al., 2008; Huang et al., 2009). If such beneficial effects are also obtained in old mice needs to be investigated. Altogether these studies highlight the potential impact of pharmacological approaches in recovering satellite cell function in diseased and aged muscles.

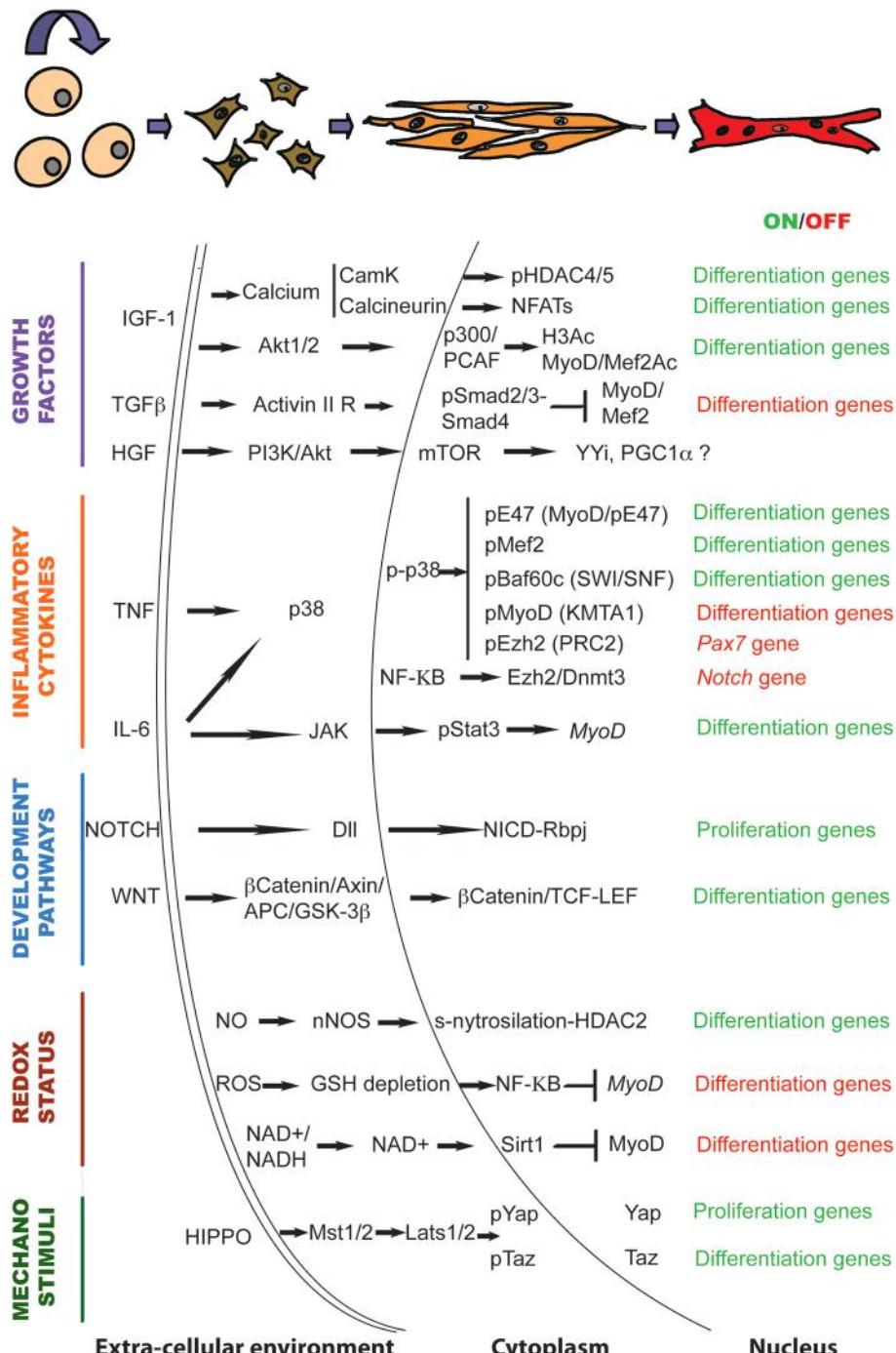


FIGURE 1 | The genetic and epigenetic signaling governing muscle regeneration. Schematic representation of the signaling pathways and downstream chromatin effectors that regulate the transition from activated satellite cells to multinucleated myotubes in response to regeneration cues.

In vivo pharmacological manipulation of a discrete chromatin signaling has in fact been the strategy adopted by the groups of Rudnicki and Sacco to stimulate the function of old satellite cells. In this case, interfering with an altered IL6/JAK/Stat3 signaling through the use of

JAK/STAT inhibitors increases the regeneration potential of old and dystrophic muscles (Price et al., 2014; Tierney et al., 2014), revealing its potential for pro-regenerative therapy to counteract the impaired regeneration observed with age.

Even if it is becoming increasingly clear that satellite cells malfunction is highly modulated by age-related changes in the surrounding microenvironment, a gap of knowledge persists on how alterations in the old regenerative environment and cell-intrinsic changes (i.e., changes in the metabolism or in the epigenetic profile) converge to modulate the function of aged satellite cells. The relative contribution of aberrant environmental cues and cell-intrinsic changes to aged satellite cells function can now be partially explained by work in Muñoz-Cánoves lab. By distinguishing old (20–24 months) from geriatric (over 28 months) mice, Sousa-Victor et al. have dissected the transcriptional changes associated to the aging process, that ultimately depends on Polycomb-mediated silencing of the p16/INK4 locus (Sousa-Victor et al., 2014). These data provide a partial explanation of why pre-senescent satellite cells are responsive to a young environment whereas full gero-conversion is an irreversible, cell autonomous, process. Future studies aimed to investigate in detail the signals and molecular mechanisms driving to this point of no return will be fundamental for the development of novel therapeutic approaches to delay muscle miss-function with age.

Remarkably, in a recent article from this special issue of *Frontiers in Aging Neurosciences*, Formicola et al. demonstrate that, contrary to what happens in limb muscles, the extra-ocular muscles (EOM) stem cell niche is resistant to age and disease (Formicola et al., 2014), probably thanks to the contribution of a population of muscle-resident cells called PICs (PW1 interstitial cells) (Mitchell et al., 2010; Pannérec et al., 2013). If this resistance to aging is due to a direct contribution of PICs to muscle regeneration or to indirect modulation of satellite cells function still needs to be investigated. It will also be interesting to understand what makes EOM PICs and maybe other EOM muscle-resident cell populations such FAPs (Fibroadipogenic precursors) more resistant to age-dependent cell-intrinsic changes. FAPs are multi-potent mesenchymal cells located in the interstitium of muscle fibers (Joe et al., 2010; Uezumi et al., 2010). During muscle regeneration they support the myogenic potential of muscle stem cells (Uezumi et al., 2010; Mozzetta et al., 2013) through the release of paracrine factors such as Follistatin, the functional antagonist of Myostatin (Mozzetta et al., 2013). In DMD at late stages of the disease, the continuous waves of degeneration and regeneration leads to the conversion of FAPS into fibro-adipocytes, responsible of the fat and fibrotic deposition that characterize DMD muscles (Joe et al., 2010; Uezumi et al., 2010; Mozzetta et al., 2013). It has been recently shown that treatment with HDACi increases the regeneration potential and prevents the fibro-adipogenic degeneration of young, but not old, dystrophic muscles (Minetti et al., 2006; Consalvi et al., 2013; Mozzetta et al., 2013).

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HDACis in young mice act in part by regulating the fate of FAPS towards the myogenic lineage, through a mechanism involving a microRNA-dependent control of SWI/SNF subunit composition (Saccone et al., 2014). On the contrary, FAPs from old dystrophic muscles are resistant to HDACi-induced chromatin remodeling at muscle loci and fail to activate the pro-myogenic phenotype (Saccone et al., 2014). Therefore, age-dependent cell-intrinsic changes in other muscle-resident populations can also alter the regeneration potential of the satellite cell pool.

Conclusions and Perspectives

Muscle regeneration is a multi-step process that entails the coordinated activation and repression of discrete transcriptional programs. In this review we aimed to highlight the key role of signaling pathways in transmitting the extra-cellular information to the chromatin of satellite cells to regulate gene expression. The temporal pattern of activation in response to extra-cellular cues, the interplay amongst the pathways and the activation and/or repression of downstream effectors are fundamental for the fine-tuned control of muscle regeneration. Deciphering the muscle-specific chromatin signaling is particularly important for the rational design of novel pharmacological approaches aimed to improve satellite cells function in old and diseased muscles. Being pharmacologically manipulable and potentially reversible, both signaling cascades and the epigenome are attractive targets for the design of novel therapeutic interventions.

Finally, numerous studies have revealed a striking similarity between adult and embryonic myogenesis, regarding the pathways and the mechanisms affecting muscle stem cell function (see Buckingham and Rigby, 2014). Here we have focused on how adult muscle stem (satellite) cells activate the differentiation program in response to regeneration stimuli. Despite less studied, we speculate similar mechanisms are also active during embryonic myogenesis and participate to the successful completion of the myogenic program in response to developmental cues.

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DNA methylation dynamics in muscle development and disease

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DNA methylation is an essential epigenetic modification for mammalian development and is crucial for the establishment and maintenance of cellular identity. Traditionally, DNA methylation has been considered as a permanent repressive epigenetic mark. However, the application of genome-wide approaches has allowed the analysis of DNA methylation in different genomic contexts revealing a more dynamic regulation than originally thought, since active DNA methylation and demethylation occur during cellular differentiation and tissue specification. Satellite cells are the primary stem cells in adult skeletal muscle and are responsible for postnatal muscle growth, hypertrophy, and muscle regeneration. This review outlines the published data regarding DNA methylation changes along the skeletal muscle program, in both physiological and pathological conditions, to better understand the epigenetic mechanisms that control myogenesis.

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Introduction

DNA methylation was the first discovered epigenetic modification (Holliday and Pugh, 1975; Riggs, 1975) being one of the best studied and most mechanistically understood. Chemically, it refers to the covalent addition of a methyl group (CH_3) into the fifth position of the cytosine DNA nucleotide resulting in the modified base 5mC, frequently considered the fifth nucleotide, and well conserved among most plant, animal and fungal models (Feng et al., 2010). Cytosine methylation in mammals is almost restricted to the symmetrical CpG context (cytosine residue followed by a guanine bound by a phosphate union) (Ramsahoye et al., 2000), a dinucleotide globally underrepresented in the genome as a consequence of the spontaneous or enzymatic deamination of 5mC to thymine in the germ line (Bestor and Coxon, 1993). DNA methylation is critical for mammalian development being traditionally considered an heritable and stable silencing mark crucial for X-inactivation (Mohandas et al., 1981; Gartler and Riggs, 1983), genetic imprinting (Reik et al., 1987; Swain et al., 1987), silencing of genomic elements such as transposons to ensure genomic stability (Walsh et al., 1998; Gaudet et al., 2003), and maintenance of constitutively repressed centromeric and pericentromeric DNA satellite repeats (Gopalakrishnan et al., 2009). In addition, DNA methylation has an important role in gene regulation that depends on the genomic CpG context: promoter methylation is associated with gene silencing (Boyes and Bird, 1992; Hsieh, 1994), gene body methylation has variable effects on gene transcription (Gelfman et al., 2013; Yang et al., 2014), and intergenic methylation may affect gene expression through enhancer regulation (Stadler et al., 2011).

DNA methylation follows a bimodal distribution across the genome defined by the inverse correlation between 5mC and CpG density: CpG-poor DNA, which comprises most of the genome, shows high levels of 5mC, whereas high CpG-dense regions, termed CpG islands (Bird, 1986; Gardiner-Garden and Frommer, 1987) located mainly in the promoter regions

of housekeeping and developmental genes, are largely resistant to DNA methylation (Takai and Jones, 2002; Illingworth and Bird, 2009). Notably, the surrounding regions to the CpG islands, named CpG island shores, are susceptible to be methylated in a tissue-specific manner correlating with changes in gene expression (Doi et al., 2009; Ji et al., 2010).

Strikingly, 20 years old seminal studies demonstrated that MyoD activation occurred in a demethylation-dependent manner (Brunk et al., 1996), establishing for the first time a direct link between DNA methylation and cellular differentiation. Since then, the importance of DNA methylation in modulating transcriptional programs during development and differentiation processes has been strongly supported by many studies. In the last few years, the development of large-scale sequencing strategies has made possible to achieve significant advances in understanding the regulatory role of DNA methylation at context-specific level and how the methylome affects cell identity. The aim of the present review is to discuss the major advances concerning the DNA methylation dynamics during myogenesis and its implications in muscle development and disease.

Shaping the Cellular Identity Through DNA Methylation

During cellular differentiation stem cells lose their plasticity and narrow their identity into particular differentiated cell types, which are usually stably maintained by epigenetic mechanisms. Embryonic stem cells (ESCs) are pluripotent cells showing very low methylation levels at CpG-rich sequences (Fouse et al., 2008), which along the differentiation processes into the three germ layers gain methylation in common but also specific regions (Meissner et al., 2008; Isagawa et al., 2011). Although ESCs completely lacking DNA methylation are viable and competent for self-renewal (Tsumura et al., 2006), they are partially blocked in the ability to initiate cellular differentiation (Jackson et al., 2004). Among others, the expression of mesodermal markers such as Brachyury, α -globin and β H1-globin are impaired or not maintained in embryo bodies (EBs) derived from ESCs lacking DNA methyltransferase activity (Jackson et al., 2004; Schmidt et al., 2012). Paradoxically, although there is a global gain of methylation during cellular differentiation, specific loci lose methylation in a cell-type dependent manner. While the gain of methylation mediates the silencing of pluripotency-associated and gamete-specific genes, as well as cell-type specific markers to avoid the expression of non-appropriate lineages, loss of methylation occurs in lineage-specific genes to define cellular identity (Nagae et al., 2011; Calvanese et al., 2012; Nazor et al., 2012; **Figure 1**). Interestingly, these demethylated sequences are not only restricted to promoter regions, but are also found in distal gene sequences and intronic regions, which might affect enhancer elements, alternative promoters and alternative splicing variants in a cell-type dependent manner (Meissner et al., 2008; Stadler et al., 2011).

Genome-wide Studies Addressing the Skeletal Muscle Methylome

Several studies comparing DNA methylation signatures between cell types and tissues identified muscle-specific differentially methylated regions. As mentioned before, CpG islands are found at over half of all human gene promoters and are often free of methylation (Ioshikhes and Zhang, 2000; Lander et al., 2001; Saxonov et al., 2006). The analysis of 17,000 CpG islands in five human samples (blood, sperm, brain, skeletal muscle and spleen) showed a common set of methylated CpG islands in all somatic samples, associated with genes that are essential for development, neurogenesis, and segment specification (Illingworth et al., 2008). In addition, this study identified 178 CpG islands specifically hypermethylated in muscle tissue, showing the skeletal muscle the highest percentage of methylated CpG islands (8.3%) (Illingworth et al., 2008). Sorensen et al. analyzed the methylation level of 200 lineage-specific differentiation markers in muscle-progenitor cells (MPCs), adipose stem cells (ASCs) and bone marrow mesenchymal stem cells (BMMSCs), using Methyl-DNA Immunoprecipitation (MeDIP) followed by microarray hybridization. The results showed that despite most of the lineage-specific promoters did not show changes in DNA methylation, important lineage-specific markers were hypermethylated in MPCs, correlating with gene silencing, such as GATA-2, AQP7 and ACACA (adipogenic lineage), RUNX2 and THY1 (osteogenic lineage), INS2 and PDX1 (pancreatic development), KRT1A (skin development) and NEFH (neurogenic lineage) (Sorensen et al., 2010). Later on, Fernandez et al. analyzed 1,505 CpGs surveying 808 gene promoters in 1,628 human samples including 22 muscle tissues. This study identified 183 muscle-specific differentially methylated CpGs (dmCpG) on gene promoters including GLI2, CREBBP, PDGFRA, CD34 and CARD15 genes (Fernandez et al., 2012). In parallel, using the methylation array Illumina Infinium 27K DNA Methylation BeadChIP, 47 genes were found specifically hypomethylated in muscle tissue, including genes encoding for proteins located in contractile fibers such as Obscurin, Myotilin, and Myh7 (Calvanese et al., 2012). At the same time, a genome-wide DNA methylation analysis was performed to compare 205 human pluripotent stem cells and 130 somatic samples, including skeletal muscle, by using the complete Illumina Infinium 450K DNA Methylation BeadChIP array. The results revealed 782 and 621 unique CpGs exclusively hypomethylated and hypermethylated, respectively, in skeletal muscle samples, being the hypomethylated regions functionally enriched in response to peptide hormone stimulus and wound healing (Nazor et al., 2012).

All these studies highlighted a specific DNA methylation signature of skeletal muscle cells compared to other cell-types, but did not address when this specific pattern was acquired. A recent study comparing human proliferating myoblasts (MBs) and differentiated myotubes (MTs), using the non-promoter-oriented RRBS method, showed no significant methylation changes during myogenic terminal differentiation (Tsumagari et al., 2013b). In this study the authors also

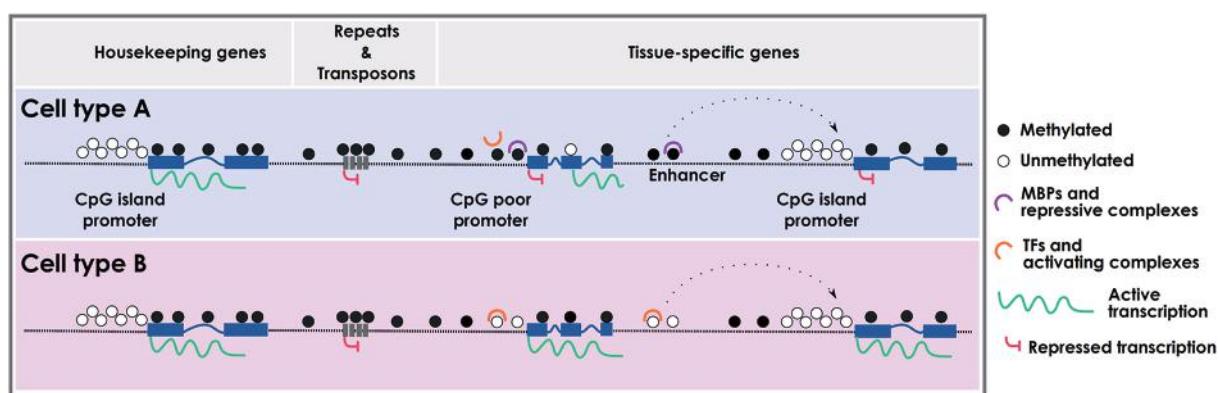


FIGURE 1 | Cell type-specific DNA methylation profiles. Schematic representation of cell type-specific methylomes. CpG island promoters are usually protected from DNA methylation and are prone to active transcription. CpG-poor regions (intergenic) and repetitive elements are typically methylated,

with the exception of enhancers and CpG-poor promoters that can be differentially methylated in a cell type-specific fashion. Intragenic regions can also be differentially methylated leading to specific cell-type transcripts. MBPs: methyl-binding proteins; TFs: transcription factors.

compared non-muscle cell cultures with myoblast/myotube cultures and found similar numbers of differentially hyper- and hypo-methylated sites (9,592 and 10,048, respectively). The regions specifically hypermethylated at MB/MT cells were strongly associated with genes encoding for transcription factors, especially homeobox and T-box genes, while muscle hypomethylation was observed at contractile fiber genes. The myogenic hypermethylation found in specific subregions of all four HOX gene clusters pointed out the involvement of DNA methylation (at 5' and internal promoters, as well as at intragenic and intergenic enhancers) in the fine-tune HOX genes regulation during development (Tsumagari et al., 2013a). In addition, the comparison of skeletal muscle methylome vs. other tissues showed that the 94% of differentially methylated sites were hypomethylated in muscle, and 47% of them were also hypomethylated in MB/MT cells, suggesting that DNA demethylation occurs gradually along the myogenic process (Tsumagari et al., 2013b). Very recently, Miyata et al. addressed the chronological alterations in DNA methylation during myogenic differentiation comparing human myoblast and myotube cultures using the Illumina Infinium 450K DNA Methylation BeadChIP array. Interestingly, they found hypermethylation in the binding sites for the transcription factor ID4 and ZNF238 during the myogenic progression, contributing to myotube formation (Miyata et al., 2014). Intriguingly, they also observed a small but global increase in DNA methylation levels during terminal differentiation occurring at genes involved in muscle contraction and muscle system process.

In summary, all these studies comparing methylation profiles between cell-types show both common and specific features among all of them. A general consensus about the importance of muscle-specific demethylation during lineage specification is prevalent among studies, and remarkable, identity-dependent methylation changes occur mostly during early cell fate decisions while fewer DNA modifications take place later during terminal cellular maturation.

DNA Demethylation-dependent Activation of Muscle-specific Genes

Seminal works of Helen Blau in the early eighties showed that the fusion of human non-muscle cells with mouse muscle cells resulted in heterokaryons expressing human muscle-specific genes, under conditions in which there was no DNA replication (Blau et al., 1983, 1985). In addition, these pioneer experiments showed that HeLa cells treated with 5-azacytidine prior to fusion also formed heterokaryons, which expressed muscle-specific genes suggesting that DNA demethylation could be involved in muscle gene activation (Chiu and Blau, 1985). Importantly, a decade later it was shown that *MyoD* activation occurred in a demethylation-dependent manner (Brunk et al., 1996), and H. Blau's laboratory demonstrated active demethylation of human *MyoD* in fused non-dividing heterokaryons (Zhang et al., 2007). Several examples in different cellular models have grounded the hypothesis that lineage-specific demethylation in tissue-specific progenitors is required for cellular differentiation, since DNA demethylation generates a transcriptionally permissive chromatin. We present below some examples of DNA demethylated myogenic genes.

5-azacytidine Triggers and Enhances Muscle Differentiation

As previously mentioned, the finding of the *MyoD* tissue-specific demethylation was the seminal work that linked for the first time DNA methylation and cell fate commitment. In 1973, Peter Jones was investigating the effect of 5-azacytidine, a new chemotherapeutic drug, in mouse embryonic fibroblasts (10T1/2 cells) when he realized that treated cells turned into a huge syncytium of multinucleated cells (Constantinides et al., 1977). After confirming the myogenic phenotype of 10T1/2 treated cells and demonstrating that the tested drug was a potent inhibitor of DNA methylation (Jones and Taylor, 1980), *MyoD* was identified as the key gene involved in muscle reprogramming (Lassar et al., 1986; Davis et al., 1987).

In addition, the same myoblastic conversion occurred in 10T1/2 fibroblasts transfected with an antisense cDNA against the maintenance DNA methyltransferase DNMT1 (Szyf et al., 1992), suggesting the involvement of DNA methylation in *MyoD* gene regulation. Analysis of treated 10T1/2 fibroblasts revealed the highly methylated state of the CpG island surrounding the *MyoD* promoter, however this CpG island was constitutively free of methylation in mouse analyzed tissues (Jones et al., 1990) raising the question whether the demethylation of *MyoD* promoter was indeed the signal required to initiate the myogenic program. The answer came when Brunk et al. showed the specific demethylation of a distal regulatory region located at–20 Kb of *MyoD* TSS during somitogenesis (Brunk et al., 1996). They observed by sodium bisulphite conversion that three CpGs located in this enhancer region were partially methylated in liver, heart, and brain mouse tissues (50–60%), and in fibroblast and neuroblastoma cells (50–80%), while they were almost totally unmethylated in forelimb or hindlimb muscles (17%) and in C2C12 myoblast cell line (8%). Moreover, they analyzed the methylation state during somitogenesis observing that the distal enhancer was heavily methylated in the presomitic mesoderm and became almost completely demethylated between somites 6 and 10 as myogenesis progressed. Intriguingly, mutations of these dmCpG did not cause precocious activation of *MyoD* in transgenic mice, which implied that DNA methylation was not sufficient for gene inactivation. Moreover, the authors showed that demethylation of *MyoD* distal enhancer occurred prior to gene activation, although *MyoD* expression did not immediately follow enhancer demethylation, suggesting that active demethylation was required but was not sufficient for *MyoD* activation (Brunk et al., 1996). In parallel and intriguingly, Takagi et al. observed an enhancement in myotube formation upon overexpression of DNMT1 in myoblast C2C12 cell line (Takagi et al., 1995). Using methods based in methylation sensitive enzymes they detected a positive correlation between *MyoD* expression and exon 1-2 hypermethylation, which would be in agreement with the idea that DNA methylation in gene bodies correlates with gene expression.

Subsequent studies also observed an enhancement of muscle differentiation in C2C12 MBs treated with 5-azacytidine, resulting in higher myotube formation with enhanced maturity (Hupkes et al., 2011). A recent study analyzed the effect of 5-azacytidine treatment on C2C12 cell cycle regulation. The results showed that inhibition of DNA methylation increased the expression of checkpoint genes involved in cell cycle progression, arrested cell cycle, and up-regulated myogenic transcription factors enhancing myogenesis, although no analysis of methylation state of myogenic genes was performed (Montesano et al., 2013).

Myogenin DNA Demethylation During Myoblast Differentiation

The correlation between muscle differentiation and DNA demethylation was further underscored by the finding that *Myogenin* promoter became demethylated at the onset of C2C12 muscle differentiation. Lucarelli et al. reported that the methylation status of a single CpG site at 340 bp upstream

from the TSS affected *Myogenin* transcription in mouse tissues and C2C12 cells. *Myogenin* expression in differentiated muscle cells correlated with lack of methylation, while methylated non-muscle tissue (spleen and brain) and proliferating MBs showed no gene expression (Lucarelli et al., 2001). In addition, the same lab showed that proliferating MBs treated with 5'-Aza-2'-deoxycytidine increased *Myogenin* expression due to a reduction in DNA methylation, highlighting the regulatory role of DNA methylation in *Myogenin* expression (Scarpa et al., 1996). Later on, Palacios et al. confirmed these results and addressed *Myogenin* promoter DNA methylation dynamics during somitogenesis showing anterior-posterior DNA demethylation correlatives to *Myogenin* expression and muscle development (Palacios et al., 2010). Mechanistically, they showed that efficient activation of *Myogenin* promoter required DNA demethylation following binding of SIX1 and MEF2 proteins to favor the competition between these two transcription factors and methylated DNA binding proteins. In this regard, Oikawa et al. demonstrated that the methyl-CpG-binding protein CIBZ suppressed myogenic differentiation by direct binding to the methylated *Myogenin* promoter, and importantly DNA demethylation prevented the binding of CIBZ repressor allowing the binding of MyoD/Pbx/Meis activator complex (Oikawa et al., 2011). Finally, Strom's laboratory also reported DNA demethylation in the *Myogenin* promoter but at non-CpG dinucleotides, claiming that demethylation occurs more rapidly at non-CpG than at CpG dinucleotides (Fuso et al., 2010). Although most studies restrict non-CpG methylation to ESCs and brain tissue (Ramsahoye et al., 2000; Lister et al., 2009; Guo et al., 2014), it has been estimated that 7% of cytosines within the sequence CCAGG or CCTGG are methylated in human skeletal muscle (Barres et al., 2009). In the near future it would be important to address the role of non-CpG methylation in muscle development.

Other Genes Becoming DNA Demethylated During Myoblast Cell Differentiation

Recently, other studies have also reported changes in DNA methylation affecting the activation of muscle-related genes. This is the case of *Desmin* gene, which contains a CpG island covering the TSS and a proximal enhancer extensively studied at the epigenetic level in MBs, MTs and peripheral blood, as non-muscle control sample. While the CpG island is fully unmethylated in all samples, the enhancer shows lack of methylation exclusively in myogenic cells, both in MBs and MTs, although unfused MBs exhibit lower level of *Desmin* expression (Lindahl Allen et al., 2009). The study of the histone modifications revealed that MTs showed increase positive/open chromatin marks (H3K4me3 and H3K4me2) compared to MBs, suggesting that DNA demethylation may provide a transcriptionally poised state that would be activated during differentiation, upon the acquisition of transcription factors and positive histone marks.

The transcription factor SIX1 is essential for the formation of multiple organs including the skeletal muscle (Grifone et al., 2005). Wu et al. reported that SIX1 was exclusively expressed in porcine adult skeletal muscle and displayed the

highest expression levels in fast muscles (Wu et al., 2011). The comparison of DNA methylation levels of SIX1 core promoter in several tissues revealed that skeletal muscle showed the lowest levels correlating with the higher gene expression, and similarly different methylation status were also observed in different skeletal muscles consistent with the reported variations in expression levels (Wu et al., 2013).

Expression of α -smooth muscle actin (α -SMA) is a key indicator of myofibroblast differentiation into fibroblast, and its accumulation in tissue remodeling and fibrosis leads to a deleterious excess of extracellular matrix components (Hinz et al., 2003; Hinz, 2007; Klingberg et al., 2013). α -SMA gene contains three CpG islands and a differential methylated state was reported in the second and third islands in α -SMA expressing myofibroblast vs. non-expressing epithelial cells (Hu et al., 2010). In addition, inhibition of DNA methyltransferase activity led to significant induction of α -SMA expression, while ectopic expression of DNMTs suppressed its expression (Hu et al., 2010). Interestingly, all these data suggest that the enhancement of DNA methylation, by DNMT activity, could be a key mechanism for reducing myofibroblast differentiation.

The Role of TET Proteins in Active Demethylation in Muscle Differentiation

The recent demonstration that Ten-eleven translocation family of protein dioxygenases (TET1-3) had the capacity to convert 5mC into 5-hydroxymethylcytosine (5hmC) raised the possibility that 5hmC might constitute a distinct epigenetic state contributing to dynamic changes in DNA methylation (Tahiliani et al., 2009; Ito et al., 2010). 5hmC was found in many tissues and cell types, although with diverse levels of abundance being enriched in ESCs and certain types of neurons (Kriaucionis and Heintz, 2009; Ito et al., 2010; Szwalbergczak et al., 2010). Recently, an ultra-sensitive and accurate isotope based HPLC-MS method was used to precisely determine the levels of h5mC in different mouse tissues revealing that skeletal muscle showed an intermediate level estimated between 0.15–0.17% of cytosine residues (Globisch et al., 2010).

As mentioned above, dynamic changes in DNA methylation occur during early development (Surani et al., 2007; Hajkova et al., 2010) and DNA demethylation takes place in a highly locus-specific fashion upon development (Ma et al., 2009; Wu and Zhang, 2010). Base-resolution analysis of 5hmC in ESCs revealed high levels of 5hmC (and reciprocally low levels of 5mC) at regions of low CpG content and near, but not on, transcription factor binding sites, suggesting that TET proteins might influence gene expression (Yu et al., 2012). TET1^{-/-} mice were viable and fertile but about 75% of the homozygous mutant pups had smaller body size (Dawlaty et al., 2011). Gene expression array analysis showed 221 significantly deregulated genes, including developmental skeletal muscle and muscle contraction genes (Dawlaty et al., 2011). The very recent generation of TET1/2/3 (TKO) ESCs showed EBs totally depleted in 5hmC with a concomitant increase in global 5mC levels (Dawlaty et al., 2014). Moreover, TKO EBs expressed reduced levels of mesodermal and endodermal markers. Furthermore, global gene expression and DNA methylation analyses of TKO

EBs revealed promoter hypermethylation and deregulation of genes implicated in embryonic development and differentiation, including skeletal muscle development genes being one of the top ten GO categories (Dawlaty et al., 2014). All these observations suggest that TET enzymes are critical during ESC differentiation by regulating promoter methylation levels of a subset of developmental regulators and lineage commitment genes, and thus enabling their activation by differentiation-induced and lineage-specific demethylation.

Very interestingly, it was reported that NOTCH1 receptor and its ligand DLL1 showed high levels of 5hmC in myogenic differentially methylated regions in skeletal muscle, cerebellum and heart (Terragni et al., 2014). Notch signaling is involved in the regeneration of injured skeletal muscle, brain function and cardiac disease, and these results suggest that hypomethylation and/or hydroxymethylation may help to control gene expression in a tissue and stage-specific manner (Terragni et al., 2014).

It has been widely reported that enhancer activity is modulated through histone modifications, histone variant deposition and nucleosome stability. Interestingly, during neuronal differentiation of P19 cells and adipogenic differentiation of 3T3-L1 cells DNA hydroxymethylation was an early event of enhancer activation (Serandour et al., 2012). Recently, a compendium of regulatory elements driving muscle differentiation was identified based on chromatin signatures and MyoD recruitment (Blum et al., 2012). In the near future, it would be worth to analyze whether the acquisition of 5hmC in myogenic-specific distal regulatory regions might activate enhancer elements leading to the expression of muscle-differentiation genes.

DNA Methylation Changes During the Skeletal Muscle Aging

Aging is accompanied by a progressive decline in adult tissue-specific stem cells functions, resulting in less effective tissue homeostasis and repair. Aging-associated mechanisms are not yet totally understood, but DNA methylation changes and other epigenetic mechanisms contribute to aging phenotypes. Early studies measuring global DNA methylation, using biochemical methods, showed a progressive depletion of 5-methylcytosine in senescent normal fibroblast (Wilson and Jones, 1983), and similar changes were also observed in aging mouse tissues and human cells (Wilson et al., 1987). Comprehensive DNA methylation analyses have been facilitated by the application of genome-wide technologies revealing numerous methylation alterations, both hyper- and hypomethylation changes, occurring in several tissues during aging (Gröniger et al., 2010; Maegawa et al., 2010; Rakyan et al., 2010; Bocke et al., 2011; Hernandez et al., 2011; Bell et al., 2012; Heyn et al., 2012; Beerman et al., 2013). Although the functional consequences of this age-related methylation drift remain unknown, the alterations in DNA methylation state would create epigenetic mosaic between aging-stem cells affecting their proliferative and differentiation capacities (Issa, 2014).

A recent genome-wide study comparing DNA methylation patterns in postmitotic skeletal muscle taken from healthy young

(18–27 years of age) and old (68–89 years of age) males showed a predominant pattern of hypermethylation in the DNA of aging skeletal muscle (Zykovich et al., 2014). Most of the methylation changes occurred intragenically, being underrepresented in promoter regions. Using the Illumina Infinium 450K DNA Methylation BeadChip array 2,114 genes were identified with at least one dmCpG site located intragenically. The Tubulin-folding cofactor D (TBCD), involved in microtubule functions, was the highest hypermethylated gene in aged-samples. Gene ontology analysis of genes with two or more intragenic dmCpG site showed that the most enriched terms and pathways were “muscle cell” ($P = 0.0004$) and “axon guidance signaling” ($P = 6.17E-10$), suggesting that the dmCpG sites identified within the aged group were relevant to muscle tissue functions and neuromuscular junctions (Zykovich et al., 2014). In fact, one of the major contributors in sarcopenia is the motor unit loss or denervation, and these results point out that the process might be affected by DNA methylation. Notably, NFATC1 gene involved both in the regulation of signaling at the neuromuscular junction, and acting as transcriptional regulator of muscle fibers in direct response to electrical stimulation via calcium/calmodulin signaling (McCullagh et al., 2004; Rana et al., 2008; Salanova et al., 2011) was hypermethylated in aged-skeletal muscles. Interestingly, DNA methylation changes on NFATC1 and other genes within the axon guidance signaling pathway may alter transcriptional events leading to the loss of proper reinnervation at the neuromuscular junction during muscle turnover resulting in muscle wasting.

Very recently, Jin et al. analyzed genome-wide DNA methylation changes in skeletal muscle between young and middle-aged pigs, as a good biomedical model for human studies, using a methylated DNA immunoprecipitation sequencing approach. Contrarily to the previous study, they found a global tendency towards loss of DNA methylation in middle-aged pigs compared to the young group, enriched also in gene-body regions (Jin et al., 2014). The intersection of genes that presented DMRs in their promoters and gene body with the 288 genes potentially involved in the human aging process (according to the Human Aging Genomic Resources database) revealed 12 known age-related genes including FoxO3 and FGFR1. FoxO3 is an essential transcription factor involved in lysosomal proteolysis in muscle, by activating autophagy and proteosomal pathways (Mammucari et al., 2007; Zhao et al., 2007). Interestingly, FoxO3 becomes upregulated in aged muscles correlating with lower gene-body methylation status (Jin et al., 2014). Contrary, FGFR1 shows an opposite function to that of FoxO3, inhibiting the atrophy of skeletal muscle (Eash et al., 2007), and it is found downregulated and hypermethylated in gene-body in middle-aged pigs. These results highlight the participation of DNA methylation changes enhancing proteolysis and protein catabolic processes during aging, which suggest an important role of epigenetic mechanisms in aged-related muscle-atrophy.

A potential issue regarding these two genome-wide skeletal muscle studies is the use of muscle biopsies that include several non-muscle cell types, which might confound to some point muscle-specific methylation signatures. In the future, it will be necessary to evaluate whether DNA methylation differences

between young and aged/sarcopenic satellite cells may affect muscle stem cell function. Notably, *MyoD* hypermethylation was reported in aged blood and brain samples (Hernandez et al., 2011; Fernandez et al., 2012) raising the question if it could be also differentially methylated in aged satellite cells. Finally, the identification of age-related DNA methylation patterns was used to build a model of biological aging (Hannum et al., 2013). Interestingly, some of the differentially methylated probes distinguishing young from old skeletal muscles were included in these age-predicting markers (Zykovich et al., 2014). In this regard, the ability to predict biological age from DNA methylation markers could be envisioned as a health assessment to prevent and diagnose aged-related diseases.

DNA Methylation Alterations in Rhabdomyosarcoma and Muscle Diseases

Epigenetic modifications in concert with genetic mechanisms regulate transcriptional activity in normal tissue but are often dysregulated in diseases. Here we discuss recent progress regarding DNA methylation alterations in muscle-related pathologies.

Rhabdomyosarcoma Tumorigenesis

Epigenetic aberrations have been well established in cancer: the genome of cancer cells is globally hypomethylated compared to normal tissues, but at the same time CpG islands in regulatory regions of tumor suppressor genes are often hypermethylated, being both events important for the origin and progression of many human cancer (Berdasco and Esteller, 2010). Rhabdomyosarcoma (RMS) is the most common soft-tissue sarcoma of childhood, with skeletal muscle presumed origin because of its myogenic phenotype. However, unlike normal MBs RMS cells differentiate poorly both *in vivo* and in culture (Keller and Guttridge, 2013). Experiments in the late eighties showed that the treatment of the RMS cell line RMZ-RC2 with the demethylating agent 5-azacytidine resulted in an increased differentiation capacity, suggesting that aberrant DNA methylation was repressing differentiation genes in this RMS cell line (Lollini et al., 1989). In addition, the comparison of DNMTs levels between normal skeletal muscles and RMS tumors showed significant higher expression of DNMTs in both alveolar and embryonal RMS subtypes, which have distinct etiologic and clinical behaviors (Chen et al., 1998). Candidate gene approaches identified DNA methylation changes in RMS tumors in *MyoD* (Chen et al., 1998), *p21WAF1* (Chen et al., 2000), *RASSF1* (Harada et al., 2002), *PAX3* (Kurmashova et al., 2005), *plakoglobin* (Gastaldi et al., 2006), *FGFR1* (Goldstein et al., 2007), *JDP2* (MacQuarrie et al., 2013), *BMP2* (Wolf et al., 2014), and *CAV1* (Huertas-Martínez et al., 2014) genes. Recently, a genome-wide analysis of promoter CpG island methylation between RMS subtypes and skeletal muscles revealed RMS-specific hypermethylation in genes associated with tissue development, differentiation and oncogenesis such as *DNAJA4*, *HES5*, *IRX1*, *BMP8A*, *GATA4*, *GATA6*, *ALX3* and *P4HTM*, implicating aberrant DNA methylation in the pathogenesis of RMS (Mahoney et al., 2012). In addition, cluster analysis showed

that embryonal and alveolar subtypes had distinct methylation patterns, with the alveolar subtype being enriched in DNA hypermethylation of polycomb target genes (Mahoney et al., 2012). Importantly, the different DNA methylation signatures between RMS subtypes might aid to define tumor subtype, clinical prognosis and treatment response of RMS tumors.

The connection between miRNAs, tissue differentiation and malignant transformation emerged long time ago (Reinhart et al., 2000; Calin et al., 2002). Although some miRNAs can act as oncogenes, miRNAs identified as de-regulated in cancer are more commonly tumor suppressors, that are down-regulated by several mechanisms including epigenetic silencing (Rota et al., 2011). In the last few years, the list of miRNAs undergoing promoter hypermethylation has been rapidly expanded in many human tumors, highlighting the transcriptional repression of miRNAs by DNA methylation as a common feature in human cancer (Lopez-Serra and Esteller, 2012). Recent evidences have shown low expression of miR-206 (Missiaglia et al., 2010), miR-1 and miR-133a (Rao et al., 2010), and miR-203 (Diao et al., 2014) in RMS cells which have been correlated with higher proliferation rates, impaired differentiation and poor overall survival. Notably, it was recently demonstrated that miR-203 was frequently down-regulated in both RMS cell lines and RMS biopsies by promoter hypermethylation, and importantly, it can be reactivated by DNA-demethylating agents inhibiting tumor growth and migration capacity, and promoting terminal differentiation (Diao et al., 2014). All these data allow envisioning the use of combined treatment including epigenetic drugs for the treatment of the aggressive RMS tumors.

Epigenetic Determinants in Other Muscle Pathologies

Most muscle pathologies are caused by gene mutations; however, for some recessive myopathies a number of affected individuals show only monoallelic mutations, pointing out that epigenetic mechanisms could be affecting the expression of the second allele. Recessive core myopathies, where the skeletal muscle Ryanodine receptor gene (*RYR1*) is mutated (Quane et al., 1993; Jungbluth et al., 2005), and dysferlinopathies, caused by mutations in Dysferlin (*DYSF*) gene (Liu et al., 1998), show a percentage of patients with only an identified mutated allele. Importantly, for core myopathies it was shown that the treatment of cultured patient skeletal-muscle MBs with 5-Aza-2'-deoxycytidine reactivated the expression of the non-mutated allele, suggesting the implication of DNA hypermethylation in the silencing of the gene (Zhou et al., 2006). However, detailed bisulphite sequencing analysis of all CpG islands located in *RYR1* and *DYSF* genes showed no differences in DNA methylation levels between healthy and affected individuals (Zhou et al., 2006; Gallardo et al., 2014). These results raised the question of which would be the epigenetic mechanism responsible for the reported *RYR1* monoallelic expression. The most plausible hypothesis according to the authors was a genomic-imprinting mechanism mediated by differentially methylated imprinting control regions, since they observed an association with the sex of the non-transmitting parent, and a tissue-specific nature of the epigenetic silencing (Zhou et al., 2006). Although other

regulatory mechanisms cannot be definitively excluded, the results suggesting an altered epigenetic regulation involved in these muscle pathologies support the use of epigenetic drugs to reverse or to ameliorate the symptoms of the disease.

Juvenile dermatomyositis (DM) is a severe chronic childhood autoimmune disease showing the affected children muscle weakness caused by chronic muscle damage (Christen-Zaech et al., 2008; Feldman et al., 2008). Comparison of genome-wide DNA methylation profiling, by Illumina Infinium Human Methylation27K BeadChip array, revealed 27 genes with significant methylation differences between normal and juvenile DM diagnosed muscle biopsies (Wang et al., 2012). Although previous gene expression studies showed alterations in the expression of genes involved in immune response, vascular remodeling and endoplasmic reticulum response to acid stress (Nagaraju et al., 2005; Chen et al., 2008), no significant methylation alterations in those genes were found in juvenile DM. However, among the 27 differentially methylated genes several HOX genes (*HOXC11*, *HOXD3* and *HOXD4*) and the developmental transcription factor *WT1* were found hypomethylated in juvenile DM samples, as well as in other types of idiopathic inflammatory myopathies (IIMs) with muscle weakness, such as juvenile polymyositis (Wang et al., 2012). The similar methylation alterations of *WT1* and homeobox genes found in IIMs provided additional evidences that the damaged muscles in these children had self-renewal capacity, stimulating the muscle stem cell pool towards muscle repair. Interestingly, these results suggest that the homeobox and *WT1* genes are epigenetically marked to facilitate the repair process in response to the muscle damaged occurring during disease pathogenesis.

Effects of Exercise on Skeletal Muscle DNA Methylation Signature

There is no doubt that physical exercise contributes to improve human health. Indeed, a large number of studies showed that exercise alters the expression of genes that affect glucose and lipid metabolism, mitochondrial function, as well as, transcription factors, myogenic regulatory factors and myokines (Pilegaard et al., 2000, 2003; Coffey and Hawley, 2007; Egan et al., 2010). Although the global effect of exercise on DNA methylation is still not well known, some studies have addressed whether DNA methylation plays a role in exercise-induced gene expression.

Barrès et al. analyzed DNA methylation levels of the *vastus lateralis* skeletal muscle from young and healthy men and women before and after 20 min of acute exercise. Luminometric methylation assay (LUMA) showed a small but very rapid global DNA methylation decrease in young muscles (Barrès et al., 2012). Although factors involved in expression of muscle-specific genes such as *MyoD* did not show methylation differences, several metabolic gene promoters such as *PGC-1 α* , *TFAM*, *PPAR γ* , *PDK4* and citrate synthase showed lower methylation, by MeDIP-PCR analysis, correlating with higher expression levels after exercise. Similarly, promoter methylation of *PGC-1 α* , *PPAR γ* , and *PDK4* was decreased in mouse *soleus* muscles 45 min after *ex vivo* contraction correlating with increased gene expression (Barrès et al., 2012). In parallel, another study

using MeDIP-ChIP analysis evaluated the effect of 6 months of moderate aerobic exercise, in persons with and without diabetes type 2 family histories. The results showed DNA methylation changes in 134 genes after 6 month long training period, independently of the familiar history (Nitert et al., 2012). Most of the genes showed decreased methylation (115 out of 134) and were involved in retinol metabolism, calcium-signaling pathway, and starch and sucrose metabolism. Among them, the transcription factors RUNX1 and MEF2A, both important for muscle physiology, increased their expression after moderate exercise correlating negatively with DNA methylation changes (Nitert et al., 2012).

Altogether, these results suggest that physical exercise may modify DNA methylation patterns, although future studies will be needed to deeper understand the involvement of epigenetic mechanisms in the beneficial effects of regular exercise on human health.

Conclusions

DNA methylation plays an important role in mammalian development, as illustrated during skeletal muscle cell fate commitment and differentiation. During development muscle stem cells acquire an unique DNA methylation signature associated with its specialized functions, and specific-myogenic factors are activated in a demethylation-dependent manner. DNA methylation patterns are not fixed but dynamic, and can be modulated by external influences. We have highlighted the recent data regarding how skeletal muscle methylome changes in response to physical exercise, aging and in muscle-related pathologies including cancer (summarized in Figure 2). The development of whole-genome approaches has contributed with important advances in understanding the

regulatory role of DNA methylation at context-specific level and how the methylome affects cell identity. However, many important questions remain open regarding the demethylation mechanisms involved in specific-myogenic demethylation, the function of non-CpG methylation in muscle cells, and the role of 5-hydroxymethylation modulating proximal and distal regulatory regions affecting gene expression. In addition, the intense and recent interest in cellular reprogramming in the field of regenerative medicine emphasizes the importance of identifying cell type-specific epigenetic signatures to ensure a safety reprogramming in stem cell-based therapies (Barrero et al., 2010). Finally, the recognition of DNA methylation as a significant contributor to normal muscle physiology and its alterations in pathological processes, as well as in aging open new avenues to envision a near future where epigenetic therapies will be included in the treatment of muscle-related diseases.

Author Contributions

EC and MS wrote the manuscript. Both authors read and approved the final manuscript.

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Abbreviations

ChIP, Chromatin immunoprecipitation; DMRs, Differentially methylated regions; DNMT, DNA methyltransferase; EB,

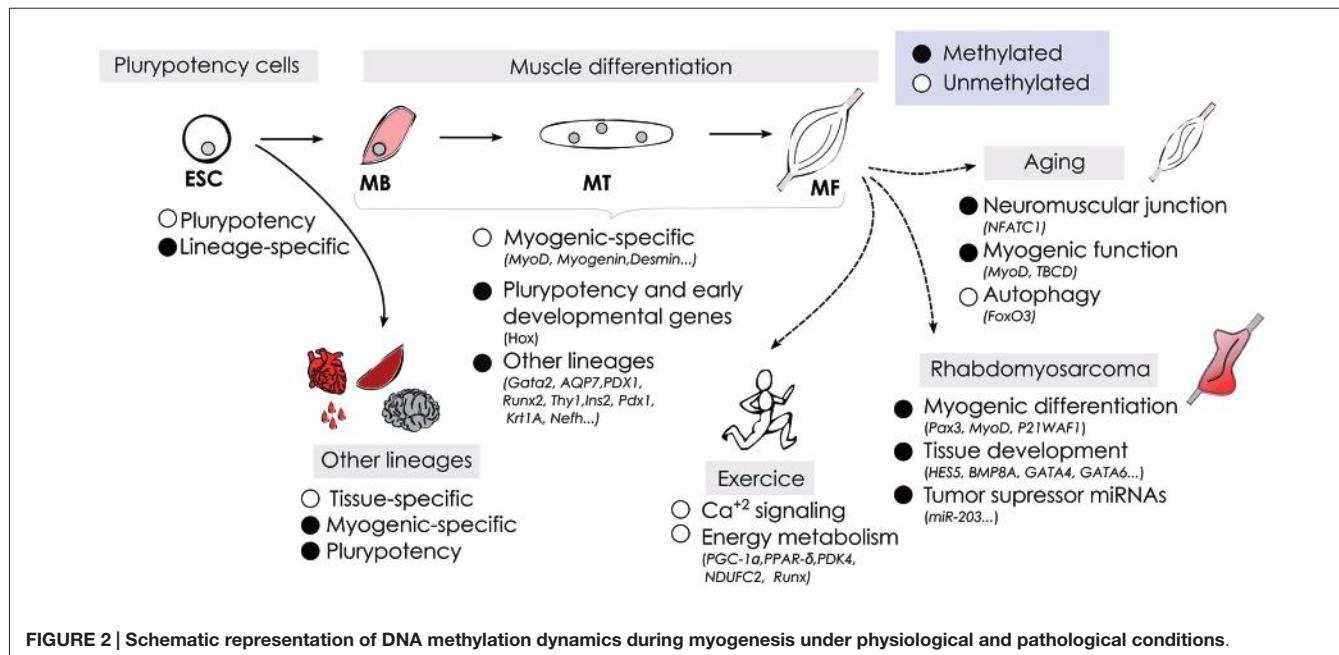


FIGURE 2 | Schematic representation of DNA methylation dynamics during myogenesis under physiological and pathological conditions.

Embryo body; ESCs, Embryonic stem cells; MB, Myoblast; MT, Myotube; MeDIP, Methyl DNA immunoprecipitation; MPC, muscle progenitor cell; RMS, Rhabdomyosarcoma; RRBS,

Representative reduced bisulphite sequencing; TET, Ten-eleven translocation; TSS, Transcription start site; 5C, 5-cytosine; 5mC, 5-methylcytosine; 5hmC, 5-hydroxymethylcytosine.

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Pericytes: multitasking cells in the regeneration of injured, diseased, and aged skeletal muscle

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INTRODUCTION

In skeletal muscle, small blood vessels called capillaries surround each myofiber (Poole et al., 2008). The capillary wall consists of endothelial cells and pericytes. The latter are wrapped by the capillary basal lamina, located on the abluminal surface of the endothelial capillary tube. The word *pericyte* derives from the Greek *kytos*, a hollow vessel, appropriately describing a cell surrounding a blood vessel.

Electron microscopy studies estimated the pericyte-to-endothelial-cell ratio for the overall coverage of microvessels in striated skeletal muscle as approximately 1:100 (Shepro and Morel, 1993). However, the analyzed samples were unstained, so the technique could not precisely distinguish pericytes from other surrounding cells. Pericytes are defined by their anatomical location in combination with several molecular markers (Kunz et al., 1994; Verbeek et al., 1994; Lindahl et al., 1997; Ozerdem et al., 2001), and the great advances in fluorescent imaging techniques suggest reanalyzing this ratio.

In most peripheral organs, pericytes are derived from the mesoderm (Armulik et al., 2011), but their origin in skeletal muscle has not been explored sufficiently. Specific pericyte subpopulations with distinct roles in the skeletal muscle have been described (Birbrair et al., 2013c). Whether pericyte subsets derive from different embryonic tissues remains unknown. CNS pericytes derive from the ectoderm (Bergwerff et al., 1998; Etchevers et al., 2001; Korn et al., 2002; Heglind et al., 2005). Brain pericytes exhibit both exclusive markers (Bondjers et al., 2006) and markers shared with skeletal muscle pericytes (Armulik et al., 2011). Whether and how pericyte functions in skeletal muscle differ from those in the brain remains to be explored. Brain

pericytes are perivascular cells that envelop and make intimate connections with adjacent capillary endothelial cells. Recent studies show that they may have a profound impact in skeletal muscle regeneration, innervation, vessel formation, fibrosis, fat accumulation, and ectopic bone formation throughout life. In this review, we summarize and evaluate recent advances in our understanding of pericytes' influence on adult skeletal muscle pathophysiology. We also discuss how further elucidating their biology may offer new approaches to the treatment of conditions characterized by muscle wasting.

Keywords: pericytes, skeletal muscle, regeneration, innervation, fat formation, fibrous tissue, angiogenesis, aging

and skeletal muscle pericytes express NG2 proteoglycan (Armulik et al., 2011; Birbrair et al., 2013c) and react to injury by forming fibrotic scars (Popa-Wagner et al., 2006; Dulauroy et al., 2012), suggesting that, independent of location, they share some properties.

Transplanting fluorescently marked embryonic tissues (e.g., mesoderm, endoderm, ectoderm, neural crest cells) into unmarked embryonic skeletal muscle may provide some clues on pericyte ancestors. Another approach would use transgenic mice for genetic tracking of cells from different embryonic tissues to pinpoint the origin of skeletal muscle pericytes and whether it differs for the recently identified subpopulations (**Figure 1**) (Birbrair et al., 2013a,c,d, 2014, 2015).

Pericytes are heterogeneous, exhibiting major differences depending on the tissue from which they have been isolated (Sims, 2000; Bondjers et al., 2006). Their functions seem to be tissue-specific, and very little is known about their function in skeletal muscle. Here, we present an overview of the current knowledge on their participation in adult muscle regeneration, reinnervation, vascularization, fibrosis, fat formation, and calcium deposition.

PERICYTE PARTICIPATION IN SKELETAL MUSCLE REGENERATION

Skeletal muscle comprises about 40% of the human body by weight. It is a highly organized network of different types of cells, neurovascular structures, and connective tissue. It may undergo physiological changes based on everyday physical activity, and due to its superficial location, it is constantly subjected to different grades of traumatic injury. Nevertheless, in young healthy

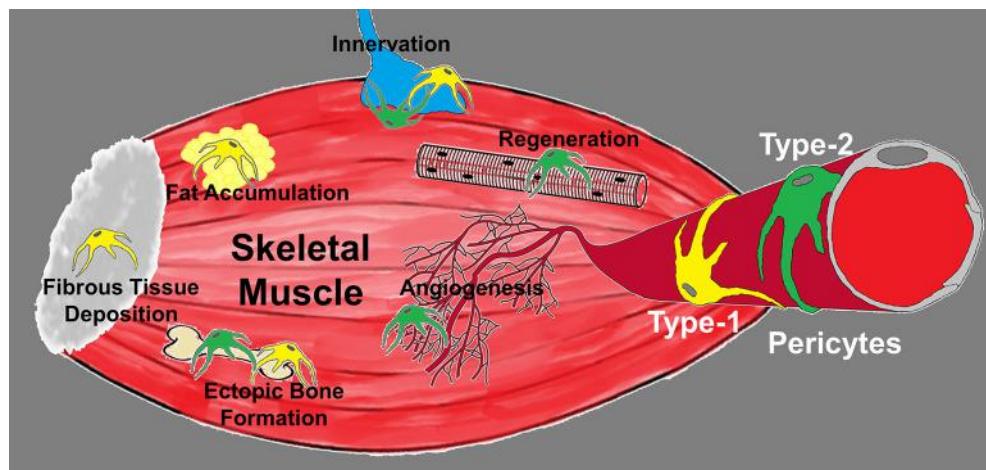


FIGURE 1 | Diagram depicting the roles of pericyte subtypes in skeletal muscle. Type-1 (yellow) and type-2 (green) pericytes are associated with blood vessels and differentially committed to tissue formation. Type-1

pericytes form adipose and fibrous tissue, while type-2 pericytes cooperate with myogenesis and angiogenesis. Their role in muscle innervation and ectopic bone deposition remains unexplored.

subjects, skeletal muscle is well recognized for its remarkably robust endogenous capacity to repair itself (Huard et al., 2002; Cossu and Biressi, 2005; Relaix and Zammit, 2012). Functional loss of skeletal muscle mass and strength is observed with aging in a process known as sarcopenia (Delbono, 2011); as a secondary effect, known as cachexia, in some cancer patients; and due to trauma, vascular injuries, or degenerative muscle disorders, such as muscular dystrophy (Janssen et al., 2002; Acharyya et al., 2005; Glass, 2010; Vilquin et al., 2011). Muscle wasting can cause severe debilitating weakness and represents a significant clinical problem with few solutions (Vilquin et al., 2011). All of these conditions would benefit from approaches that promote skeletal muscle regeneration, but they would require complete understanding of the complexity of the cellular mechanisms involved.

The most well-studied myogenic cells in skeletal muscle are the muscle-specific stem cells called *satellite cells*, which are located between the basal lamina and sarcolemma of individual myofibers (Charge and Rudnicki, 2004; Zammit et al., 2006; Boldrin and Morgan, 2013). These cells participate in skeletal muscle repair in response to injury, but they are scarce and difficult to isolate (Berardi et al., 2014). Other problems include poor survival, incompatibility with systemic delivery, and host rejection (Giordano and Galderisi, 2010). Hence, researchers have explored and identified other skeletal muscle cells with high myogenic potential. They include muscle-derived stem cells (MDSCs; Lee et al., 2000; Qu-Petersen et al., 2002; Lavasani et al., 2006; Urich et al., 2009; Drowley et al., 2010); CD133+ progenitor cells (Torrente et al., 2004; Peault et al., 2007; Negroni et al., 2009); endothelial cells (Zheng et al., 2007); PW1+ interstitial cells (Mitchell et al., 2010; Pannerec et al., 2013); muscle side population (SP) cells (Gussoni et al., 1999; Asakura and Rudnicki, 2002; Bachrach et al., 2004); and pericytes (Dellavalle et al., 2007, 2011; Birbrair et al., 2013a,d).

Note that, besides cell therapy, all these cells may contribute to endogenous skeletal muscle formation, defined as the creation

of new myofibers by proliferation, recruitment, migration and fusion of mononucleated cells (Yin et al., 2013).

The exact relationship between the different skeletal muscle cell types with myogenic potential and whether they all derive from one source have yet to be established. Future studies must define their specific contributions to muscle formation. One approach might be to use mouse models in which only one of these cell populations is ablated. Such a study showed that satellite cells are essential for skeletal muscle regeneration (Lepper et al., 2011; McCarthy et al., 2011; Murphy et al., 2011; Sambasivan et al., 2011). However, efficient skeletal muscle formation also depends on the interaction of cell types without myogenic potential, such as connective tissue fibroblasts (Barnes and Glass, 2011). In future studies ablating each of these cell populations separately, followed by skeletal muscle regeneration analysis, we may learn that they interact physically and release factors directly affecting each other's myogenic capacity.

Pericytes are myogenic *in vitro* (Dellavalle et al., 2007). When injected into injured skeletal muscle, they induce a higher muscle regenerative index than enriched myoblasts (Crisan et al., 2008b). Intra-arterial transplantation in animal models of muscular dystrophies demonstrated that they largely engrafted and may improve skeletal muscle function (Sampaoli et al., 2003, 2006). Tedesco et al. (2012) found that transplantation of genetically modified pericytes resulted in functional amelioration of the dystrophic phenotype.

A more interesting question is whether endogenous pericytes contribute to skeletal muscle formation. Fate-tracking experiments with alkaline phosphatase (AP) Cre-ER mice proved that pericytes associated with blood vessels contribute to postnatal skeletal muscle growth by type; for example, less in tibialis anterior than diaphragm. They can assume satellite cells' position and become satellite cells (Dellavalle et al., 2011). Through unknown mechanisms, pericytes' contribution increases greatly during skeletal muscle regeneration in response to chemical injury (Dellavalle et al., 2011). Whether pericytes expand in the

skeletal muscle following physical injury (for instance, in response to exercise) remains unknown; and, if so, whether the mechanisms are similar to those activated in response to chemical injury must be addressed (Boppert et al., 2013).

The molecular mechanisms activating and orchestrating pericytes' transition from quiescence to regenerating capacity in the skeletal muscle are also unknown. Again, whether selective ablation of pericytes from skeletal muscle will prevent or otherwise affect regeneration will clarify whether they can be replaced by other cell types with myogenic capacity. We propose that due to their ability to secrete several growth factors, pericytes may be required to induce other cell types to adopt a myogenic fate (Sato and Rifkin, 1989; Shepro and Morel, 1993; Davis et al., 1996; Yamagishi et al., 1999; Brown et al., 2001; Reinmuth et al., 2001; Hirschi et al., 2003; Niimi, 2003; Armulik et al., 2005; Paquet-Fifield et al., 2009; Shimizu et al., 2011). A global analysis of candidate growth factors secreted by skeletal muscle pericytes that promote skeletal muscle regeneration is required.

Our recent work reported the presence of two pericyte subpopulations in the skeletal muscle. Type-1 (Nestin-GFP-/NG2-DsRed+) and type-2 (Nestin-GFP+/NG2-DsRed+) pericytes are in close proximity to blood vessel endothelial cells and co-localize with other pericytic markers (Birbrair et al., 2013c), but only type-2 is myogenic and participates in skeletal muscle regeneration (Birbrair et al., 2013a). Type-1 may not express the specific receptors needed to mediate the signaling pathways required for myogenic differentiation. Future studies should reveal the specific signaling pathways and why only one subpopulation can be induced to a myogenic fate. We also found that only type-2 pericytes can enter the satellite cell compartment and express satellite cell marker Pax7 (Birbrair et al., 2013d).

The host microenvironment is critical for their myogenicity; for example, in older mice, the muscular regenerative capacity of type-2 pericytes is limited (Birbrair et al., 2013d), suggesting that it might be improved by modifying the deleterious aged muscle microenvironment. Approaches aimed at changing the old skeletal muscle environment have been reported. For instance, 3D hydrogel was used to rejuvenate pericytes derived from aged skeletal muscle, and their myogenic capacity improved (Fuoco et al., 2014).

To what extent impaired type-2 myogenicity leads to myofiber loss or skeletal muscle atrophy as compared to the effects of other myogenic cells in the skeletal muscle has yet to be defined. No one has yet studied whether intrinsic pericyte changes may impair skeletal muscle regeneration with aging, as recently reported in satellite cells (Bentzinger and Rudnicki, 2014; Bernet et al., 2014; Cosgrove et al., 2014; Sousa-Victor et al., 2014). Further, are pericyte autonomous changes with aging reversible? Is one pericyte subtype more prone to senescence or apoptosis? Does the aging environment select for a pericyte subtype with poorer myogenic potential? Does the number of distinct pericyte subpopulations change with aging and in diseased dystrophic skeletal muscle?

The only marker differentially expressed in skeletal muscle pericytes is Nestin-GFP, which is also expressed in satellite cells (Birbrair et al., 2011). Thus, only the combination of Nestin-GFP and NG2-DsRed expression distinguishes the pericyte subpopulations reported in the muscle interstitium (Birbrair et al., 2013c).

Future studies to determine whether the absence of endogenous type-2 pericytes compromises skeletal muscle regeneration, as with satellite cells (Barnes and Glass, 2011; Dellavalle et al., 2011; Lepper et al., 2011; McCarthy et al., 2011), will require discovery of novel markers selectively expressed in a pericyte subpopulation. To define them, investigators must first characterize the expression profiles of type-1 and type-2 pericytes. Better characterization may also advance therapies that target specific receptors. For instance, β -agonist therapy has shown potential in skeletal muscle repair (Beitzel et al., 2004); whether skeletal muscle pericytes express beta adrenergic receptors in response to these drugs is not known, although pericytes from other tissues do so (Kelley et al., 1988; Elfant et al., 1989; Ferrari-Dileo et al., 1992; Zschauer et al., 1996; Rucker et al., 2000; Quignard et al., 2003; Mendez-Ferrer et al., 2010a,b; Daly and McGrath, 2011; Lee et al., 2014).

Recent studies demonstrate that pericytes are involved in skeletal muscle regeneration as described above (Crisan et al., 2008b; Dellavalle et al., 2011; Birbrair et al., 2013a). However, the molecular mechanisms underlying the recruitment of pericytes in this complex process are unknown.

PERICYTES IN SKELETAL MUSCLE REINNervation

Muscle innervation is essential for maintaining mass and function with aging (Delbono, 2003, 2011). Denervation can result from trauma, immobility, unloading, infections, vasculitis, neuropathies, autoimmune processes, neoplasms, amyotrophic lateral sclerosis, and aging (Polak et al., 1988; Nikawa et al., 2004; Argiles et al., 2006; Glass, 2010; Bonaldo and Sandri, 2013). Loss of the nerve supply causes robust progressive skeletal muscle degeneration (Nikawa et al., 2004; Batt et al., 2006; Aagaard et al., 2010; Ohlendieck, 2011). Focal injuries to the peripheral nerves are often followed by complete recovery due to the capacity of peripheral axons to reoccupy neuromuscular junctions on denervated muscle fibers (Young, 1974; Rich and Lichtman, 1989; Nguyen et al., 2002). Even without nerve injury, the nerve terminal is constantly remodeled during degeneration and regeneration of skeletal muscle fibers, as this neuromuscular connection is needed for functional recovery of skeletal muscle (Li and Thompson, 2011). Rounds of denervation and reinnervation are evidenced by the random distribution of myofiber types across the skeletal muscle and their increased clustering with age (Larsson, 1995; Andersen, 2003; Rowan et al., 2012). Over the years, complete functional recovery is significantly reduced (Verdu et al., 2000; Kawabuchi et al., 2011). Whether these changes are controlled by the muscular, neural, or both systems is not known. Myofiber denervation has been demonstrated in mice and elderly humans (Hashizume et al., 1988; Kanda and Hashizume, 1989, 1992; Einsiedel and Luff, 1992; Doherty et al., 1993; Johnson et al., 1995; Zhang et al., 1996; Delbono, 2003; Aagaard et al., 2010; Valdez et al., 2010; Chai et al., 2011).

Regenerating axons grow through a complex microenvironment during the reinnervation process (Rich and Lichtman, 1989). They probably recognize and attach to postsynaptic sites guided by non-synaptic muscle fiber membranes and the membranes of glial cell processes (Kang et al., 2007). Schwann cells are widely believed to support axonal growth and to provide

important cues that enable nerve fibers to reach the vacant synaptic sites (Chen et al., 2007). They also provide such important growth factors as neurotrophins, which ensure correct reinnervation after experimental nerve transection (Sendtner et al., 1992; Funakoshi et al., 1993; Friedman et al., 1996; Frostick et al., 1998; Brushart et al., 2013). Regenerating axons are guided by processes extended by terminal Schwann cells at the denervated synaptic location (Kang et al., 2014). Distinct Schwann cell populations have been described, including non-myelinating, myelinating, terminal, and perisynaptic Schwann cells, and terminal Schwann-like cells of sensory neuritis (Kwan, 2013). How their roles differ in skeletal muscle reinnervation remains poorly understood. The release of neurotrophic factors may be differentially regulated in these subpopulations. Schwann cells from young and old mice have been shown to differ both morphologically and in their ability to cover the motor endplate (Chai et al., 2011). The number of cells at the neuromuscular junction increases after skeletal muscle denervation, and based on Schwann cell markers, not all are Schwann cells (Magill et al., 2007). Future studies should determine the identity of the other cells and how they contribute to reinnervation. Whether pericytes participate remains largely unexplored.

Previous studies have demonstrated that CNS perivascular pericytes can form cells that express glial markers (Dore-Duffy et al., 2006; Bonkowski et al., 2011; Jung et al., 2011; Nakagomi et al., 2011). In skeletal muscle, cells in the interstitial space differentiate into glial lineage (Romero-Ramos et al., 2002; Alessandri et al., 2004; Kondo et al., 2006; Schultz and Lucas, 2006; Arsic et al., 2008; Birbrair et al., 2011). We recently demonstrated that under optimized skeletal muscle culture conditions, only type-2 pericytes form oligodendrocyte progenitors (Birbrair et al., 2013b,c), which produce mature oligodendrocytes and Schwann cells (Zawadzka et al., 2010). Whether type-2 pericytes contribute to Schwann cell populations generally and to the newly formed Schwann cells that participate in skeletal muscle reinnervation specifically remains to be addressed.

Also, to what degree do these endogenous pericytes influence the reoccupation of synaptic sites during reinnervation? Recently, cells isolated from adult human skeletal muscle were shown to differentiate into myelinating Schwann cells and to ameliorate a critical-sized sciatic nerve injury in a murine model. Denervated skeletal muscles from treated mice exhibit substantially decreased atrophy, and the motor endplates at the postsynaptic sites reorganize (Lavasani et al., 2014). Although this study provides evidence for the therapeutic capability of skeletal muscle-derived cells *in vivo*, it did not identify these cells. As the skeletal muscle cell environment is heterogenous, future work should focus on identifying muscle-derived cells that can repair the sciatic nerve after injury. We reported that only type-2 pericytes have gliogenic potential in the skeletal muscle (Birbrair et al., 2013c), so they are immediate candidates for testing. Tracking pericyte fate by an inducible Cre system after skeletal muscle denervation will be required to address pericyte potential to form Schwann cells.

As there are several types of Schwann cells (Kwan, 2013), it would be interesting to explore whether pericytes form a specific subtype. The Wnt/beta-catenin signaling pathway is active in NG2+ cells differentiating into NG2 glia cells in the brain (White

et al., 2010). As pericytes express NG2 proteoglycan and form NG2 glia cells (Birbrair et al., 2013b,c), this pathway might be activated to induce skeletal muscle reinnervation.

To understand the role of pericytes in neuromuscular junction regeneration, future studies should test the effect of ablating them after sciatic nerve injury. Mouse models using viral thymidine kinase to genetically deplete pericytes are viable and have been used recently (Cooke et al., 2012; Lebleu et al., 2013). As Schwann cells have the capacity to dedifferentiate into immature cells after sciatic nerve injury (Yang et al., 2012), do they ever become pericytes?

We envision that determining exactly what happens after denervation may provide cellular targets for pharmacological manipulation to improve skeletal muscle reinnervation. At present, the role of pericytes in this process is only speculative.

PERICYTE CONTRIBUTION TO VESSEL FORMATION IN SKELETAL MUSCLE

Blood supply to the skeletal muscle can be compromised after vascular injury, bone fracture, and crush injury (Blaisdell, 2002) and by complications due to such cardiovascular and metabolic diseases as atherosclerosis, heart failure, diabetes, and obesity (Baumgartner et al., 2005; Varu et al., 2010; Chi et al., 2011). In addition, the number of capillaries and arteries feeding the skeletal muscle decreases with age (Conley et al., 2000; Behnke et al., 2006), compromising its perfusion (Wahren et al., 1974; Irion et al., 1987). Compared to young mice, the capacity of old mice to form new blood vessels (angiogenesis) is impaired (Rivard et al., 1999; Shimada et al., 2004; Yu et al., 2006).

When the blood supply to tissue is partially obstructed, oxygen content decreases, leading to ischemia (Forsythe et al., 1996; Heil and Schaper, 2004). Collateral arteries and anastomoses can partially restore blood flow (Heil and Schaper, 2004), attenuating the damage caused by hypoxia, but even short-term ischemia induces necrosis, leading to inflammatory reactions. After only 5 h, an ischemic environment causes necrosis in 90 percent of skeletal muscle (Labbe et al., 1987). If revascularization fails, it can lead to limb amputation (Conrad et al., 2011). While exercise is a potent stimulus for new vessel formation in adult skeletal muscle (Booth and Thomason, 1991; Egginton, 2009), most of the conditions leading to skeletal muscle ischemia preclude exercise.

Angiogenesis is a complex process in which new blood vessels form from existing ones. It involves extensive interplay between cells and growth factors, extracellular matrix proteins, proteases, and adhesion molecules (Folkman, 1971; Caduff et al., 1986; Kilarski et al., 2009). The exact cellular mechanisms of physiological angiogenesis in skeletal muscle remain poorly understood. It requires the proliferation and migration of endothelial cells to line the interior of the blood vessels (Rousseau et al., 2000; Li et al., 2005; Lamalice et al., 2006). Macrophages and other inflammatory cells (Barbera-Guillem et al., 2002; Shireman, 2007) infiltrate the tissues after ischemia (Sica, 2010; Alexander et al., 2011) and, together with fibroblasts and myofibers (Gustafsson et al., 1999; Steinbrech et al., 1999), secrete such angiogenic molecules as vascular endothelial growth factor (VEGF), placenta growth factor (PIGF), fibroblast growth factor 2 (FGF2), and platelet-derived

growth factor (PDGF; Lewis and Murdoch, 2005; Murdoch and Lewis, 2005; Murdoch et al., 2005), which are necessary to construct new blood vessels and to restore blood perfusion.

Pericytes also participate in the formation of new blood vessels (Egginton et al., 1996; Hellstrom et al., 1999; Gerhardt and Betsholtz, 2003; Bergers and Song, 2005). This participation includes phenotype changes, migration, alignment, and contacts with endothelial cells. Pericytes are the first cells to invade newly vascularized tissues (Diaz-Flores et al., 1991; Nehls et al., 1992; Reynolds et al., 2000). The hypoxic state stimulates pericyte migration and angiogenesis (Murata et al., 1994). They adopt angiogenic phenotype by shortening their processes and increasing their somatic volume (Diaz-Flores et al., 1992b, 1994b). Autoradiographic studies show that the proliferation of these activated pericytes increases (Schoefl, 1963; Cavallo et al., 1972, 1973; Sholley et al., 1977; Burger and Klintworth, 1981; Diaz-Flores et al., 1992b, 1994a,b). Pericytes guide and determine where the newly formed blood vessels spread (Nehls et al., 1992; Tsuzuki and Sasa, 1994; Ozerdem et al., 2001; Morikawa et al., 2002; Ozerdem and Stallcup, 2003) and promote endothelial cell survival (Amselgruber et al., 1999; Morikawa et al., 2002; Darland et al., 2003; Kale et al., 2005). They can form tubes (Moldovan et al., 2000; Anghelina et al., 2002, 2004, 2006; Ozerdem and Stallcup, 2003) and penetrate endothelial cells. They prevent vessel regression (Benjamin et al., 1998, 1999; Enge et al., 2002). Pericytes express PDGF receptors and respond to PDGF (Balabanov et al., 1996). Their recruitment is crucial for vessel maturation, as the lack of PDGF disrupts vessel development (Lindahl et al., 1997; Hellstrom et al., 2001). Following exercise, NF- κ B, a strong inducer of angiogenesis, is activated in a pericyte sub-population (Hyldahl et al., 2011). Under hypoxic conditions, VEGF from pericytes can stimulate other pericytes to proliferate and migrate (Yamagishi et al., 1999).

The process of angiogenesis depends on appropriate cell signaling based in the tissue microenvironment, so most of our data comes from *in vivo* studies. Dissecting and occluding the femoral artery to induce brief ischemia is a common model for studying physiological angiogenesis (Shireman and Quinones, 2005; Westvik et al., 2009).

Therapeutic angiogenesis has been pursued as a potential treatment for ischemic disorders (Isner and Asahara, 1999; Ferrara and Kerbel, 2005; Giacca and Zacchigna, 2012). Its goal is to stimulate blood vessels to grow new blood vessels (Folkman, 1995; Isner, 1996; Ferrara and Kerbel, 2005). Several cell types have been used to induce neovascularization (Kalka et al., 2000; Hamano et al., 2001; Shintani et al., 2001; Iwase et al., 2005; Swijnenburg et al., 2005). Pericyte transplantation induces angiogenesis and improves blood flow to ischemic hindlimbs in animal models (He et al., 2010; Dar et al., 2012), and based on their role in forming and stabilizing engineered blood vessels, they have been proposed for angiogenic therapy. Surprisingly, not all pericytes can induce angiogenesis. Only type-2 has angiogenic potential *in vitro*, and, *in vivo*, angiogenesis occurs when type-2, but not type-1, pericytes are injected with endothelial cells in a Matrigel plug (Birbrair et al., 2014). Type-2 can also recover blood flow in a mouse model of hindlimb ischemia (Birbrair et al., 2014), but the mechanism remains to be elucidated (Birbrair

et al., 2014). Due to the short recovery time, the femoral artery is probably rebuilt by anastomoses of the proximal stump with new collateral blood vessels after intramuscular pericyte injection (Schaper and Scholz, 2003).

Pericyte subtypes in human skeletal muscle have not yet been identified and isolated, and whether their angiogenic potential differs remains unknown.

To study the physiological roles of different cell populations, genetic strategies to ablate specific cell types have been developed. Several transgenic mice now provide effective means to genetically ablate pericytes (Cooke et al., 2012; Lebleu et al., 2013), satellite cells (Dellavalle et al., 2011; Lepper et al., 2011; McCarthy et al., 2011), fibroblasts (Barnes and Glass, 2011), and macrophages (Ferenbach et al., 2012; Weisser et al., 2012) for a defined period. These studies will elucidate the exact role of each cell population in inducing and regulating skeletal muscle angiogenesis. For example, to determine whether endogenous pericytes are necessary for skeletal muscle angiogenesis after ischemia, transgenic mice with pericyte depletion, such as NG2-tk (Cooke et al., 2012; Lebleu et al., 2013), should be examined. Cell ablation studies must consider that besides pericytes, oligodendrocyte progenitors express NG2 (Encinas et al., 2011).

Understanding the molecular mechanisms of ischemia induced endogenous angiogenesis is critical. Genetically modified mice have been widely applied to study the signals required for postnatal hindlimb angiogenesis. Knockout mice allowed several groups to test whether such signals are necessary for skeletal muscle angiogenesis. Neovascularization of impaired ischemic limbs was found in mice deficient in angiotensin II type-1 receptor (Sasaki et al., 2002), endothelial-derived nitric oxide synthase (eNOS; Murohara et al., 1998), matrix metalloproteinase-9 (Johnson et al., 2004), caveolin-1 (Sonveaux et al., 2004), adiponectin (Shibata et al., 2004), PIGF (Carmeliet et al., 2001), and IL-10 (Silvestre et al., 2000). Are these molecules expressed in pericytes? The specific cell types essential to angiogenesis after hindlimb ischemia remain unclear. In addition, analysis of global knockout mutant mice is complicated by unrelated side effects in other tissues, which can be avoided only by performing conditional mutagenesis. When investigators can control the timing and location of somatic mutations in adult mice, they will be able to determine the roles of specific signaling molecules in different cell populations and the functional consequences of deleting single genes in specific cell types, such as pericytes, during skeletal muscle angiogenesis. The clinical need for interventions in ischemic illnesses leading to revascularization and the encouraging recent findings that pericytes have the potential to improve blood perfusion will stimulate these efforts.

PERICYTES IN SKELETAL MUSCLE FIBROSIS

Fibrosis is an incompletely understood process characterized by excessive accumulation of extracellular matrix components, such as collagen (Wynn, 2008). It occurs under chronic disease conditions and may affect skeletal muscle (Wynn, 2007). Fibrous tissue may prevent full functional recovery of skeletal muscle (Kasemkijwattana et al., 1998; Kaariainen et al., 2000; Huard et al., 2002; Jarvinen et al., 2005; Gharaibeh et al., 2012), which, under normal conditions, can repair itself after injury. Fibrosis

directly contributes to progressive skeletal muscle dysfunction in several chronic diseases, such as Duchenne muscular dystrophy (Mann et al., 2011; Brandan and Gutierrez, 2013; Morales et al., 2013; Acuna et al., 2014); and its treatment is currently considered important for muscular dystrophies. Furthermore, one of the causes of age-related skeletal muscle stiffness, weakness, and atrophy is increased infiltration of fibrous tissue (Ryall et al., 2008; Thompson, 2009; Kragstrup et al., 2011; Walston, 2012). The regenerative potential of muscle stem cells is limited by the formation of fibrous tissue, which lacks innervation and contractile properties (Juhas and Bursac, 2013). Studies performed in mouse models clearly associate skeletal muscle fibrosis with aging (Goldspink et al., 1994; Huard et al., 2002; Jarvinen et al., 2002; Brack et al., 2007; Zhu et al., 2007; Graham et al., 2010; Trenz et al., 2010). Understanding the cellular and molecular mechanisms underlying skeletal muscle fibrosis is essential to developing effective antifibrotic therapies.

While collagen accumulation is a major feature of skeletal muscle fibrosis (Mohan and Radha, 1980; Alnaqeeb et al., 1984; Goldspink et al., 1994; Haus et al., 2007; Kragstrup et al., 2011), the source of the collagen-producing cells in various conditions is less clear. Many have been proposed, including resident fibroblasts (Thiery et al., 2009; Lieber and Ward, 2013), muscle-derived stem cells (Li and Huard, 2002), myoblasts (Li et al., 2004; Alexakis et al., 2007), endothelial cells (Zeisberg et al., 2007), pericytes (Birbrair et al., 2013d), fibroadipogenic progenitors (FAPs; Joe et al., 2010; Uezumi et al., 2011), fibrocytes (Herzog and Bucala, 2010), and even nerve-associated cells (Hinz et al., 2012). However, the exact role of these cells in skeletal muscle fibrosis is unclear, and the origin of collagen-producing cells has not been confirmed using the same methodologies (*in vitro* or *in vivo*). Furthermore, skeletal muscle fibrosis induced in different ways may recruit different cell populations. For instance, some fibrosis mouse models are reversible, and collagen production may be part of the repair process. Given the large number of possible cell sources, future studies will have to use modern molecular techniques, such as fate-mapping, to create strategies to reverse skeletal muscle fibrosis.

Chronic activation of PDGFR α results in widespread organ fibrosis (Olson and Soriano, 2009), indicating that PDGFR α + cells may have a role in skeletal muscle fibrosis. Type-1 pericytes and FAPs express this receptor (Joe et al., 2010; Uezumi et al., 2010, 2011; Birbrair et al., 2013a), and like pericytes, FAPs line the skeletal muscle vasculature (Joe et al., 2010), suggesting their roles may overlap (Birbrair et al., 2013a). The extent of perivascular PDGFR α + cells' contribution to skeletal muscle fibrosis has not been demonstrated. Future studies should use fate-mapping of endogenous skeletal muscle PDGFR α + cells exposed to distinct conditions leading to fibrosis. Determining whether PDGFR α + pericytes and PDGFR α + FAPs are lineage-related and whether their roles in skeletal muscle fibrosis vary would also be interesting.

As with PDGFR α , the selective overexpression of a disintegrin and metalloprotease 12 (ADAM12) in the skeletal muscle increases fibrosis and suppresses regeneration (Jorgensen et al., 2007). In an elegant study, researchers used an inducible, tetracycline-dependent, cell-fate mapping system. They generated

triple transgenic mice that expressed tetracycline transactivator under control of the ADAM12 locus, the conditional reporter RosaYFP, and the recombinase Cre under control of the tetracycline transactivator. Labeling the cells derived from ADAM12+ cells was temporally controlled by administering doxycycline, which prevents Cre expression, and they inducible genetic ablation of ADAM12+ cells in the skeletal muscle. Their findings revealed that pericytes expressing ADAM12 during development, located in very close proximity to blood vessel endothelial cells, give rise to most of the collagen-producing cells during skeletal muscle injury (Dulauroy et al., 2012). However, fibrosis formation was analyzed in healthy, young skeletal muscle after injury. Future studies should explore whether the collagen-producing cells in the skeletal muscle of mdx and old mice have the same ancestors.

A new finding complements the evidence that pericytes are the source of collagen-producing cells in the fibrous tissue deposited in old skeletal muscle. Birbrair et al. (2013d) found that the pericytes involved in scar formation in the skeletal muscle of old mice differ from those associated with skeletal muscle regeneration; only type-1 pericytes contribute (Birbrair et al., 2013d). Future studies should use this pericyte subpopulation as a cellular target to reduce fibrosis in older mammals.

The cellular source of fibrosis in chronic diseases, such as Duchenne muscular dystrophy, remains unknown. To what extent type-1 pericytes contribute to collagen-producing cells in the skeletal muscle in comparison with other cell populations that give rise to those cells is also unclear. The detailed fate-mapping and lineage-tracing experiments that confirm pericyte participation in skeletal muscle fibrosis have not been done for other possible cellular sources of collagen-producing cells.

The basic molecular mechanisms involved in fibrous tissue deposition in the skeletal muscle are not completely understood. The well-studied cytokine transforming growth factor β (TGF β), which is released from injured myofibers, seems to be essential to fibrous tissue formation (Massague, 2012). It binds to transmembrane receptor TGF β receptor type II, recruiting TGF β receptor type I to the complex. Both receptors have serine/threonine kinase activity and form heteromeric complexes in the presence of the activated ligand. TGF β binding to the extracellular domains of type I and type II receptors initiates signaling cascades across the cell membrane by inducing transphosphorylation. It subsequently activates the type I receptor at the glycine/serine (GS)-rich domain, which acts as a phosphorylation site with receptor kinase activity (Kang et al., 2009). The type 1 receptor then catalyzes activation of the intracellular SMAD transcription factors (Massague et al., 2005), which stimulate transcription of specific target genes, leading to the production of extracellular matrix proteins and fibrosis formation (Lieber and Ward, 2013) that interfere with skeletal muscle regeneration and function (Gharaibeh et al., 2012). In contrast, inhibiting TGF β reduces fibrosis and promotes muscle regeneration (Fukushima et al., 2001; Sato et al., 2003).

TGF β is involved in a range of biological processes (Heldin et al., 2009; Padua and Massague, 2009; Hawinkels and Ten Dijke, 2011; Dooley and Ten Dijke, 2012; Pardali and Ten Dijke, 2012). Thus, detailed understanding of which cells respond to

its signaling is required for the design of effective therapeutic approaches without undesirable side effects. At least *in vitro*, type-1 pericytes respond to TGF β , increasing type I collagen production (Birbrair et al., 2013d), while type-2 did not seem to respond under the same conditions (Birbrair et al., 2013d). Future experiments should test whether TGF β signaling is required for pericytes to participate in skeletal muscle scarring *in vivo*. One indication for this requirement is that TGF β induces ADAM12 expression (Solomon et al., 2010; Dulauroy et al., 2012), which plays an important role in pericytes' fibrotic response.

Another member of the TGF β protein superfamily, myostatin (McPherron et al., 1997), also known as GDF-8, not only controls skeletal muscle growth, but also regulates the progression of fibrosis (Li et al., 2008). Connective tissue growth factor (CTGF) is another molecule that has been shown to reproduce many of the profibrotic effects of TGF β in skeletal muscle. Elevated levels of CTGF have been detected in skeletal muscle from mdx mice, dystrophic dogs, and patients with Duchenne muscular dystrophy (Sun et al., 2008; Vial et al., 2008). Whether pericytes express receptors and respond to myostatin and CTGF has yet to be explored. TGF β can also induce production of PDGFs (Bonner, 2004). As pericytes express receptors to these ligands (Hellstrom et al., 1999), whether this signaling pathway plays a role in skeletal muscle fibrosis *in vivo* should be explored. Fibroblasts that express MMP9 and PlGF help to recover the vascular network structure by diminishing collagen deposition in the skeletal muscle of old dystrophic mice (Gargioli et al., 2008). Whether the expression of these factors differs between the two pericyte subtypes is unknown.

For full functional recovery of skeletal muscle affected by chronic diseases, aging, and trauma, fibrosis must be limited. Effective repair of skeletal muscle under these conditions cannot be achieved yet. More studies are needed to define the cellular and molecular mechanisms and functional significance of fibrosis in healthy, young and diseased, old skeletal muscles. Although pericytes play an important role in this process, detailed fate-mapping and lineage-tracing experiments would significantly advance the field.

PERICYTE CONTRIBUTION TO FAT ACCUMULATION IN SKELETAL MUSCLE

Accumulation of ectopic adipocytes in skeletal muscle is typical of such disorders as obesity, sarcopenia, and dystrophies and provides an accurate assessment of the severity of Duchenne muscular dystrophy (DMD) (Wren et al., 2008). Increased fat is also observed in the skeletal muscle of older adults (Goodpaster et al., 2004; Goodpaster and Wolf, 2004; Visser et al., 2005).

The origin of these fat cells has been revealed only recently. A group of cells in the perimysium, particularly the perivascular space (Greco et al., 2002), where fat accumulation is most evident, express platelet-derived growth factor receptor α (PDGFR α), the major contributor to ectopic fat cell formation in skeletal muscle. These cells are quiescent in intact muscle but proliferate efficiently in response to damage. PDGFR α + cells differ from satellite cells and are located in the muscle interstitial space between myofibers, close to blood vessels (Joe et al., 2010; Rodeheffer, 2010; Uezumi et al., 2010). Skeletal muscle pericytes can differentiate *in vitro*

toward adipogenic lineage (Farrington-Rock et al., 2004; Crisan et al., 2008a) but, like PDGFR α + cells, do not generate myofibers, and only type-1 express PDGFR α . When purified type-1 pericytes are delivered intramuscularly in a mouse model of fatty infiltration, ectopic white fat is generated (Birbrair et al., 2013a). This approach clearly identifies their adipogenic potential, but only lineage-tracing will demonstrate that type-1 pericytes become fat cells in skeletal muscle *in vivo* under physiological conditions.

Whether perivascular PDGFR α + cells have a physiological role in the various illnesses characterized by muscular ectopic fat accumulation, such as myopathies and obesity, and whether modifying cell properties by manipulation and grafting would influence their fate *in vivo* are unclear. Although genetic tracing techniques were used to track these cells in other tissues (Lee and Granneman, 2012), confirming their capacity to become fat cells in skeletal muscle will require lineage-tracing studies. Testing whether depleting specific PDGFR α + perivascular cells would prevent fat formation in skeletal muscle would also be interesting.

Is activating PDGFR α important for the adipogenetic role of PDGFR α + perivascular cells in skeletal muscle? Most of the primary functions of PDGF α and platelet-derived growth factor receptor α (PDGFR α), were unknown because Pdgfa and Pdgfra knockout mice die either as embryos or shortly after birth. Recent experiments using conditional gene ablation and gain-of-function transgenics (Gnessi et al., 1993; Bostrom et al., 1996; Soriano, 1997; Fruttiger et al., 1999; Karlsson et al., 1999, 2000) showed that PDGFR α receptors are crucial for the proper development of several tissues (Crosby et al., 1998; Bostrom et al., 2002; Ostman, 2004). After ligand binding, the kinase domains of PDGFR α phosphorylate tyrosine residues of the receptor's cytoplasmic domain, which act as docking sites for phosphatidylinositol 3-kinase, STATs, SRC family kinases, SHP2 phosphatase, and phospholipase Cy (Vignais and Gilman, 1999; Lakner et al., 2010; Xiong et al., 2010; Lin et al., 2014). These pathways regulate such transcription factors as SREBP, FOXO, c-MYC, and AP1, which are involved in cell growth, proliferation, differentiation, survival, and migration (Besancon et al., 1998; Tsatsanis and Spandidos, 2000; Guida et al., 2007; Erovic et al., 2012). They have also been linked to diseases characterized by fat accumulation in blood vessel walls, such as atherosclerosis (Tedgui and Mallat, 2006; Artwohl et al., 2009; Feinberg, 2013; Li et al., 2013). However, the function of PDGFR α ligands and their receptors in skeletal muscle adipogenesis remains unclear. The pericyte marker neural/glial antigen 2 (NG2) proteoglycan (Ozerdem et al., 2001) binds to PDGFR α (Goretzki et al., 2000) and may function as its co-receptor with a potential effect on the respective cell-surface signaling receptor (PDGFR α) (Grako and Stallcup, 1995; Grako et al., 1999). Future studies may determine whether the fate of PDGFR α + pericytes changes when they are exposed to PDGFR α and whether their differentiation potential remains unchanged after exposure to PDGFR α -Fc chimeric receptors, which compete with their receptors for ligands *in vitro*. Loss-of-function and gain-of-function assays may demonstrate whether PDGFR α in pericytes regulates fat formation in skeletal muscle.

Systemic factors, such as hormone levels and nutrients, may play a role in regulating PDGFR α + cells' adipogenic potential. For instance, a high-glucose medium was reported to

enhance adipogenic differentiation of skeletal muscle-derived cells (Aguiari et al., 2008), suggesting that the microenvironment may determine the fate of cells that sense changes in skeletal muscle physiology. The ability to target skeletal muscle PDGFR α + perivascular cells exclusively will open new therapeutic strategies for skeletal muscle diseases caused by, or associated with, severe adipose tissue accumulation.

Pericyte participation in fat infiltration of skeletal muscle has been confirmed (Birbrair et al., 2013a), providing a cellular target susceptible to pharmacological modulation and signaling manipulation. This strategy will require more detailed analyses.

PERICYTES AND ECTOPIC BONE FORMATION IN SKELETAL MUSCLE

Heterotopic ossification, the ectopic formation of bone and/or cartilage in soft tissues, such as skeletal muscles outside the periosteum, happens only in genetic disorders, such as fibrodysplasia ossificans progressiva and progressive osseous heteroplasia (Adegbite et al., 2008; Yu et al., 2008; Kaplan et al., 2012). In the skeletal muscles of mdx mice (dystrophin-deficient mouse model of Duchenne muscular dystrophy) (Kikkawa et al., 2009; Mu et al., 2013), this debilitating condition may be induced by the inflammation associated with trauma. However, other causes have been reported (Thorseth, 1968; Sirvanci et al., 2004; McCulloch and Bush-Joseph, 2006; Bek et al., 2009; Kim and Choi, 2009; Chouhan et al., 2012; Kalenderer et al., 2012).

The biological mechanism leading to osteoinduction in the skeletal muscle under physiological conditions has not been identified, and the exact cellular origin of heterotopic ossification is not well characterized. Nevertheless, recent sophisticated studies have made advances. The use of Cre/loxP technology allows investigators to track specific cell lineages (Liu et al., 2004; Maes et al., 2010). Several studies used murine models harboring real-time visual transgenes and Cre/loxP technology as a powerful way to identify which cells in skeletal muscle give rise to bone-forming cells (Kan et al., 2009, 2013; Lounev et al., 2009; Medici et al., 2010; Chakkalakal et al., 2012). Injury may provoke a local inflammatory reaction, and cytokines released into the blood might prompt circulating immune cells to differentiate into osteoblasts. However, using CD19-Cre, LCK-Cre, and Lyz-Cre transgenic mice, researchers have shown that B cells, T cells, and macrophages/monocytes, respectively, do not generate them (Kan et al., 2009). Somite-derived cells were excluded using Nestin-Cre reporter mice, and myoblasts, which are more committed to the myogenic lineage, were excluded using Myf5-Cre (Kan et al., 2009) and MyoD-Cre (Lounev et al., 2009) transgenic mice. These results are consistent with the fact that, during the generation of ectopic bone, the early immune response in skeletal muscle lesions kills myoblasts (Shore and Kaplan, 2010).

Recent investigations have suggested that cells residing in the skeletal muscle interstitial space contribute to some of the ectopic bone tissue (Woszczyna et al., 2012). However, their precise identity was not determined (Bosch et al., 2000). Histological analyses of heterotopic lesions from patients with fibrodysplasia ossificans progressiva demonstrate positive staining for endothelial markers, such as the angiopoietin receptor, Tie2, in ectopic chondrocytes and osteoblasts, suggesting a possible role

for endothelial cells (Lounev et al., 2009; Medici et al., 2010). However, this marker is not specific to endothelial cells; in fact, it is also expressed in pericytes (Park et al., 2003; Cai et al., 2008). Cells expressing Tie2 receptor respond to inflammatory triggers, differentiate into osteogenic lineage, and contribute greatly to heterotopic bone in animal models of fibrodysplasia ossificans progressiva (Lounev et al., 2009; Woszczyna et al., 2012). Lineage-tracing studies using Tie2-Cre reporter mice have also pointed to these cells in generating the chondrocytes and osteoblasts found in skeletal muscle lesions (Lounev et al., 2009; Medici et al., 2010; Chakkalakal et al., 2012). Other analyses revealed that skeletal muscle osteogenic progenitors, distinct from satellite cells, express PDGFR α (Oishi et al., 2013). They undergo osteogenic differentiation both *in vitro* and *in vivo* in response to osteogenic conditions and/or BMP stimuli (Uezumi et al., 2010; Oishi et al., 2013) and have been observed surrounding ectopic bone tissues after trauma in humans. In skeletal muscle, PDGFR α + cells accumulate around blood vessels (Uezumi et al., 2014) and include type-1 pericytes (Birbrair et al., 2013a), suggesting that a pericyte subpopulation may also form ectopic bone in skeletal muscle. Blood vessels could be a source of osteogenic progenitor cells, which differentiate into osteoblasts, for example, when inflammatory cytokines are released by macrophages. However, this hypothesis has not been tested experimentally.

Skeletal muscle pericytes have chondrogenic potential *in vitro* (Crisan et al., 2008a), and Li et al. (2011) showed that cells residing in the skeletal muscle fascia have strong chondrogenic potential but lack pericyte marker CD146. Crisan et al. (2008b) also reported that vascular pericytes may differentiate into osteoblasts. Levy et al. (2001) and other groups suggested a similarity between osteoprogenitors in the skeletal muscle and pericytes isolated from intramuscular connective tissue (Diaz-Flores et al., 1992a; Gronthos and Simmons, 1996; Reilly et al., 1998; Kuznetsov et al., 2001; Levy et al., 2001; Crisan et al., 2008a). Whether they contribute to the heterotopic ossification that occurs in skeletal muscle *in vivo* is not known. Although in bone marrow pericytes are capable of bone formation (Shi and Gronthos, 2003; Sacchetti et al., 2007; Mendez-Ferrer et al., 2010b), their characteristics may vary significantly between tissues (Armulik et al., 2011).

A recent lineage-tracing study using GLAST-CreER mice identified GLAST-expressing cells as precursors that contribute to heterotopic ossification (Kan et al., 2013). GLAST (glutamate aspartate transporter) is expressed in various central nervous system (CNS) cells, such as Muller, Bergmann glia, astrocyte, and neural stem cells (Danbolt et al., 1992; Levy et al., 1993; Lehre et al., 1995; Shibata et al., 1997; Izumi et al., 2002; Slezak et al., 2007; Ehm et al., 2010), but can also be found in other cell types, such as pericytes in the spinal cord (Goritz et al., 2011). Whether GLAST is expressed in cells outside the CNS is not known, and verifying whether skeletal muscle pericytes express GLAST would be especially interesting. Supporting this idea, most GLAST-creER labeled cells in skeletal muscle interstitium were closely associated with vasculature (Kan et al., 2013). This study did not specify the cellular origin, but approximately 35% of the ectopic bone-producing cells in the lesions clearly belong to a GLAST-expressing lineage. Is a specific pericyte subpopulation responsible? What is its contribution compared to other cell

types? The possibility that osteoprogenitor cells might originate from perivascular cells highlights the strong association between angiogenesis and the heterotypic ossification of skeletal muscle (Hegyi et al., 2003).

The basic molecular mechanisms involved in ectopic calcification in skeletal muscle are not known. Critical inductive factors and a permissive environment may affect specific cell types and contribute to heterotopic ossification (Chalmers et al., 1975; Baird and Kang, 2009). A recent study suggests that the same mechanism that induces vascular calcification gives rise to osteoprogenitor cells (Yao et al., 2013). Elucidating these mechanisms is important since we have no effective and safe therapy to prevent this condition. Regulatory molecules acting on the perivascular cells necessary for the development of traumatic heterotopic ossification should be further investigated.

The bone morphogenetic proteins (BMP) of ALK2 ligands, such as BMP2, BMP4, and BMP9, might be primary inducers of heterotopic ossification; mixed with Matrigel and injected into the skeletal muscles of mice, they have osteoblastic activity (Chen et al., 2012; Nishimura et al., 2012), and they are highly expressed in human lesions with heterotopic ossification (Gannon et al., 1997; Grenier et al., 2013). Transgenic mouse models of heterotopic ossification with specific signaling molecules, such as BMPs, deleted should be used in different cellular targets to verify whether those molecules are essential for *in vivo* ectopic bone formation in skeletal muscle in physiological conditions. Future studies should determine whether BMP receptors are expressed in pericytes. Additionally, the fate of pericyte subtypes exposed to BMPs should be investigated to determine whether their differentiation potential remains unchanged after exposure to BMPR-Fc chimeric receptors, which compete with pericyte receptors for ligands. Future efforts should focus on the activation of osteogenic potential by such less-studied molecules as the growth factor Nell-1, which induces osteogenic differentiation in pericytes (Zhang et al., 2011). The discovery of such signals and a better understanding of the exact role of pericytes in skeletal muscle ectopic calcification would support development of therapeutic strategies to treat this clinically significant condition.

CONCLUSIONS

Pericytes play several critical roles in skeletal muscle repair, and elucidating how their tissue-formation capabilities contribute to skeletal muscle pathophysiology will be important to future treatments. Based on their molecular markers and specific functions, muscular pericytes have been identified as heterogeneous, and at least two subpopulations have been described. Taking their diversity into account, information regarding pericytes will be crucial in advancing our understanding of skeletal muscle disease and aging.

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Potential of adipose-derived stem cells in muscular regenerative therapies

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Regenerative capacity of skeletal muscles resides in satellite cells, a self-renewing population of muscle cells. Several studies are investigating epigenetic mechanisms that control myogenic proliferation and differentiation to find new approaches that could boost regeneration of endogenous myogenic progenitor populations. In recent years, a lot of effort has been applied to purify, expand and manipulate adult stem cells from muscle tissue. However, this population of endogenous myogenic progenitors in adults is limited and their access is difficult and invasive. Therefore, other sources of stem cells with potential to regenerate muscles need to be examined. An excellent candidate could be a population of adult stromal cells within fat characterized by mesenchymal properties, which have been termed adipose-derived stem cells (ASCs). These progenitor adult stem cells have been successfully differentiated *in vitro* to osteogenic, chondrogenic, neurogenic and myogenic lineages. Autologous ASCs are multipotent and can be harvested with low morbidity; thus, they hold promise for a range of therapeutic applications. This review will summarize the use of ASCs in muscle regenerative approaches.

Keywords: adipose, stem cells, muscle, regeneration, reprogramming, transdifferentiation, transplantation

Introduction

One major challenge of modern medicine is to repair damaged tissues. Self-adult stem cells for regenerative therapies could overcome transplantation limitations such as organ rejection or shortage of organs available for donation. Furthermore, many degenerative diseases such as muscular dystrophies have no cure and the possibility to replace damaged muscle of the whole body by transplantation, nowadays, is not feasible. In dystrophic patients, continuous myogenic regeneration is sustained by resident stem cells. However, stem cell pool is eventually depleted, which correlates with aggravation of the disease and deterioration of patients. Consequently, cellular regenerative therapies that may replenish myogenic stem cell populations and counteract muscle loss by producing healthy muscle could represent a new treatment to improve quality of life and prevent early death for dystrophic patients. The potential use of adult adipose-derived stem cells (ASCs) for muscle regeneration will be discussed.

Myogenic Progenitors from Different Sources

Intensive research has been done to identify different types of myogenic progenitors in muscles that could be isolated, manipulated and expanded *in vitro* for therapeutic use

reviewed in (Motohashi and Asakura, 2014). The classical stem cell from muscle is the satellite cell (Mauro, 1961; Montarras et al., 2005; Scharner and Zammit, 2011), however, in recent years several groups have described other myoprogenitors in muscle tissue such as muscle side-population cells (Gussoni et al., 1999; Asakura and Rudnicki, 2002), muscle-derived stem cells (Qu-Petersen et al., 2002), interstitial cells (Mitchell et al., 2010), and muscle-derived CD133 + stem cells (Benchouir et al., 2007). Other cells present in muscles called fibroadipogenic progenitors have been shown to crosstalk with satellite cells to enhance myogenesis (Joe et al., 2010; Uezumi et al., 2010). Although muscle tissue seems to contain a high concentration of progenitors (it is estimated that 550 satellite cells are present in 1 mg of muscle tissue, Bentzinger et al., 2012), muscle biopsies are difficult to obtain and are small, therefore the yield of progenitors that can be obtained seems to be insufficient for regenerative therapies. One approach to solve this hurdle would be to expand progenitor population's *ex-vivo*, but unfortunately it has been shown to result in low engraftment rates and failure to improve muscle strength in Duchenne muscular dystrophy (DMD) patients (Mendell et al., 1995; Vilquin, 2005). Interestingly, growing those progenitors in different extracellular matrices and hydrogel platforms that better mimic *in vivo* niche, could overcome this problem (Gilbert et al., 2010). Nevertheless, satellite cells from Duchenne dystrophic patients proliferate less, undergo rapid senescence (Cossu and Mavilio, 2000) and are difficult to obtain due to fiber fragility (Boldrin and Morgan, 2012). In addition, muscle biopsies are still painful and can cause irreversible damage to donors.

Therefore, additional sources of myogenic stem cells have been explored such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPS). Ectopic expression of two regulators of myogenic transcription, BAF60c and MyoD, were able to differentiate hESC towards myogenic lineage, resulting in three-dimensional contractile structures called myospheres (Albini et al., 2013). Whether those myospheres could engraft in damaged muscle and contribute to regeneration was not tested, nevertheless they could be a tool for drug discovery to identify molecules that potentiate myogenesis. In addition, myospheres generated from dystrophic patients' iPS cells could open the way for tailored treatments in the future. Overexpression of paired-box transcription factors such as PAX3 (master regulator of embryonic myogenic program) in mouse ESCs, and PAX7 (involved in maintenance of satellite cell compartment) in human ESC and iPS cells, converted these cells to myogenic lineage. Those cells were then transplanted into immunodeficient Duchenne muscular dystrophic mice, engrafted successfully to supply dystrophin and repaired muscle strength. Importantly, in case of PAX7-ESCs/-iPS the stem cell pool was restored (Darabi et al., 2008, 2011, 2012). However, safety use of these genetically modified embryonic or pluripotent cells needs to be carefully addressed before they can be used in clinical setting. Some concerns using these former approaches are related to lentiviral usage for genetic modification of cells and the need to fully reprogram them in order to avoid teratoma

formation and tumor progression when transplanted to patients.

A safer and ethical-acceptable alternative could be the use of adult multipotent stem cells such as mesenchymal stem cells (MSCs), which are self-renewable and still able to differentiate to several lineages *in vitro*. MSCs were first identified in bone marrow (BM) by Friedenstein and colleagues (Friedenstein et al., 1966, 1968), and subsequently MSCs have also been found in many other adult tissues such as adipose, synovial membrane, dermis, periosteum, dental pulp, peripheral and menstrual blood, liver, spleen and lung (da Silva Meirelles et al., 2006; Kassis et al., 2006; Zou et al., 2010). One of most extensively studied MSC is the BM-derived, which has been shown to differentiate towards myogenic lineage *in vitro* (Pittenger et al., 1999). BM transplants contributed to muscle regeneration in cardiotoxin-injured muscles and in *mdx* mice (a model for DMD; Ferrari et al., 1998; Bittner et al., 1999; Gussoni et al., 1999; Fukada et al., 2002; Bossolasco et al., 2004). However, engraftment efficiency was low and further research estimated that muscle repair by BM-derived cells did not exceed 1% of total muscle fibers during the lifespan of transplanted *mdx* mice. In addition, this procedure did not contribute to ameliorate muscular dystrophy symptoms (Ferrari et al., 2001). Most of these works used whole BM populations and not purified MSCs; thus, it is possible that limited myogenic potential was due to little MSCs presence in total BM cells, being less than 0.01% in harvests that approximately yield 6×10^6 cells per mL (Pittenger et al., 1999). In order to increase myogenic engraftment of transplanted cells, BM-derived MSCs were expanded and genetically modified *ex-vivo* to express PAX3. When transplanted in dystrophic mice, PAX3 BM-derived MSCs were able to activate myogenic program and to fuse successfully with injured muscles, showing higher engraftments (10.8 + 3.6%) than previous works. Surprisingly and unfortunately, the expected functional recovery was not observed (Gang et al., 2009).

Another MSC type is adipose-derived mesenchymal stem cell, which is now termed ASC. ASCs were characterized by Patricia Zuk and colleagues, who isolated ASCs from lipoaspirates, expanded them *in vitro* and showed their multipotentiality towards not only adipogenic but also osteogenic, chondrogenic, neurogenic and myogenic lineages upon culture with defined media (Zuk et al., 2001). Could ASC represent a new and powerful source of myogenic progenitors for regenerative therapies?

Adipose Stem Cell Characterization

Compared to harvesting BM-derived MSCs, which requires drilling into the bone, ASCs can be obtained by liposuction, which is a less invasive procedure and may be more attractive for donors due to its positive esthetic results. In addition, stem cell yields are higher from adipose tissue, with 1 g containing an average of 2×10^6 cells with 10% being ASCs (Aust et al., 2004; Oedayrajsingh-Varma et al., 2006). For this reason, adipose tissue represents an abundant and

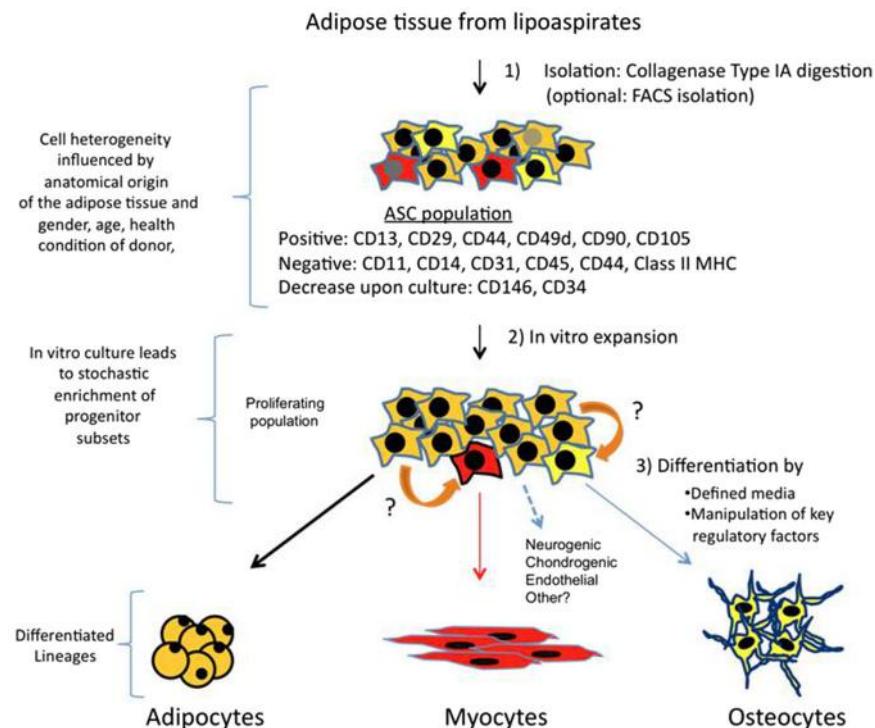


FIGURE 1 | Isolation, expansion and differentiation of ASCs. Adipose tissue is obtained from lipoaspirates but it can also be obtained from other surgeries. Upon digestion with collagenase type IA followed by centrifugation; the pellet obtained is known as SVF. SVF is a mixture of several types of progenitors and more differentiated cells. A majority of cells (90–100%) are positive for mesenchymal stem cell surface markers, and this fraction is the ASCs. Other markers such as CD146 and CD34 are more controversial, and may represent subsets of other progenitors, which vary in their ratios depending on anatomical origin of fat and other parameters related to donors. *In vitro* culture of this proliferating

population, which can arrive to 70 PDs, can also alter percentages of specific progenitors. Therefore, differential expression of CD markers and their fluctuations may represent a heterogenic composition of ASCs, which contain subsets of multipotent cells that can respond to differentiation cues of other lineages (represented in red and yellow). Adipocyte is the main lineage obtained from ASCs, however, upon culture with defined media, or by ectopically expressing specific factors, myogenic, osteogenic and other lineages can also be obtained. It is also possible that progenitors can transdifferentiate (indicated by orange arrows) in response to lineage specific cues.

practical source of multipotent stem cells for autologous and heterologous cell transplantation approaches compared to other tissues.

The protocol for ASCs isolation comprises an enzymatic digestion of adipose tissue or lipoaspirate, with collagenase type IA, followed by centrifugation (Figure 1). The pellet is called stromal vascular fraction (SVF), which contains a heterogeneous population of cells: fibroblast, red blood cells, smooth muscle cells, pericytes, and preadipocytes. Freshly isolated SVF can be plated with growth media (typically DMEM + 10% FBS) where a population of adherent cells proliferates and can be expanded for several passage doublings (PDs) in most cases without karyotype abnormalities (more than 70 PDs reported by; Rodriguez et al., 2005). This adherent population is considered to be ASCs, which is characterized to be positive for a specific set of surface mesenchymal markers: CD13, CD29, CD44, CD49d, CD90, CD105 and negative for hematopoietic markers such as CD11, CD14, CD31, CD45 and CD144 (Zuk et al., 2002; Katz et al., 2005; Mitchell et al., 2006; Yoshimura et al., 2006; Varma et al., 2007;

Zannettino et al., 2008). Other CD markers have been more controversial and with expressions showing variable percentages amongst total ASCs population. For instance, many works report that CD146 is not expressed by ASCs, however there is data suggesting that a subset of ASCs express CD146 and localize to areas surrounding blood vessels (Crisan et al., 2008; Zannettino et al., 2008; Cai et al., 2011), arguing in favor that a pericyte multipotent population resides in adipose tissue. A CD34 + CD146- population with multipotent abilities has been detected as well in the outer adventitial ring of vasculature (Traktuev et al., 2008; Zimmerlin et al., 2010). Therefore, all these works suggest that several lineage precursors may be forming part of ASCs population (Zeve et al., 2009) and that their relative composition present in isolated SVF may reflect different anatomic origins of adipose tissues, as well as other characteristics associated to donors such as age, body mass index, gender and health conditions. In other words, the particular niche of adipose tissue has an important role to determine type and quantities of progenitors, and a more detailed analysis needs to be performed in order to fully understand

which subtypes of progenitors are better suited for a specific regenerative aim.

Furthermore, it is important to note that upon culture for several weeks some CD markers have been reported to increase (CD105, CD166) while others such as CD146 and CD34 decrease dramatically (Mitchell et al., 2006; Varma et al., 2007). This can be explained by enrichment of certain cell types during culture where diverse parameters such as composition of basal media, supplements, plating substrates or cell confluence could have an impact on this issue. The media used for amplifying proliferating ASCs is a regular 10% FBS in DMEM, and using other media with specific growth factors and cytokines has not been tested systematically to understand how it could impact on maintaining or selecting a certain progenitor population. Conveniently, longterm cultured ASCs express very little amounts of major histocompatibility complex class I proteins and do not express class II proteins (HLA-DP, DM and DR), which are crucial for antigen presentation. As a result, absence of these molecules could give an advantage for ASCs therapeutic usage, allowing evasion of host's immune response in heterologous transplantations (Lin et al., 2012). Whether ASCs would upregulate human leukocyte antigen (HLA) proteins upon myogenic differentiation is not known.

***In vitro* Skeletal Muscle Differentiation Potential of ASCs**

The ability of ASCs to differentiate *in vitro* towards myogenic lineage has been reported by several groups. Culture of ASCs with myogenic differentiation media has resulted in ASCs adopting an elongated morphology, similar to differentiating myoblasts, and expression of early (MyoD1, myogenin) and late (myosin heavy chain) markers of muscle differentiation (Zuk et al., 2001; Mizuno et al., 2002; Zheng et al., 2006). These and other works show variable differentiation efficiencies in terms of onset and fusogenic ability, which may be the result of using different inductive media (**Table 1**). Co-culture experiments with myoblasts have shown that secreted factors and cell-to-cell contacts induce ASCs myogenic conversion and fusion with mouse C2C12 and primary myoblasts (Lee and Kemp, 2006; Di Rocco et al., 2006; Eom et al., 2011). Variations in fusogenic ability were observed when hASCs were treated with myogenic differentiation media before co-culture, and the highest efficiency was obtained when ASCs were already at late stages of myogenic differentiation and expressing myosin heavy chain (MyHC) and dystrophin (Eom et al., 2011).

Importantly, for regenerative purposes as a treatment for dystrophies, ASCs should also be able to fuse with dystrophic myotubes, for this reason, Vieira and colleagues showed that *in vitro* human ASCs could indeed fuse with DMD-derived myoblasts to form myotubes that recovered dystrophin expression (Vieira et al., 2008a). Therefore, these experiments support the use of allogeneic ASCs not only for their conversion towards the myogenic lineage and thus contributing to counteract muscle loss, but also as providers of WT dystrophin

and other potential beneficial factors that may be missing in dystrophic muscles. The use of coatings and hydrogels has shown to enhance myogenic differentiation from ASCs (Choi et al., 2012). However, at present, a universal media composition for a robust myogenic differentiation of ASCs is missing.

Table 1 summarizes the results showed in these works and indicates the composition of differentiation media used in each of them.

***In vivo* Skeletal Muscle Differentiation Potential of ASCs**

All these *in vitro* works indicate that different stimuli can potentiate and promote ASCs differentiation towards myogenic lineage, ranging from hormones and growth factors present in media (or secreted by cells), to cell-to-cell contacts or even by other mechanophysical inputs such as plating surfaces. However, whether ASCs could contribute to myogenic regeneration *in vivo* was not clear until the work of Rodriguez et al. (2005). In this work, the authors characterized *in vitro* human ASCs from different young donors and prior to transplantation in *mdx* mice, they identified two multipotent populations, one characterized to be fast adherent and expandable *in vitro* for more than 200 passages (chosen for transplantation experiments), and a slower adherent population that showed senescent features upon long term culture. Interestingly, fast-adherent ASCs at low-passages were HLA-I positive and HLA-II negative whereas at later passages were HLA-I low and HLA-II negative. Therefore, to assess whether absence of major histocompatibility complex could be advantageous to evade host immune's system, the transplantations were performed in *mdx* mice that were treated or not with 10 mg of cyclosporine A/kg (daily i.p. injection) to immunosuppress the host. Human ASCs (1.5×10^5) from long-term passages (160 PDs) were injected in left tibialis anterior (TA) muscle. Transplanted animals showed expression of dystrophin at several days post-transplantation, and human nuclei (elegantly revealed by FISH with a specific probe for human centromeres) were observed in central and peripheral locations of muscles, indicating that human-derived ASCs contributed to the regenerative process. After 6 months, dystrophin expression was well distributed along muscle fibers, and even present in adjacent gastrocnemius muscle, suggesting that cell migration from injection site to other dystrophic muscles could have occurred or that dystrophin delivery was achieved by another unknown mechanism. Furthermore, in immunocompetent mice, regeneration took place successfully with no evidence of CD3 positive-lymphocytic infiltration. In contrast, hASC from early passages (that express HLA-I antigens) elicited an immune response in the host and could not restore dystrophin expression. Whether hASCs contributed to regeneration not only by fusion but also by converting themselves to myogenic lineage *in vivo* was not clear, although *in vitro* conversion of ASCs to myogenic lineage suggests that both mechanisms (fusion to myotubes and also myogenic conversion of ASC themselves) could have occurred. In this regard, the work of Liu et al. (2007), suggests that not only fusion but also myogenic conversion of hASC

TABLE 1 | Summary of ASC's myogenic differentiation, engraftment and functional assessment.

	Differentiation, engraftment, functional assessment	Myogenic differentiation media
In vitro		
Zuk et al. (2001)	12% myogenic differentiation of hASCs from lipoaspirates.	DMEM, 10% FBS, 5% horse serum, 0.1 µM dexamethasone 50 µM hydrocortisone, 1% Antibiotic-antimycotic
Mizuno et al. (2002)	At 6 weeks of differentiation, approx. 15% of hASCs expressed MyoD and 8.5% expressed Myosin heavy chain.	Same as Zuk et al. (2001) without dexamethasone.
Zheng et al. (2006)	After 6 weeks of differentiation, mouse ASCs expressed desmin monitored by immunofluorescence.	Same as Zuk et al. (2001).
Eom et al. (2011)	C2C12-GFP/hASC cocultures; at day 21, myotubes showed 50% nuclei from hASC origin.	LG-DMEM, 10% FBS, 3 µM 5-azacytidine (Sigma), 10 ng/ml FGF-2 (Sigma) for 24 h. Followed by 10% FBS, 10 ng/ml FGF-2 in LG-DMEM. Finally, hASC 5 days-filtered supernatant (conditioned media) was used.
Di Rocco et al. (2006)	Primary myoblasts and mASC-GFP direct cocultures showed 10-fold higher myotube incorporation of ASCs than transwell cocultures. Green myotubes expressed Troponin T by immunofluorescence.	DMEM high glucose, 5% horse serum, 2 mM L- glutamine, 1% penicillin-streptomycin.
Lee and Kemp (2006)	Positive fusion of hASCs with C2C12 myoblasts as myotubes expressed nestin from human origin monitored by immunofluorescence.	DMEM-high glucose (Gibco), 2% heat-inactivated horse serum (Gibco), and 1% penicillin/streptomycin.
Vieira et al. (2008a)	Coculture of DMD-derived myoblasts with GFP-hASCs. Fusion successful monitored by dystrophin expression in green myotubes by immunofluorescence.	hASCs passage 4 were cultured DMEM-HG, 10% FBS, 0.1 µM dexamethasone (Sigma), 50 µM cortisol (Sigma) and 5% HS (Gibco) for 45 days.
In vivo		
Rodriguez et al. (2005)	hASC injected in tibialis anterior muscles from mdx mice. Dystrophin was detected in up to 50% of the myofibers analyzed per section 10 d after transplantation. 10 to 50 d post-transplantation, 73 to 85% increase in peripheral nuclei from hASCs; 27 to 15% decrease in central nuclei.	Skeletal Muscle Cell Differentiation medium (PromoCell).
Di Rocco et al. (2006)	mASC from 6-week-old GFP+ mice were injected, after hind limb ischemia, in the left adductor muscle of GFP negative mice. After one week, GFP positive fibers represented up to 20% of the total area of sections (38.33 ± 8.82 GFP-positive fibers per mm ² of section area calculated as an average from 8 experiments). Dystrophin was detected in up to 10% of the myofibers analysed on sections (11.7 ± 2.94 dystrophin positive fibers per mm ² of section area, average from 6 experiments).	DMEM high glucose, 5% horse serum, 2 mM L-glutamine, 1% penicillin-streptomycin).
Zheng et al. (2006)	mASCs-LACZ injected in muscles of mdx mice. Few cells engrafted.	Same as Zuk et al. (2001).
Liu et al. (2007)	Cardiotoxin-injured mdx muscles injected locally with Flk1+ ASC: dystrophin expression restored in many fibers. Central nuclei decreased compared to controls at 4 weeks post-transplantation: 48.1% vs. 72.8% (n=5), p = 0.05, and at 12 weeks post-transplantation: 43.3% vs. 74.2%, n = 5, p = 0.05. Decreased creatinine kinase concentrations in Flk1+ ASCs-treated mice compared to controls at 12 weeks post transplantation: 5,321.6 +/– 1,289.75 vs. 13,746.8 +/– 5,373.75 UI ⁻¹ , n = 5, p = 0.005).DMEM containing 2% fetal bovine serum (FBS), 5% horse serum (Gibco), 50 µM hydrocortisone (Sigma), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco).	
Vieira et al. (2008b)	Systemic delivery of hASC in SJL mice (tail vein injection). Human Dystrophin found in approx. 50% +/– 2% ($p = 3.623 \times 10^{-13}$, Student's t test, n = 7) of the fibers.The treated group showed an improvement of 15.2% +/– 7.0% in their performance, whereas the untreated worsened 6.12% +/– 6.0% ($p = 0.013$, Student's t test, n = 7).	Same as in Vieira et al. (2008a), during 10 days before transplantation to SJL mice. At this stage, hASCs cells express MyoD.
Goudenege et al. (2009)	Tibialis anterior muscles of Rag2 ^{−/−} /γC ^{−/−} mice injected with MyoD-hASCs show between 27 and 38 spectrin-positive fibers per section whereas ASCs injected muscles show less than 10 spectrin-positive fibers per section.	1.5 g/l glucose Dulbecco's modified Eagle's medium supplemented with 10 µg/ml insulin and 5 µg/ml transferrin when the cells reached 90% confluence.

(Continued)

TABLE 1 | Continued

	Differentiation, engraftment, functional assessment	Myogenic differentiation media
Vieira et al. (2012)	Systemic injections of hASC in GRMD dogs showed variable dystrophin expression by WB (11 to 19% where 100% corresponds to normal human muscle). Immunofluorescence for dystrophin showed compatible results. hASC local injection in muscles of GRMD dogs did not engraft (hASCs not present in muscles 1 month after transplantation).	Same as Zuk et al. (2001).

Composition of myogenic differentiation media used in different works is also indicated.

occurs *in vivo* since expression of myogenic markers (MyoD, Myogenin, MyHC) from human origin could be detected by RT-PCR with specific primers. In this work, a subset of hASCs expressing endothelial marker Flk1 were transplanted by two different methods in two different regeneration models: tail vein injection (systemic) in cardiotoxin-injured TA muscles or intramuscular injection (local) in *mdx* mice. They both resulted in successful engraftment, partial recovery of sarcolemmal expression of dystrophin, decreased necrosis after 4 weeks and lower levels of muscle creatinine kinase in blood after 12 post-transplantation weeks. Importantly, the satellite stem cell pool seemed to be replenished from hASC since positive PAX7 cells co-stained for the human protein β2M. Another work (Vieira et al., 2008b), also transplanted hASCs by tail-vein injection in a SJL mice, a model of limb girdle muscular dystrophy 2B which is milder than Duchenne's. Different from *in vitro* co-culture experiments where highest fusion was observed using hASCs differentiated to late myogenic stages (Eom et al., 2011), undifferentiated hASC, but not hASC previously differentiated to myogenesis *in vitro*, were able to engraft successfully in muscles and provide expression of dysferlin and dystrophin of human origin. This data suggests that a progenitor state is more suitable to use in transplantation approaches, perhaps this undifferentiated population is more competent to receive regenerative signals from *in vivo* niche than more differentiated cells do, and this "instructive" step may be necessary in order to fuse to injured muscle. Also, it is possible that myogenic-differentiated ASCs are not able to sustain a proliferating pool of cells that could give cycles of regeneration upon time, which may be required to achieve robust and successful engraftments. These authors also reported that inflammatory process shown by those dystrophic muscles from SJL mice, did not improve upon hASC transplantation, in contrast to other works, which have reported a beneficial immunoprotective effect of ASC transplantation, for instance in rat brains upon a hemorrhagic stroke (Kim et al., 2007) and in an osteoarthritic mice model (ter Huurne et al., 2012). Nevertheless, mice showed improved muscle functionality in several motor ability tests up to 6 months of transplantation.

To assess the viability of cell transplantation approaches in humans, it is crucial to validate whether they can be feasible in larger animal models. This issue is important in order to predict pathogenesis and treatment outcomes. An alternative bigger model than mice and rats for muscle regeneration is the golden retriever muscular dystrophy (GRMD) dog. This

model reproduces full spectrum of human DMD and it has been used for successful transplantation studies with systemically injected mesoangioblasts, which is a type of mesenchymal stem cell present in walls of large vessels. In these experiments, dystrophin expression was recovered as well as partial muscle functionality (Sampaoli et al., 2006). However, the dogs were immunosuppressed and treated with steroids, which have been shown a beneficial impact on muscle functionality. For this reason, Vieira et al., 2012, assayed the transplantation of hASCs in GRMD dogs that were not immunosuppressed. hASCs were injected systemically into the dog cephalic vein (5×10^7 cells/kg in 0.1 ml of Hank's buffered salt solution) and engrafted successfully in the muscles. Expression of human dystrophin was validated up to 6 months after transplantation but could not be found after 12 months, suggesting that hASCs were not able to replenish the stem cell pool. The percentage of human dystrophin expressed in muscles was small (ranging from 6% to 19%) as monitored by immunofluorescence and immunoblotting. Unfortunately, histopathological features were not improved and beneficial effects on disease symptoms were difficult to be concluded. Therefore, from these data one can speculate that perhaps multiple injections, for instance every 6 months, and larger amounts of cells may be required to maintain exogenous dystrophin expression at levels that give a positive functional output. Surviving animals did not present adverse immune responses or other complications after 24 months, which might indicate ASC transplantation to be a safe procedure. In contrast, locally injected hASC were not able to engraft successfully, opposite to what had been described for *mdx* mice (Rodriguez et al., 2005; Liu et al., 2007).

Table 1 summarizes the results showed in these works and indicates quantifications of ASCs contribution to muscle differentiation and regeneration.

MyoD-Driven Conversion of ASCs

From all these works it is suggested that ASCs can be reprogrammed to myogenic lineage *in vitro* and *in vivo* by extracellular cues, highlighting a possible therapeutic use of ASCs as a new source of myogenic progenitors. However, efficiency of this conversion is not 100%, suggesting that there is a subpopulation of ASCs receptive to myogenic-commitment cues and a non-responsive population. In order to increase levels of myogenic conversion, Goudenege et al., 2009, genetically manipulated ASCs to express MyoD1, a transcriptional factor crucial for myogenic differentiation. MyoD1-pioneering ability

was suggested years ago by Weintraub and colleagues, who ectopically expressed MyoD1 in fibroblasts, fat, liver and nerve cell lines resulting in activation of myogenic gene expression (Weintraub et al., 1989, 1991). Fibroblasts harboring an inducible MyoD1 system and μ dystrophin gene were able to convert to myogenic lineage and contribute to muscle regeneration in *mdx* mice (Kimura et al., 2008). In ASCs, overexpression of MyoD1 resulted in upregulation of CD56, a surface marker of myogenic progenitors committed to differentiate (Capkovic et al., 2008), from 2% to 30% of total ASCs (Goudenege et al., 2009). Those MyoD1-expressing hASCs could efficiently form multinucleated myotubes *in vitro* upon culture with differentiation media (containing 10 μ g/ml insulin and 5 μ g/ml transferrin when cells reached 90% confluence), as well as to fuse with human DMD-derived myoblasts *in vitro*. Upon transplantation in a mouse model for regeneration (cryoinjury in TA muscles of $Rag2^{-/-}\gamma C^{-/-}$ immunodeficient mice), hASC engraftment was monitored by immunofluorescence against human Lamin A/C, revealing that hASC localized between fibers and also integrated in regenerating muscles. To assess hASC contribution to regenerative process, several human specific muscle proteins (spectrin, dystrophin, and δ -sarcoglycan) were detected by immunofluorescence at the membrane of different fibers. Muscles transplanted with MyoD1-hASCs seemed to contain higher number of positive human-derived myogenic marker-expressing fibers than WT-hASCs, indicating that MyoD1 expression in ASCs results in higher regenerative *in vivo* potential. This data argues in favor of manipulating ASCs *in vitro* with transcriptional factors to force ASCs commitment towards myogenic lineage, since using these manipulated ASCs result in a higher contribution to muscle regeneration. Whether those MyoD1-ASCs contribute to functional recovery of damaged muscles remains to be tested and perhaps MyoD1-hASC should also be assayed in DMD models such as *mdx* mice and GRMD dogs.

Epigenetics of ASC-Derived Myocytes

During adult stem cell differentiation as well as in the progression from a pluripotent to a multipotent state, expression of proliferative and pluripotent genes is erased, and expression of differentiation or lineage-specific genes from previously silent loci is activated. This process is controlled by concerted action of signaling cues with ubiquitous and cell-specific transcription factors that govern a changing epigenetic landscape during cellular differentiation. In other words, distinct epigenetic signatures can be associated to a precise cellular stage (Hussein et al., 2014; Tee and Reinberg, 2014).

DNA methylation at CpG sites is one of best-studied epigenetic modifications, which has been associated to gene repression (Heard et al., 1997; Walsh et al., 1998; Gaudet et al., 2003), although it is also found in gene bodies of highly transcribed genes (Jjingo et al., 2012). DNA methylation fluctuates during early mammalian development (Reik, 2007), but it is quite stable in most of the cells, which show an average of 70–80% of methylated CpGs. Nevertheless, global analyses in different cell types and tissues have shown that a

dynamic regulation occurs for almost 22% of autosomal CpGs at enhancers of lineage specific regulators (Hon et al., 2013; Ziller et al., 2013; Carrió et al., 2015), suggesting that DNA methylation at CpG sites is an epigenetic feature that determines cellular identity.

Therefore, to verify a certain lineage reprogramming, conversion, or proper differentiation, instead of monitoring expression of a few markers of these processes, global characterization of gene expression and epigenetic patterns seems to be a more complete approach to validate whether a full or partial conversion has been achieved. Berdasco and colleagues pursued this technique and characterized global DNA methylation status of myogenic lineages derived from hASC by Infinium methylation arrays interrogating 27,578 CpG sites (Berdasco et al., 2012). Their results indicate that methylome of ASCs-derived myocytes is more similar to the one of undifferentiated ASCs than to the one of primary myocytes from human biopsies. ASCs methylome shared the lowest similarity with a rhabdomyosarcoma cell line, a pediatric muscle tumor. Importantly, methylomes of ASCs and ASCs-myogenic derivatives did not show hypermethylation of tumor suppressor genes, a hallmark of DNA methylation profiles of cancer cells. Hence, these results argue in favor of ASCs as a safe cell population for therapeutic purposes. Intriguingly, these data show that although ASC-derived myocytes and primary myocytes present overlapping methylomes, ASC-derived myocyte methylome is more similar to the one of undifferentiated ASCs. Therefore, this result could imply several interpretations such as incomplete conversion towards myogenic lineage of all ASCs, or that just a subset of isolated ASCs was converted to myogenic lineage while the rest were resistant to this conversion. It could also imply that many of the observed 2,313 differential methylated regions between ASC-derived myocytes and primary myocytes, are not essential for myogenic differentiation, which indeed took place in ASC-derived myocytes supported by positive expression of α -sarcomeric actin, actinin and troponin by immunofluorescence. In any case, other epigenetic mechanisms different from DNA methylation may be crucial for lineage plasticity contributing to reprogram ASCs towards myogenic lineage. In fact, a direct link between DNA methylation state and differentiation capacity is not obvious as for instance mesenchymal stem cells from adipose, BM and skeletal muscle all show lineage-specific genes hypomethylated (Sørensen et al., 2010).

A comparative epigenomic profiling in murine and human models of adipogenesis, which monitored genome-wide distribution of several histone modifications, CTCF and adipose specific transcriptional factor peroxisome proliferator-activated receptors (PPAR γ), was able to identify two novel players, promyelocytic leukaemia zinc finger (PLZF) and serum response factor (SRF), with anti-adipogenic activity (Mikkelsen et al., 2010). A positive correlation was found between gene expression values and presence of H3K27ac in enhancer regions, giving to this modification a predictive value when present. This work also suggested that gain of histone acetylation accompanying PPAR γ binding, distinguishes functional PPAR γ binding sites from nonproductive ones. Surprisingly, a major number of

PPAR γ binding sites were different in mouse and human, and many were associated to rodent-specific transposable elements. This work shows that adipogenesis may not be 100% equivalent in mice and humans, implying that regulation of ASC plasticity may be different as well. Whether PLZF and SRF could play a role in early stages of myogenic determination of ASCs remains to be elucidated.

Considerations for Use of ASCs in Muscle Regenerative Therapies

Previously mentioned studies support the idea that ASCs are a promise for muscle regenerative therapies: they can differentiate towards myogenic lineage, they can be obtained in great quantities by simple liposuction, and they may represent a safer alternative to embryonic or induced pluripotent stem cells, which can produce tumors if full reprogramming is not achieved. Autologous transplantations of ASCs have been safely used for reconstructive surgery and wound healing therapies for several years (Ross et al., 2014), nevertheless, clinical studies are scarce to confirm long-term safeness of ASC transplants (Barkholt et al., 2013). Whether allogeneic ASC transplants in humans would work for muscular dystrophy treatment is not known, but successful engraftments of ASCs in muscles of immunocompetent mice support the idea that they might.

However, for successful muscular regenerative therapies it is necessary that transplanted ASCs not only engraft in muscles but also that differentiate efficiently resulting in a positive functional output. At present, ASCs' efficiency to differentiate towards myogenic lineage is low and their contribution to recover muscle function in *in vivo* regenerative models is not consistent. Stem cell research from skeletal muscle and hematopoietic fields have reported that stem cells are heterogeneous regarding self-renewal, proliferation and lineage output (Copley et al., 2012; Malecova and Puri, 2012), supporting a view of mixture of progenitors in those tissues. Adipose tissue is emerging as a new crucial player in the endocrine field, and the former idea that it is just a reservoir of energy, in form of lipid deposits, is changing. Therefore, it is not surprising to find different types of progenitors also present in this complex tissue. ASCs comprise a mix of multipotent progenitors, which seem to vary in their composition depending on several factors such as donor characteristics or their anatomical origin. Perhaps, anatomical location defines subsets of stem cells, which may respond better to signals of that particular environment. Supporting this idea, ASCs derived from epicardial adipose tissue differentiate poorly towards adipocyte lineage, whereas they express myocardial and endothelial markers *in vitro*. Furthermore, when transplanted into injured hearts they are able to differentiate into cardiac cells, engraft successfully and promote paracrine effects to induce local vascularization in mouse, rat and porcine models of myocardial infarction (Bayes-Genis et al., 2010, 2013). Consequently, not all adipose tissue from the body has the same characteristics and more research is needed to identify molecular determinants that define their specificities. It is quite plausible that extracellular niche plays an important role in this issue; however signaling pathways that control stem cell pools in adipose tissue as well

as their crosstalks with adjacent normal or diseased organs are still poorly understood. In regenerating dystrophic muscles of *mdx* mice, recently identified fibroadipogenic progenitors play a crucial role by enhancing muscle progenitor differentiation as well as modulating the inflammatory response that leads to fibrosis (Mozzetta et al., 2013). Also, parabiotic experiments suggest that age-related changes in systemic environment and the niche in which progenitor cells reside prevent complete activation of these cells for successful muscle regeneration (Conboy et al., 2005). Thus, these and other works show that tissue homeostasis involves interplay of local and systemic factors, and it is far more complex than previously anticipated.

In vivo skeletal muscle regenerative experiments using ASCs suggest that there is a subset of cells with increased myogenic capacity, which argues in favor of a myoprogenitor population present in ASCs. Therefore, identifying this myoprogenitor ASCs with specific surface markers could allow their isolation and expansion for a successful therapeutic use. Encouragingly, for bone regenerative purposes, Chung and colleagues have identified a population of CD90-high expressing ASCs, which show increased osteogenic capacity in mice models of bone regeneration (Chung et al., 2013). However, regarding ASC-derived myoprogenitors it is not clear which marker may identify them. Myoprogenitor marker CD56 is only present in 2% of cultured ASCs (Goudenege et al., 2009), which seems a small percentage of cells to account for the observed ASCs myogenic capacity. Intriguingly, in ASCs there is a 29% of CD146+ cells (Zannettino et al., 2008), but whether those cells contain type II pericytes with ability to regenerate muscles after injury (Birbrair et al., 2013) is not clear. Flk1+ cells purified from ASCs showed high myogenic differentiation potential, and although Flk1 is considered to be an endothelial marker (Yamaguchi et al., 1993), cardiac stem cells seem to express Flk1 (Iida et al., 2005). Perhaps, single cell analyses similar to those performed for muscle stem cells (Sacco et al., 2008) could help to identify and characterize a group of myoprogenitor markers in ASCs that could be instrumental to isolate a progenitor fraction with enhanced myogenic properties more suitable for muscle regenerative goals.

Other works suggest that transdifferentiation of ASCs towards myogenic lineage is also possible, although whether this transdifferentiation includes reprogramming to a myoprogenitor or direct differentiation is not clear. While transcriptional programs that direct adipocyte and myocyte differentiation are well studied and many players have been identified such as insulin-like growth factor 1 (IGF-1), bone morphogenetic protein (BMP), transforming growth factor beta (TGF β), retinoic acid, integrins, Ncadherin, galectin, cAMP response element-binding protein (CREB), PPARG, p38 kinase, MyoD, BAF60c, myocyte enhancer factor-2 (Mef2), ASH2 (Rosen and MacDougald, 2006; Perdigero et al., 2009; Cristancho and Lazar, 2011; Dilworth and Blais, 2011; Giordani and Puri, 2013), early molecular events that determine which fate ASCs will follow are mostly unknown. Shed new light on this issue and showed that inhibition of Rho-ROK signaling pathway in differentiating myocyte precursors (C2C12 in presence of IGF1), blocked myogenic differentiation in favor of adipogenesis, while excessive Rho activation in adipocyte precursors (L1–3T3)

blocked adipogenesis Sordella et al. (2003). The phosphorylation status of p190-B RhoGAP would be a sensor of this pathway. Other works have shown that in a mesenchymal stem cell population, a dominant negative RhoA expression induces adipogenesis whereas a constitutively active RhoA drives osteogenic differentiation (McBeath et al., 2004). These works highlight important roles of Rho pathway modulating cell fate decisions in MSC. Whether this pathway controls adipose vs. myogenic fate of ASC in response to IGF1 remains to be elucidated.

One of the signaling pathways involved in cell fate during development and also in cancer disease is Sonic Hedgehog. Eric Olson's group has described that constitutive activation of Sonic Hedgehog signaling in adipose lineage results in skeletal muscle tumors that resemble embryonal rhabdomyosarcomas (Hatley et al., 2012). This work supports a transdifferentiation hypothesis of an adipose progenitor towards muscle lineage, and importantly it reminds us the need to make sure ASC progenitors used for therapy are not harboring mutations or activated signals that can lead to cancer. *In vitro* and *in vivo* assays show that ASCs are not intrinsically tumorigenic cells, however they could enhance tumorigenic behavior of c-Met+ breast cancer cells by eliciting an inflammatory microenvironment that sustained tumor growth (Eterno et al., 2014). In other words, using ASC in esthetic procedures for breast reconstruction after cancer could enhance recurrence at least in c-Met+ tumors. Thus, more research is needed to better understand ASC biology upon transplantation and ensure safeness for clinical applications.

Epigenetic modifications are catalyzed by enzymes that can be targeted by drugs; hence, these drugs have an impact on

cell fate. For instance, use of demethylating drug 5-Azacytidine in fibroblasts resulted in their conversion to chondrocytes, adipocytes and contractile muscle striated cells (Taylor and Jones, 1979) illustrating the broad effect this drug had on demethylation of many lineage regulatory loci. Therefore, in order to control a specific lineage conversion at will, it is necessary to find more precise strategies. In this regard, CRISPR-CAS technology holds great potential for targeted approaches to gene therapy. Perhaps it could be used for several objectives ranging from recruiting desired epigenetic enzymes to specific lineage regulatory loci to activate known myogenic regulators in ASCs, or to target dystrophin gene in order to fix its mutations in autologous DMD-cultured ASCs. Alternatively, this strategy could also be used to activate expression of myogenic booster genes (Engvall and Wewer, 2003) in ASCs, which could then fuse to muscles to alleviate muscular dystrophy symptoms.

In conclusion, several works have shown promising data on ASCs contribution to muscle regeneration. ASCs safety is supported by *in vivo* transplantation experiments and epigenetic data. Identification of new protocols and tools that allow enrichment, expansion and manipulation of ASC-derived myoprogenitors could help to boost muscle regeneration.

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Exceptional longevity and muscle and fitness related genotypes: a functional *in vitro* analysis and case-control association replication study with SNPs *THRH rs7832552*, *IL6 rs1800795*, and *ACSL1 rs6552828*

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There are several gene variants that are candidates to influence functional capacity in long-lived individuals. As such, their potential association with exceptional longevity (EL, i.e., reaching 100+ years) deserves analysis. Among them are rs7832552 in the thyrotropin-releasing hormone receptor (*TRHR*) gene, rs1800795 in the interleukin-6 (*IL6*) gene and rs6552828 in the coenzyme A synthetase long-chain 1 (*ACSL1*) gene. To gain insight into their functionality (which is yet unknown), here we determined for the first time luciferase gene reporter activity at the muscle tissue level in rs7832552 and rs6552828. We then compared allele/genotype frequencies of the 3 abovementioned variants among centenarians [$n = 138$, age range 100–111 years (114 women)] and healthy controls [$n = 334$, 20–50 years (141 women)] of the same ethnic and geographic origin (Spain). We also studied healthy centenarians [$n = 79$, 100–104 years (40 women)] and controls [$n = 316$, 27–81 years (156 women)] from Italy, and centenarians [$n = 742$, 100–116 years (623 women)] and healthy controls [$n = 499$, 23–59 years (356 women)] from Japan. The *THRH rs7832552 T*-allele and *ACSL1 rs6552828 A*-allele up-regulated luciferase activity compared to the C and G-allele, respectively ($P = 0.001$). Yet we found no significant association of EL with rs7832552, rs1800795 or rs6552828 in any of the 3 cohorts. Further research is needed with larger cohorts of centenarians of different origin as well as with younger old people.

Keywords: centenarians, polymorphisms, luciferase reporter, gene association study, muscle and sarcopenia

Introduction

The oldest old population (≥ 85 years) is rapidly expanding among westerners (Waite, 2004; Robine and Paccaud, 2005). However, aging is associated with an increased risk of loss of functional independence (Christensen et al., 2008). In this regard, centenarians (people aged 100+ years) are the paradigm of healthy aging, as they have usually postponed (or even avoided, in some cases) major age-related diseases, as well as the onset of disability, until they were well into their nineties (Terry et al., 2008). Thus, the search for the gene variants that might influence the likelihood of reaching exceptional longevity (EL, i.e., becoming a centenarian) might help identify targets of "anti-aging" interventions.

In old people, functional independence is dependent on physical fitness, which in turn is determined by several phenotypes such as mainly cardiorespiratory fitness and muscle performance (Garber et al., 2011). Concerning the latter, aging is inevitably associated with a decline in muscle mass and function, i.e., sarcopenia, with an acceleration of this process increasing the risk of mortality (Metter et al., 2004; Ruiz et al., 2008). Thus, those gene variations that can be associated with preservation of muscle mass/function at advanced ages, e.g., the K153R polymorphism in the myostatin (*MSTN*) gene (Garatachea et al., 2013a) or the I/D polymorphism in the angiotensin converting enzyme (*ACE*) (Garatachea et al., 2013b) as well as other genetic variations linked with muscle aerobic capacity (e.g., the R577X mutation in the α -actinin-3 (*ACTN3*) gene), might also influence the likelihood of reaching EL (Fiuza-Luces et al., 2011).

There are other potential candidates to influence muscle phenotypes in long-lived individuals and as such their potential association with EL deserves analysis. In this regard, a recent genome wide scan (GWAS) analysis of 379,319 SNPs in US Caucasians of both genders (aged 50 years on average) revealed an association of lean body mass with 2 SNPs in tight linkage disequilibrium within the thyrotropin-releasing hormone receptor (*TRHR*) gene, rs16892496 and rs7832552 (Liu et al., 2009). These results were further corroborated in independent cohorts of older Caucasians of both genders, aged 63 (men) and 61 years (women). The functional significance of these 2 variants remains however to be determined. Another candidate is the -174C/G polymorphism (rs1800795) in the interleukin-6 (*IL6*) gene, where the G-allele is associated with higher transcription *in vitro* (Fishman et al., 1998) and *in vivo* conditions (Bennnermo et al., 2004). IL6 is a multifunctional cytokine that might be also involved in muscle regeneration (Serrano et al., 2008). Pereira et al recently found that the GG genotype, which is associated with lower IL6 levels and thus with "anti-inflammatory" profile, was associated with better physical performance in community-dweller elderly women (≥ 65 years) (Pereira et al., 2013).

As for cardiorespiratory fitness [which is usually determined with peak oxygen uptake ($VO_{2\text{peak}}$)], a recent GWAS study in sedentary Caucasians found that, among 324,611 SNPs, the strongest association with the $VO_{2\text{peak}}$ response to exercise was found to acyl coenzyme A synthetase long-chain 1 (*ACSL1*) gene polymorphism rs6552828 (Bouchard et al., 2011). The *ACSL1*

gene is a candidate to explain individual variability in $VO_{2\text{peak}}$, as well as in some health-related phenotypes, owing to its potential role in aerobic metabolism at the adipocyte, cardiomyocyte, liver and skeletal muscle fiber level (Martin et al., 1997; Coleman et al., 2000; Hall et al., 2003; Mashek et al., 2006; Ellis et al., 2010), yet its functional significance has not been assessed.

In order to analyze their functionality at the muscle level, we measured for the first time luciferase gene reporter activity in *TRHR* rs16892496 and rs7832552, and also in *ACSL1* rs6552828. Only rs7832552 in the *TRHR* gene was genotyped in this study because it is in tight linkage disequilibrium with rs16892496 (Liu et al., 2009). Based on the hypothesis that common genetic polymorphisms influencing physical fitness may also have an impact on the ability to reach EL, we then compared allele/genotype frequencies of the abovementioned SNPs—together with the *IL6* rs1800795 SNP (whose functional significance is already known, as mentioned above)—among Spanish centenarians (*cases*) and healthy controls matched by ethnic and geographic origin and also in 2 other geographically and ethnically-independent replication cohorts (from Italy and Japan).

Methods

Functional Analysis: Luciferase Reporter Gene

The fragment, including the allele, was directly inserted into the pGL3-promoter at the restriction recognition sites *MluI/NheI* in the 5' and *XhoI* in the 3' (see below -in bold) of the sequences obtained from the genomes of:

- (i) one individual homozygous for the rs16892496 A-allele and one individual homozygous for the rs16892496 C-allele (see below -underlined)

rs16892496 AA

GCTAGCTATGAAAGATCTACGTTAAAACATAAGGTT
AAGCTGTGCAGTGATACAGAAGAGACAAGAAAGTGG
TACTTACTGTGCATAAGGTTGAAGAGCAAGCCCCCA
GTGGGATACAAGTCACTCTCAGGCTTGAAGGAAATGAG
TAGGCATTCACTAGGCCAACATAAAATACAAGAAGA
CCCTCCAGTCTGCAGAAGTAGTCAATGACTCGAG

rs16892496 CC

GCTAGCTATGAAAGATCTACGTTAAAACATAAGGTT
AAGCTGTGCAGTGATACAGAAGAGACAAGAAAGTGG
TACTTACTGTGCATAAGGTTGAAGAGCAAGCCCCCC
GTGGGATACAAGTCACTCTCAGGCTTGAAGGAAATGAG
TAGGCATTCACTAGGCCAACATAAAATACAAGAAGA
CCCTCCAGTCTGCAGAAGTAGTCAATGACTCGAG

- (ii) one individual homozygous for the rs7832552 C-allele and one homozygous for the rs7832552 T-allele (see below -underlined)

rs7832552-CC

GAGCTCATTAGCCTTGTGACAAAAGCAACGCACCTCC
ATTTTGACACAGTACTTGACTTTATTTGCTACTGC
CTTGACCTCAAAGGAATGTGATAGTGTGAGGTACGA

ATGCTCTTAATAAACAGGATCGATCAAGGGTGCTTG
ACTCTTGTGTTCATGTGCAAGTATACTGGCTTTTT
TGTGCCTCAACAAAACCATCAAGAGTCTCGAG

rs7832552-TT

GAGCTCATTAGCCTGTGACAAAAGCAACGCACCTCC
ATTTTGCACACAGTACTGACTTATTTGCTACTG
CTTGACCTCAAAGGAATGTGATAGTGTGAGGTATGA
ATGCTCTTAATAAACAGGATCGATCAAGGGTGCTTG
CTCTTGTGTTCATGTGCAAGTATACTGGCTTTTTG
TGCCTCAACAAAACCATCAAGAGTCTCGAG

- (iii) one individual homozygous for the rs6552828 A-allele and one individual homozygous for the rs6552828 G-allele (see below -underlined)

rs6552828-AA

GAGCTCCAAGACATTATGCCAAAAGAAACAAACAG
ATAAAATTGGTGTGCATAAACTTAAACCAACCACCAAG
ATATCTAAAGAGGAATACAGCACAGTGTGGAAG
AAAGTACAGAAATAGTATTGAGATCCTAGATGCAGC
CGGACGCGGTGGCTATGCCTGTAATCCCAGCACTT
TGGGAAGCCGAGGCAGGGTGGATCACCCCTCGAG

rs6552828-GG

GAGCTCCAAGACATTATGCCAAAAGAAACAAACAG
ATAAAATTGGTGTGCATAAACTTAAACCAACCACCAAG
ATATCTAAAGAGGAATACAGCACAGTGTGGAAG
AAAGTACAGAAATAGTATTGAGATCCTAGATGCAGC
CGGACGCGGTGGCTATGCCTGTAATCCCAGCACTT
TGGGAAGCCGAGGCAGGGTGGATCACCCCTCGAG

We used mice skeletal muscle C2C12 cell lines to study muscle-specific expression. We performed cell cultures, transfections and dual-luciferase reporter assays following the procedures previously reported by our group (He et al., 2011). We used the pRL-SV40 vector as an internal control for variations in transfection efficiency, and the pGL3-promoter vector without an insert as a negative control. The transfected cells were harvested after 48 h, and assayed for firefly and renilla luciferase activity with the dual-luciferase reporter assay system (Promega Biotech, Beijing, China) using a luminometer following the manufacturer's recommendations (TecanGenios Pro, Männedorf, Switzerland). From each measurement, we divided firefly by renilla luciferase activity reading to calculate relative luciferase activity. We performed the experiments in triplicates and expressed relative luciferase activity values as the means \pm SD of the 3 different measurements.

Subjects

We obtained approval from the local ethics committees [European University of Madrid (Spain), University of Pavia (Italy), and National Institute of Health and Nutrition, (Japan) Medical Research Institute and Keio University (Japan)] and the study followed the tenets of the Declaration of Helsinki for Human Research. Written consent was also obtained from each participant.

Spanish Cohort

Two groups of Spanish subjects were assessed: (i) 138 cases (centenarians, aged 100–111 years, 114 women); and (ii) 334

healthy controls (aged 20–50 years, 141 women). All the subjects were of the same Caucasian (Spanish) descent for ≥ 3 generations. The major diseases among the centenarians were osteoarthritis (66%), hypertension (57%), dementia (51%) and cardiovascular disease (CVD, 29%). The DNA of a convenience sample of 355 younger disease-free controls with no reported family history of high longevity (>90 years) was collected during 2008–2012 in the European University of Madrid.

Italian Cohort

Two groups of subjects from Northern Italy (mainly from Lombardy and Piedmont) were studied: (i) 79 cases (healthy centenarians, aged 100–104 years, 40 women); and (ii) 316 healthy controls (aged 27–81 years, 156 women). All patients and controls were Caucasian whites of Italian descent for ≥ 3 generations. The Italian centenarians were free of major age-related diseases, i.e., severe cognitive impairment, clinically evident cancer, CVD, renal insufficiency or severe physical impairment (Emanuele et al., 2010). Controls were free of CVD or cerebrovascular disease, cancer, dementia, chronic autoimmune/ inflammatory disorders, renal or hepatic failure, and major psychiatric conditions.

Japanese Cohort

Two groups of subjects of the same Asian (Japanese) descent were assessed: (i) 742 cases (centenarians, aged 100–116 years, 623 women); and (ii) 499 healthy controls (aged 23–59 years, 356 women). The group of cases was gathered from 2 cohorts, which are described in detail elsewhere (Gondo et al., 2006): the Tokyo Centenarians Study (TCS) and the Semi-Supercentenarians Study in Japan (SSC-J). The prevalence of hypertension, CVD and dementia among the Japanese centenarians was of 63.6, 28.8, and 59.4%, respectively. Controls from both genders (aged <60 years, and free of diagnosed CVD and chronic renal failure) were recruited during years 2008–2012.

Genotyping

As mentioned above, only one *THRH* SNP, rs7832552, was genotyped in the 3 cohorts (and not rs16892496) because the genotype distributions of both SNPs are completely linked according to available HapMap for both European and Asian populations (sorted as a Supplementary file 1) and previous research has shown that both SNPs are in strong linkage disequilibrium ($r^2 = 0.98$). All genotyping was performed only for research purposes with the researchers who performed the genotyping being blinded to the participants' identities. For quality control, a random $\sim 20\%$ of the samples of each cohort were genotyped again, with no differences in the results compared with the initial genotyping.

Spanish Cohort

DNA was extracted from the participants' buccal cells (saliva samples) using a standard phenol chloroform protocol and the genotype analyses were performed in the Biomedicine laboratory at the European University, Madrid (Spain). The DNA samples were diluted with sterile water and stored at -20°C until analysis. Genotyping was performed by Real-Time PCR and using the TaqMan® rs7832552, rs6552828, and rs1800795

SNP genotyping assays with a Step One Real-Time PCR System (Applied Biosystems, Foster City, CA).

Italian Cohort

Genomic DNA was purified from blood leukocytes using the QiaAmp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Genotyping was performed at the Cellular Pathophysiology and Clinical Immunology Laboratory (University of Pavia) using the TaqMan® rs7832552, rs6552828, and rs1800795 SNP genotyping assays (Applied Biosystems, Foster City, CA, USA).

Japanese Cohort

Total genomic DNA was extracted from blood leukocytes with a QIAamp DNA Blood Mini or Maxi Kit (Qiagen, Tokyo, Japan). Genotyping of rs7832552, rs6552828, and rs1800795 was performed at the Institute of Health and Sports Science and Medicine (Juntendo University) with Real Time Thermocycler (LightCycler 480, Roche Applied Science, Mannheim, Germany) using TaqMan SNP genotyping assay method. PCR 384-well plates were read on LightCycler 480 using the end-point analysis mode. Allelic discrimination analysis was performed with a LightCycler 480 SW software version 1.5.1.62 (Roche Applied Science, Mannheim, Germany).

Statistical Analysis

One-way analysis of variance was used to compare the relative luciferase activity in the different plasmids of each SNP. Allele frequencies were calculated by gene-counting. We tested Hardy-Weinberg equilibrium (HWE) using χ^2 -test. Genotype/allele frequencies of cases vs. controls within each cohort (Spanish, Italian, and Japanese) were compared using the χ^2 -test with Yates' correction and the association between genotypes/alleles and EL within each of the 3 cohorts was analyzed with logistic regression analysis after adjusting for sex. All statistical analyses were performed using the PASW (v. 18.0 for WINDOWS, Chicago) and corrected for multiple comparisons using the Bonferroni's method -that is, the threshold P -value was obtained by dividing 0.05 by the number of studied polymorphisms ($P = 0.05/3 = 0.017$).

Results

Functional Analysis

The results of luciferase report analyses are presented in **Figure 1**. All the SNPs we studied showed functional significance, as reflected by differences in luciferase activity between the 3 SNP constructs (all $P \leq 0.001$); the *THRH* rs16892496 A-allele up-regulated luciferase activity compared to the C-allele (upper panel), the *THRH* rs7832552 T-allele up-regulated luciferase activity compared to the C-allele (middle panel), and the *ACSL1* rs6552828 A-allele up-regulated luciferase activity compared to the G-allele (lower panel).

Spanish Cohort

Rate of genotyping success was as follows: *THRH* rs7832552, 97.2% in cases and 100% in controls; *ACSL1* rs6552828, 97.2% in cases and 99.1% in controls; *IL6* rs1800795, 100% in cases

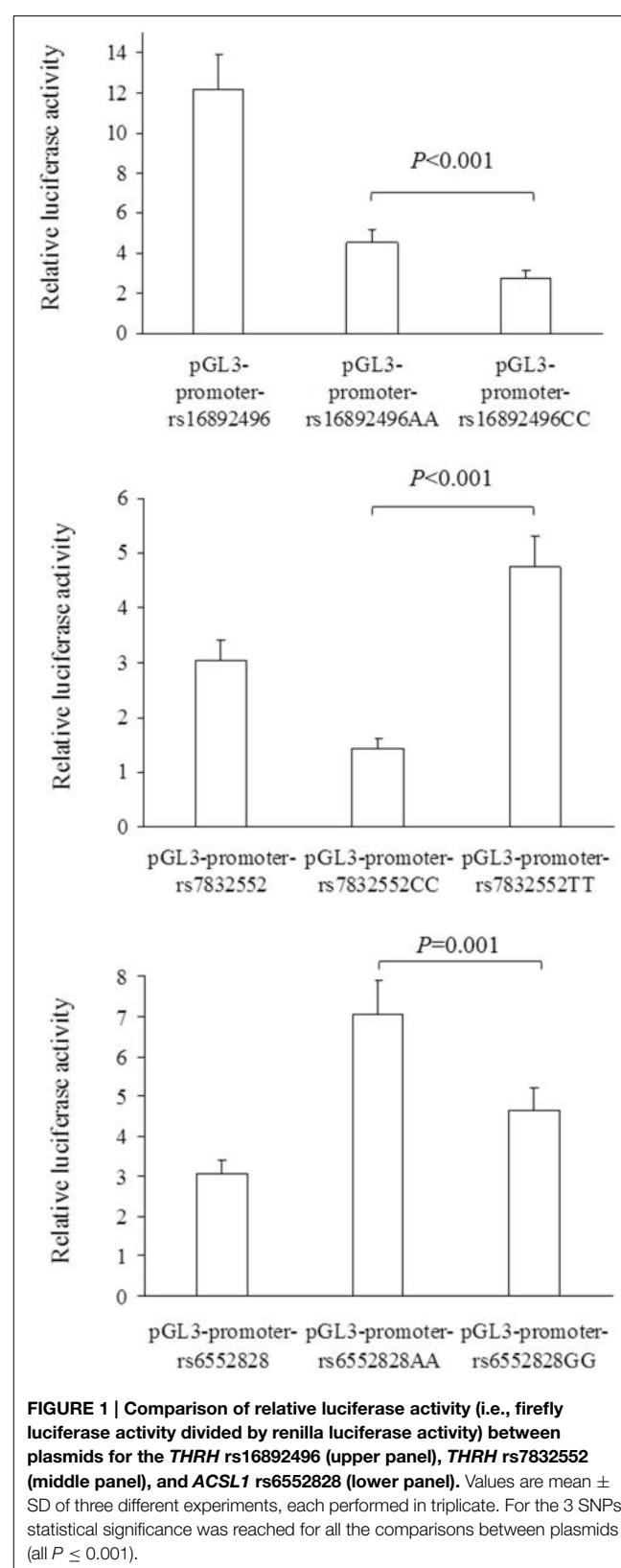


FIGURE 1 | Comparison of relative luciferase activity (i.e., firefly luciferase activity divided by renilla luciferase activity) between plasmids for the *THRH* rs16892496 (upper panel), *THRH* rs7832552 (middle panel), and *ACSL1* rs6552828 (lower panel). Values are mean \pm SD of three different experiments, each performed in triplicate. For the 3 SNPs, statistical significance was reached for all the comparisons between plasmids (all $P \leq 0.001$).

and 94.9% in controls. The distribution of all genotypes was consistent with the HWE in both groups ($P > 0.05$), except for *IL6* rs1800795 in the control group ($P < 0.01$).

The results of genotype/allele frequency distributions as well as of binary logistic regression adjusted by sex are shown in **Table 1** and summarized below. The allele ($\chi^2 = 1.21, P = 0.27$) or genotype frequency distributions of *THRH* rs7832552 did not differ between cases and controls ($\chi^2 = 2.74, P = 0.25$). Using logistic regression analysis, no significant associations were found between EL and rs7832552, including when analyzing both sexes separately (data not shown). No differences were found for *IL6* rs1800795 in allele ($\chi^2 = 1.01, P = 0.32$) or genotype distributions ($\chi^2 = 3.89, P = 0.14$), with no significant association with EL after adjusting for sex or when analyzing both sexes separately -data not shown) or for *ACSL1* rs6552828 ($\chi^2 = 2.28, P = 0.13$ for allele distribution and $\chi^2 = 3.30, P = 0.19$ for genotype distribution, with no significant association with EL after adjusting for sex or when analyzing both sexes separately -data not shown).

Italian Cohort

Rate of genotyping success was 100% for all gene variants. The distribution of all genotypes was consistent with the HWE in both groups ($P > 0.05$), except for *THRH* rs7832552 in the control group ($P = 0.04$).

The results of genotype/allele frequency distributions as well as of binary logistic regression adjusted by sex are shown in **Table 2** and summarized below. The allele ($\chi^2 = 0.003, P = 0.95$) and genotype frequency distributions of *THRH* rs7832552 did not differ between groups ($\chi^2 = 0.26, P = 0.88$) and no significant association was found between this polymorphism and EL using logistic regression adjusted by sex, or when analyzing both sexes separately (data not shown). Similar results were found for *IL6* rs1800795 (allele frequency: $\chi^2 = 0.82, P = 0.36$; genotype frequency: $\chi^2 = 1.054, P = 0.59$) and *ACSL1* rs6552828 (allele frequency: $\chi^2 = 0.56, P = 0.46$; genotype frequency: $\chi^2 = 0.67, P = 0.72$), with no significant association between these two polymorphisms and EL using logistic regression adjusted by sex, or when analyzing both sexes separately (data not shown).

Japanese Cohort

Rate of genotyping success was as follows: *THRH* rs7832552, 97.0% in cases and 100% in controls; *IL6* rs1800795, 98.7% in cases and 100% in controls; *ACSL1* rs6552828, 95.9% in cases and 99.2% in controls. The distribution of all genotypes was consistent with the HWE in both groups ($P > 0.05$), except for rs6552828 in centenarians ($P = 0.02$).

The results of genotype/allele frequency distributions as well as of binary logistic regression adjusted by sex are shown in **Table 3** and summarized below. We found no differences between cases and controls for *THRH* rs7832552 (allele frequency: $\chi^2 = 0.012, P = 0.91$; genotype frequency: $\chi^2 = 0.592, P = 0.74$), rs1800795 (allele frequency: $\chi^2 = 0.07, P = 0.78$; genotype frequency: $\chi^2 = 0.07, P = 0.78$), or rs6552828 (allele frequency: $\chi^2 = 0.020, P = 0.89$; genotype frequency: $\chi^2 = 3.78, P = 0.15$), with no significant association between any of the SNPs and EL using logistic regression adjusted by sex or when analyzing both sexes separately (data not shown).

TABLE 1 | Genotype/allele frequencies of *THRH* rs7832552, *ACSL1* rs6552828 and *IL6* rs1800795 and results of logistic regression analysis, in the Spanish cohort.

		Controls		Cases		OR	95%CI	P-value
		N	%	N	%			
rs7832552								
Codom	CC	174	52.1	61	44.2	1.00		0.14
	CT	135	40.4	67	48.5	1.52	0.97–2.37	
	TT	25	7.5	10	7.3	1.71	0.72–4.09	
Dom	CC	174	52.1	61	44.2	1.00		0.048
	CT-TT	160	47.9	77	55.8	1.54	1.00–2.37	
Reces	CC-CT	309	92.5	128	92.8	1.00		0.44
	TT	25	7.5	10	7.2	1.40	0.60–3.24	
Overdom	CC-TT	199	59.6	71	51.5	1.00		0.11
	CT	135	40.4	67	48.5	1.42	0.92–2.19	
Log-additive		–	–	–	–	1.41	0.99–1.99	0.055
Allele	C	483	72	189	68	1.00		0.06
	T	185	28	87	32	1.37	0.98–1.97	
rs6552828								
Codom	GG	126	38.1	65	47.1	1.00		0.41
	GA	153	46.2	54	39.1	1.36	0.86–2.16	
	AA	52	15.7	19	13.8	1.25	0.65–2.40	
Dom	GG	126	38.1	65	47.1	1.00		0.19
	GA-AA	205	61.9	73	52.9	1.33	0.87–2.05	
Reces	GG-GA	279	84.3	119	86.2	1.00		0.82
	AA	52	15.7	19	13.8	1.07	0.58–1.98	
Overdom	GG-AA	178	53.8	84	60.9	1.00	0.83–1.99	0.25
	GA	153	46.2	54	39.1	1.29	0.86–1.60	
Log-additive		–	–	–	–	1.18	0.86–1.60	0.30
Allele	G	405	61	184	67	1.00		0.29
	A	257	39	92	33	0.84	0.61–1.15	
rs1800795								
Codom	GG	170	53.6	65	45.5	1.00		0.20
	GC	101	31.9	59	41.3	0.65	0.41–1.04	
	CC	46	14.5	19	13.3	0.82	0.43–1.58	
Dom	GG	170	53.6	65	45.5	1.00		0.09
	GC-CC	147	46.4	78	54.5	0.70	0.45–1.07	
Reces	GG-GC	271	85.5	124	86.7	1.00		0.96
	CC	46	14.5	19	13.3	0.98	0.53–1.84	
Overdom	GG-CC	216	68.1	84	58.7	1.00		0.09
	GC	101	3.9	59	41.3	0.68	0.44–1.06	
Log-additive		–	–	–	–	0.83	0.62–1.12	0.23
Allele	G	443	70	189	66	1.00		0.20
	C	193	30	97	34	1.24	0.90–1.71	

95%CI, 95% confidence interval; Codom, codominant; Dom, dominant; OR, odds ratio; Overdom, overdominant; Reces, recessive.

TABLE 2 | Genotype/allele frequencies of *THRH* rs7832552, *ACSL1* rs6552828, and *IL6* rs1800795 and results of logistic regression analysis, in the Italian cohort.

	Controls		Cases		OR	95%CI	P-value
	N	%	N	%			
rs7832552							
Codom	CC	137	43.4	34	43.1	1.00	0.88
	CT	154	48.7	40	50.6	1.06	0.64–1.72
	TT	25	7.9	5	6.3	0.81	0.26–2.28
Dom	CC	137	43.4	34	43.0	1.00	0.96
	CT-TT	179	56.6	45	57.0	1.02	0.62–1.69
Reces	CC-CT	291	92.1	74	93.7	1.00	0.64
	TT	25	7.9	5	6.3	0.77	0.30–2.14
Overdom	CC-TT	162	51.3	39	49.4	1.00	0.76
	CT	154	48.7	40	50.6	1.09	0.68–1.75
Log-additive	–	–	–	–	1.03	0.57–1.64	0.81
Allele	C	428	68	108	68	1.00	0.88
	T	204	32	50	32	0.98	0.67–1.43
rs6552828							
Codom	GG	121	38.3	34	43.0	1.00	0.72
	GA	139	44.0	33	41.8	0.85	0.50–1.46
	AA	56	17.7	12	15.2	0.77	0.35–1.55
Dom	GG	121	38.3	34	43.0	1.00	0.44
	GA-AA	195	61.7	45	57.0	0.83	0.51–1.37
Reces	GG-GA	260	82.3	67	84.8	1.00	0.59
	AA	56	17.7	12	15.2	0.85	0.43–1.65
Overdom	GG-AA	177	56	46	58.2	1.00	0.72
	GA	139	44	33	41.8	0.92	0.54–1.51
Log-additive	–	–	–	–	0.84	0.46–1.47	0.80
Allele	G	381	60	101	64	1.00	0.40
	A	251	40	57	36	0.86	0.56–1.23
rs1800795							
Codom	CC	76	24.1	22	27.9	1.00	0.59
	CG	160	50.6	41	51.9	0.89	0.48–1.60
	GG	80	25.3	16	20.2	0.70	0.34–1.45
Dom	CC	76	24.1	22	27.8	1.00	0.48
	CG-GG	240	76.9	57	72.2	0.81	0.45–1.44
Reces	CC-CG	236	74.7	63	79.8	1.00	0.35
	GG	80	25.3	16	20.2	0.75	0.41–1.39
Overdom	CC-GG	156	49.4	38	48.1	1.00	0.84
	CG	160	50.6	41	51.9	1.06	0.64–1.75
Log-additive	–	–	–	–	1.01	0.56–1.65	0.89
Allele	C	312	49	85	54	1.00	0.32
	G	320	51	73	46	0.84	0.57–1.19

95%CI, 95% confidence interval; Codom, codominant; Dom, dominant; OR, odds ratio; Overdom, overdominant; Reces, recessive.

TABLE 3 | Genotype/allele frequencies of *THRH* rs7832552, *ACSL1* rs6552828, and *IL6* rs1800795 and results of logistic regression analysis, in the Japanese cohort.

	Controls		Cases		OR	95%CI	P-value
	N	%	N	%			
rs7832552							
Codom	CC	135	27.1	191	26.3	1.00	0.87
	CT	230	46.1	351	48.3	1.06	0.81–1.41
	TT	134	26.8	185	25.4	1.00	0.73–1.37
Dom	CC	135	27.1	191	26.3	1.00	0.76
	CT-TT	364	72.9	536	73.7	1.04	0.80–1.35
Reces	CC-CT	365	73.2	542	74.5	1.00	0.77
	TT	134	26.9	185	25.4	0.96	0.74–1.25
Overdom	CC-TT	269	53.9	376	51.7	1.00	0.60
	CT	230	46.1	351	48.3	1.06	0.84–1.34
Log-additive	–	–	–	–	1.00	0.85–1.17	0.99
Allele	C	500	50.1	733	50.4	1.00	0.99
	T	498	49.9	721	49.6	0.99	0.85–1.78
rs6552828							
Codom	AA	186	37.6	247	34.6	1.00	0.12
	GA	229	46.3	369	51.8	1.19	0.92–1.53
	GG	80	16.2	97	13.6	0.85	0.59–1.21
Dom	AA	186	37.6	247	34.6	1.00	0.45
	GA-GG	309	62.4	466	65.4	1.10	0.86–1.40
Reces	AA-GA	415	83.8	616	86.4	1.00	0.12
	GG	80	16.2	97	13.6	0.77	0.56–1.07
Overdom	AA-GG	266	53.7	344	48.2	1.00	0.07
	GA	229	46.3	369	51.8	1.24	0.99–1.57
Log-additive	–	–	–	–	0.98	0.82–1.16	0.77
Allele	A	601	61	863	61	1.00	0.78
	G	386	39	563	39	0.976	0.83–1.16
rs1800795							
	GG	498	99.8	731	99.9	1	
	GC	1	0.2	1	0.1	0.84	0.05–14.09
Allele	G	997	100	1463	100		
	C	1	0	1	0		

95%CI, 95% confidence interval; Codom, codominant; Dom, dominant; OR, odds ratio; Overdom, overdominant; Reces, recessive. Of note, logistic regression was not performed in the Japanese cohort for rs1800795 owing to the virtual absence of C-carriers in both groups.

Discussion

The main findings of our study are two-fold. First, all the studied SNPs showed functional significance, as reflected by the results of the luciferase constructs. This is the first attempt to determine (with an *in vitro* approach) the potential functional

consequences of the rs16892496, rs7832552, and rs6552828 SNPs, with the *A*-allele, *T*-allele and *A*-allele up-regulating luciferase activity compared to the other alleles, respectively. The *THRH* rs7832552 and *ACSL1* rs6552828 SNPs are intronic genomic variants and, as such, could potentially alter the stability and/or alternative splicing of mRNA, as well as transcription factor binding (Tabor et al., 2002; Knight, 2005; Mercado et al., 2005; Sasabe et al., 2007). However, we found no association between *THRH* rs7832552, *IL6* rs1800795, and *ACSL1* rs6552828 and EL.

Although more research is obviously needed, we found no evidence that higher *THRH* expression (as theoretically associated with the *T*-allele or *CT-TT* genotypes vs. *CC*) might favor EL. Yet a GWAS study reported that the *TRHR* rs7832552 SNP was associated with lean body mass in US Caucasians (Liu et al., 2009). Subjects carrying the theoretically highest expressing (*TT*) genotype had 2.55 kg higher lean body mass compared to the other subjects. There is some scientific rationale in postulating that higher TRHR expression might help preservation of muscle mass in long-lived individuals: TRHR stimulates the hypothalamic-pituitary-thyroid axis, thereby leading to the release of thyroxin, a hormone that plays an important role in the development of skeletal muscle as well as in attenuating age-related changes in tissue function (Larsson et al., 1994). Although no association was found here, the *IL6* rs1800795 might be also a candidate to influence EL. Carriage of the "low-producing" *C*-allele has been positively associated with longevity in Turkish population (Kayaalti et al., 2011), whereas the high-producing *GG*-genotype has been linked with higher survival in elderly females from Sweden (Cederholm et al., 2007). On the other hand, the rs1800795 polymorphism has been linked with longevity in Italian centenarians from Treviso (Albani et al., 2009) although this finding was not replicated in other European cohorts (Di Bona et al., 2009) (including from other parts of Italy, Albani et al., 2009), or in US elders (Walston et al., 2009). As for *ACSL1* rs6552828, our data do not show an association of this polymorphism with EL despite the involvement of this gene in aerobic metabolism in the heart, liver, adipose and skeletal muscle tissues (Martin et al., 1997; Coleman et al., 2000; Hall et al., 2003; Mashek et al., 2006; Ellis et al., 2010). Recent research did not report an association between rs6552828 and an important age-related disease condition, the metabolic syndrome (Phillips et al., 2010).

A strength and novelty from our design stems from the use of a luciferase construct study to assess functionality of the 3 SNPs at the specific muscle tissue-level. However, our study has several limitations. First, besides the fact that we did not assess the functionality of the SNPs *in vivo* in blood samples and especially in muscle biopsies (which is understandable due to ethical reasons), we used convenience samples, which increases the risk of bias induced by population stratification. The SNPs rs1800795 and rs7832552 did not meet HWE in Spanish and Italian controls, respectively. In this regard, deviation from HWE does not necessarily reflect genotyping errors (Leal, 2005; Zou and

Donner, 2006), with ~10% of all genotype–phenotype association studies actually showing failure of 1+ genotype distributions to meet HWE (Trikalinos et al., 2006). Second, we selected 3 SNPs based on previous GWAS, i.e., those showing associations of rs16892496 and rs7832552 with lean body mass (Liu et al., 2009) and of rs6552828 with $\text{VO}_{2\text{peak}}$ (Bouchard et al., 2011). Besides differences in terms of population-specificity (ethnic/geographic origin, age) between these 2 GWAS and our cohorts, an additional problem is that GWAS are generally successful to find very penetrant dominant genetic variants, but less useful to discover rarer variants, with a likely modest effect on some phenotypes, such as those that could be potentially associated with EL. Finally, a potential confounder of genetic association studies is differences in date of birth, e.g., the centenarians and controls of our study were born in the early 1900s and after 1930, respectively (Lewis and Brunner, 2004). In this regard, the potential demographic biases of longevity studies like ours performing cross-sectional comparisons of genotype/allele frequencies between controls and long lived individuals could be overcome by adding demographic information to genetic data (Yashin et al., 1999; Passarino et al., 2006; Dato et al., 2007). Thus, genetic-demographic methods should be applied in future studies in the field because they allow the estimation of hazard rates and survival functions in relation to candidate genes (Yashin et al., 1999; Passarino et al., 2006; Dato et al., 2007).

In summary, despite the potential functional consequences of the SNPs we studied (rs16892496, rs7832552, and rs6552828) none of them was associated with EL. Similarly, no association was found for rs1800795. More research is needed in the field with other cohorts, using larger population samples, as well as younger elderly (e.g., aged 65–85 years) to assess the potential link between these genetic variants and the human aging process.

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Supplementary Material

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fnagi.2015.00059/abstract>

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Forever young: rejuvenating muscle satellite cells

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A hallmark of aging is alteration of organismal homeostasis and progressive decline of tissue functions. Alterations of both cell intrinsic functions and regenerative environmental cues contribute to the compromised stem cell activity and reduced regenerative capability occurring in aged muscles. In this perspective, we discuss the new evidence supporting the hypothesis that skeletal muscle stem cells (MuSCs) are intrinsically defective in elderly muscles. In particular, we review three recent papers leading to identify fibroblast growth factor receptor-1, p38 mitogen-activated protein kinase, and p16INK4a as altered signaling in satellite cells from aged mice. These pathways contribute to age-related loss of MuSCs asymmetric polarization, compromised self-renewal capacity, and acquisition of pre-senescent state. The pharmacological manipulation of those networks can open novel strategies to rejuvenate MuSCs and counteract the functional decline of skeletal muscle during aging.

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Extended lifespan raises the issue of handling age-related disorders, which profoundly affect the quality of life of an increasing number of people. At the physiological level, the most relevant feature of aging is the functional decline of tissue functions (Oh et al., 2014).

In particular, in the elderly, muscle mass declines progressively by means of a process named sarcopenia, making skeletal muscle one of the more compromised tissues during aging. Beyond the protein breakdown associated with the loss of sarcomeric proteins, aged muscles display compromised regenerative capacity associated with altered environmental cues (Kim and Choi, 2013; Sayer et al., 2013).

Muscle regeneration is achieved by the interplay between adult stem cells, named muscle satellite cells (MuSCs), and other cellular types (i.e., macrophages and muscle interstitial cells) that participate in the orchestration of regeneration. Muscle niche derived and systemic cues contribute to regulate muscle homeostasis and functionality (Chakkalakal et al., 2012; Bentzinger et al., 2013). Changes of those three compartments are described throughout lifetime and account for the decline of functional capacities in the elderly (Jang et al., 2011). Upon muscle injury, MuSCs that are located in a niche between the basal lamina and the sarcolemma, become activated and recapitulate myogenic differentiation to replenish damaged muscle (Collins et al., 2005; Cheung and Rando, 2013). Additionally, environmental cues finely regulate this process driving efficient muscle regeneration (Sinha et al., 2014). In order to ensure optimal performance, it is critical that several properties of MuSCs are finely regulated and coordinated. Amongst these properties are survival, self-renewal, fine-tuning between exit from quiescence and proliferative

expansion, and eventually commitment toward myogenic differentiation (Bentzinger et al., 2013). All these processes are altered in the elderly leading to compromised muscle functionality.

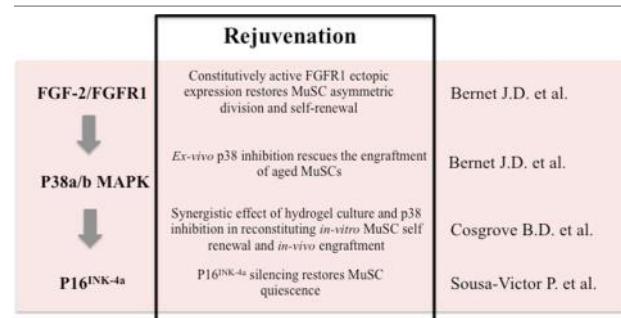
Beyond the notion provided by parabiosis experiments that circulating systemic factors are able to restore muscle regeneration in aged mice (Conboy et al., 2005), recent evidence supports the hypothesis that MuSCs are intrinsically defective in aged muscles. These new findings open the possibility to target this stem cell compartment to counteract functional decline of muscle during aging. Here, we will provide a general comment on three breakthrough studies from Bernet et al. (2014), Sousa-Victor et al. (2014) and Cosgrove et al. (2014) discussing the relative contribution to muscle regeneration of cell-autonomous vs. cell non-autonomous factors during aging.

In their work Bernet et al. and Cosgrove et al. provide evidence that constitutive activation of the p38 MAPK in aged MuSCs leads to a decline in their self-renewal and regenerative capacity. Both groups demonstrated that partial pharmacological inhibition of p38 is sufficient to restore the ability of MuSCs to participate efficiently in muscle regeneration and to maintain the stem cell pool. Interestingly, Bernet et al. identify an alteration of the FGF-2/FGFR1 axis as a feature of aged MuSC dysfunction, as observed previously by Brack and colleagues (Chakkalakal et al., 2012). Although in the paper by Chakkalakal the authors suggest that increased activity of FGFR1 results in the disruption of MuSC quiescence in aged muscles, the Bernet study supports the hypothesis that FGF-2 increase in the aged niche is a compensatory response to the loss of function of FGFR1 activity observed in aged MuSCs. In particular, they show that while in young MuSCs the FGF2/FGFR1 axis drives asymmetric division through activation of p38 only in the committed daughter cell, in aged MuSCs this balance is altered. Indeed, the insensitivity to FGF signaling in the elderly MuSCs results in constitutive activation of p38 with loss of asymmetric polarization and impaired self-renewal capacity. Likewise, FGFR1 ligand independent, constitutive activation restores MuSC asymmetric cell division.

With elegant experiments of autologous and serial MuSC transplantation Cosgrove et al. demonstrate the intrinsic defect of elderly derived MuSCs in association with increased p38 activity. The authors demonstrate a synergistic interaction of biochemical and biophysical factors, respectively pharmacological inhibition of p38 and a hydrogel culture system, which contribute to reconstitute the proliferative capability and self-renewal as assayed by *in vitro* and *in vivo* engraftment. The effect of p38 inhibition in driving stem cell renewal was already demonstrated by Palacios et al. (2010), supporting the notion that pharmacological intervention with p38 inhibitors may support muscle regeneration. Moreover, this paper provides a useful strategy to overcome the bottleneck of *in vitro* stem cell expansion in cell therapies using specific soft biomaterial that mimics the muscle niche.

In the same month Sousa-Victor and colleagues came out with a study demonstrating that geriatric MuSCs fail to support muscle regeneration and display defective activation. Serial transplantation experiments supported the conclusion that this defect is a cell intrinsic feature of geriatric MuSCs. They identify the master regulator of senescence p16^{INK4a} as a key determinant

TABLE 1 | Schematic representation of the rejuvenation strategies used in the discussed papers.



responsible for a quiescence-senescence switch (a process named geroconversion) operating in geriatric MuSCs in coincidence with their impaired regenerative potential. Indeed, genetic inactivation of p16^{INK4a} locus was sufficient to recover the cells from the senescence-associated cell cycle arrest and restore their self-renewal capacity, leading to the reconstitution of the stem cell pool after muscle damage. The novelty of this study relies on the finding that geriatric stem cells are associated with the progressive accumulation of DNA damage and senescence-associated markers that in turn contribute to the loss of reversible quiescence mediated by p16^{INK4a}. Indeed, in geriatric MuSCs, the p16^{INK4a} locus is constitutively de-repressed due to altered PRC1 complex function.

These studies demonstrate that in addition to the regenerative environment that profoundly affects the niche and stem cell function, there is another level of tissue homeostasis regulation that is intrinsic to adult stem cells. The cell autonomous functionality declines in the elderly due to de-regulated p38 signaling and accumulation of DNA damage and senescence-associated features. This evidence suggests new avenues to reverse the dysfunctional status of MuSCs from aged tissues. For instance, constitutive FGFR1 signaling can restore MuSCs asymmetric division and self-renewal, and pharmacological blockade of p38 signaling can promote MuSCs self-renewal and engraftment by silencing p16^{INK4a}, thus reversing geroconversion and allowing MuSCs to support muscle regeneration (Table 1). Intriguingly, the activation of p38 signaling has been associated with senescence (Wang et al., 2002) as well as increasing levels of p16^{INK4a} (Serrano et al., 1997; Iwasa et al., 2003) in cell types other than muscle stem cells highlighting the notion that a more complex signaling network that may be context dependent controls senescence (Xu et al., 2014). The p38 signaling pathway has been demonstrated to be involved in IL-6 induced STAT3 transcriptional activation (Zauberman et al., 1999; Riebe et al., 2011). Intriguingly, the recent finding that increases in JAK-STAT signaling inhibits MuSCs function during aging further provides evidence for the pivotal role of p38 in driving muscle regeneration (Price et al., 2014; Tierney et al., 2014). Future studies should determine the molecular relationship between these new players of muscle aging—DNA damage, p38 signaling and p16^{INK4a} in order to devise treatments aimed at reversing MuSC senescence.

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Myogenic-specific ablation of Fgfr1 impairs FGF2-mediated proliferation of satellite cells at the myofiber niche but does not abolish the capacity for muscle regeneration

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Skeletal muscle satellite cells (SCs) are Pax7⁺ myogenic stem cells that reside between the basal lamina and the plasmalemma of the myofiber. In mature muscles, SCs are typically quiescent, but can be activated in response to muscle injury. Depending on the magnitude of tissue trauma, SCs may divide minimally to repair subtle damage within individual myofibers or produce a larger progeny pool that forms new myofibers in cases of overt muscle injury. SC transition through proliferation, differentiation and renewal is governed by the molecular blueprint of the cells as well as by the extracellular milieu at the SC niche. In particular, the role of the fibroblast growth factor (FGF) family in regulating SCs during growth and aging is well recognized. Of the several FGFs shown to affect SCs, FGF1, FGF2, and FGF6 proteins have been documented in adult skeletal muscle. These prototypic paracrine FGFs transmit their mitogenic effect through the FGFRs, which are transmembrane tyrosine kinase receptors. Using the mouse model, we show here that of the four Fgfr genes, only Fgfr1 and Fgfr4 are expressed at relatively high levels in quiescent SCs and their proliferating progeny. To further investigate the role of FGFR1 in adult myogenesis, we have employed a genetic (Cre/loxP) approach for myogenic-specific (MyoD^{Cre}-driven) ablation of Fgfr1. Neither muscle histology nor muscle regeneration following cardiotoxin-induced injury were overtly affected in Fgfr1-ablated mice. This suggests that FGFR1 is not obligatory for SC performance in this acute muscle trauma model, where compensatory growth factor/cytokine regulatory cascades may exist. However, the SC mitogenic response to FGF2 is drastically repressed in isolated myofibers prepared from Fgfr1-ablated mice. Collectively, our study indicates that FGFR1 is important for FGF-mediated proliferation of SCs and its mitogenic role is not compensated by FGFR4 that is also highly expressed in SCs.

Keywords: satellite cells, fibro/adipogenic progenitors, fibroblast growth factor, Pax7, MyoD^{Cre}, alpha7 integrin, cardiotoxin injury, muscle spindles

Introduction

Skeletal muscle is composed of multinucleated myofibers that are established during embryogenesis by fusion of myoblasts. Addition of myofiber nuclei (myonuclei) or formation of new myofibers during postnatal and adult life depend on satellite cells (SCs), Pax7⁺ myogenic progenitors that are localized between the basal lamina and the plasmalemma of the myofiber (Mauro, 1961; Seale et al., 2000; Yablonka-Reuveni, 2011). During postnatal growth, at least some SCs are proliferative and contribute progeny that fuse with the enlarging myofibers (Moss and Leblond, 1971; Schultz, 1996; Halevy et al., 2004; White et al., 2010). In mature muscles, SCs are typically quiescent, but can be activated in response to muscle injury (Schultz et al., 1978; Montarras et al., 2013). Depending on the magnitude of tissue trauma, SCs may divide minimally to repair subtle damage within individual myofibers or produce a larger progeny pool that forms new myofibers in cases of overt muscle injury (Grounds and Yablonka-Reuveni, 1993; Hawke and Garry, 2001). In addition to generating myogenic progeny that fortify myofibers, at least some SCs can self-renew, thereby meeting the defining criteria of bona fide resident stem cells (Collins et al., 2005; Day et al., 2007; Kuang et al., 2007; Sacco et al., 2008).

At the molecular level, SCs and their progeny are tightly regulated by highly orchestrated temporal expression of transcription factors and cell cycle regulators, providing a balance between SC quiescence, proliferation, differentiation and renewal (Bentzinger et al., 2010; Yablonka-Reuveni and Day, 2011; Yin et al., 2013). To monitor progression through these stages, researchers have relied on distinct marker signatures, in particular, temporal expression of the paired box transcription factor Pax7, and the myogenic regulatory factors MyoD and myogenin (Yablonka-Reuveni and Rivera, 1994; Zammit et al., 2006; Yablonka-Reuveni et al., 2008; Yablonka-Reuveni, 2011). Proliferating progeny maintain Pax7 expression as their quiescent progenitors, but distinctly, are also MyoD-positive (Zammit et al., 2004). A decline in Pax7, along with the induction of myogenin, marks progeny that have entered into the differentiation phase and subsequently may fuse into myotubes (Shefer et al., 2006; Day et al., 2009). Re-emergence of cells that express Pax7, but not MyoD, defines a self-renewing population of SCs known as reserve cells (Halevy et al., 2004; Zammit et al., 2004; Day et al., 2007).

Satellite cell transition through proliferation, differentiation and renewal is not only governed by the molecular blueprint of the cells, but is also regulated by the extracellular milieu at the SC niche (Allen et al., 1984; Allen and Boxhorn, 1989; Anderson, 2006; Brack and Rando, 2007; Shefer and Yablonka-Reuveni, 2008; Yin et al., 2013; Wang et al., 2014). Isolated myofibers maintained in conditions where the SCs and their progeny are retained at their native position, have offered a unique *in vitro* means to investigate the effect of growth factors on SC behavior at their native niche (Bischoff, 1986a; Yablonka-Reuveni and Rivera, 1994; Yablonka-Reuveni et al., 1999a). Using this approach, hepatocyte growth factor (HGF) and selective members of the fibroblast growth factor (FGF) family have been shown to enhance SC proliferation (Bischoff, 1986a,b;

Yablonka-Reuveni et al., 1999a,b; Kastner et al., 2000; Wozniak and Anderson, 2007), while transforming growth factor beta (TGF β 1) has been found to repress proliferation (Bischoff, 1990; Yablonka-Reuveni and Rivera, 1997b). Our particular interest in the role of the FGFs and their receptors in regulating SC dynamics through life (Yablonka-Reuveni and Rivera, 1994, 1997b; Yablonka-Reuveni et al., 1999a,b; Kastner et al., 2000; Shefer et al., 2006; Kwiatkowski et al., 2008) has prompted the research described in the current study.

The FGFs are key players in the processes of proliferation and differentiation of a wide range of cells and tissues. Over 20 FGFs, classified as paracrine (FGFs 1–10, 16–18, 20, 22), endocrine (FGFs 15/19, 21, 23) and intracrine (FGFs 11–14) types, have been discovered to date (Mason, 2007; Itoh and Ornitz, 2011; Ohta and Itoh, 2014). Selective paracrine FGFs have long been known to act as mitogens of SCs [i.e., FGF1, FGF2, FGF4, and FGF6, but not FGF5, FGF7, and FGF8 (Sheehan and Allen, 1999; Kastner et al., 2000)]. Importantly, several of these paracrine FGFs that can promote SC proliferation (FGF1, FGF2, FGF6) have been detected at the transcript and the protein levels in adult skeletal muscle (Yamada et al., 1989; Alterio et al., 1990; Le Moigne et al., 1990; Oliver et al., 1992; Clarke et al., 1993; Dusterhoft et al., 1999; Kastner et al., 2000; Zhao and Hoffman, 2004; Fon Tacer et al., 2010; Chakkalakal et al., 2012). In particular, FGF2 (formerly known as basic FGF) has been used extensively as the FGF of choice in many studies of SCs in single myofibers (Yablonka-Reuveni and Rivera, 1994, 1997b; Yablonka-Reuveni et al., 1999a,b; Shefer et al., 2006) and as a routine medium supplement in primary cultures (Rando and Blau, 1994; Motohashi et al., 2014). Apart from its mitogenic effect, FGF2 has been suggested to directly repress myoblast differentiation, thereby supporting expansion of the proliferative pool (Clegg et al., 1987; Olwin et al., 1994).

Studying SCs in isolated myofibers under conditions that retain SCs at the myofiber niche, we previously showed that SCs from senile mice (29–33 months) could not enter a proliferative state without FGF2 supplementation, whereas SCs from young mice (3–6 months) did not require exogenous FGF2 (Shefer et al., 2006). In accordance with our findings, a recent study reported that FGF2 is required to remove age-associated proliferative inhibition of SCs (Li et al., 2014). We also demonstrated that an FGF2 activity-blocking antibody drastically reduced SC activation/proliferation in isolated myofibers from young rodents (Yablonka-Reuveni and Rivera, 1994). Collectively, our studies indicate that FGF2 is required for SC proliferation and that FGF2 (or FGF2-mediated signaling) becomes rate limiting in SC function in old age, and this may be an underlying factor in the age-associated decline in SC numbers observed in some limb muscles (Brack et al., 2005; Shefer et al., 2006, 2010, 2013). However, it has been reported that excess FGF2 harbored in the myofibers of aging mice leads to SC depletion due to detrimental proliferation (without self-renewal), rather than retention of the quiescent state (Chakkalakal et al., 2012). Hence, means for direct ablation of FGF2 signaling are needed to assist in determining its role in SC performance during aging.

As the paracrine FGFs mediate their biological responses by binding to cell surface FGF receptors (FGFR1, FGFR2, FGFR3,

FGFR4), FGFR impairment offers one possible approach for studying the effect of FGF2 signaling on SC performance. The FGFRs share a common “generic” structure consisting of an extracellular region containing three immunoglobulin-like domains (Ig-1, Ig-2, Ig-3), a transmembrane domain, and an intracellular domain containing a tyrosine kinase core. FGF binding to the FGFR extracellular domain induces receptor dimerization and activation of the tyrosine kinase domain, which can initiate key downstream intracellular signaling pathways: RAS-RAF-MAPK, PI3K-AKT, STAT, and PLC γ (Eswarakumar et al., 2005; Mason, 2007; Lanner and Rossant, 2010; Goetz and Mohammadi, 2013). While the FGFRs are encoded by four separate genes (Fgfr1, Fgfr2, Fgfr3, Fgfr4), alternative splicing variants, alongside the temporal and spatial regulation of expressed FGF and FGFRs and the involvement of additional co-factors, increase the complexity and specificity of FGF signaling (Ornitz, 2000; Zhang et al., 2006; Mason, 2007; Itoh and Ornitz, 2011; Goetz and Mohammadi, 2013). Out of the four FGFRs, typically only FGFR1 and FGFR4 have been considered in the context of adult myogenesis, due to their relative higher transcript levels observed in freshly isolated SCs and myogenic cultures [(Sheehan and Allen, 1999; Cornelison et al., 2000; Kastner et al., 2000; Jump et al., 2009; Chakkalakal et al., 2012); current study]. Furthermore, to date only FGFR1 and FGFR4 have been documented at the protein level in SCs or their progeny (Cornelison et al., 2001; Kwiatkowski et al., 2008; Cassano et al., 2011). While our overexpression studies have suggested different modes of function for FGFR1 and FGFR4 (Kwiatkowski et al., 2008), it is unknown whether these two FGFRs can compensate for each other during SC myogenesis. Pharmacological-based abrogation of FGFR-signaling has been employed in order to elucidate the role of FGFR1 in the context of SC dynamics (Chakkalakal et al., 2012; Bernet et al., 2014). However, the inhibitory drug used, SU5402 (Mohammadi et al., 1997), can theoretically target all FGFRs based on its effect on blocking FGFR tyrosine kinase function. Indeed, SU5402 has been used as a general inhibitor of FGF signaling in different species regardless of the expressed FGFR (Udayakumar et al., 2003; Delaune et al., 2005; Dvorak et al., 2005; Abe et al., 2007; Thomsen et al., 2008; Vatsveen et al., 2009; Franzdottir et al., 2010; Fukui and Henry, 2011; Li et al., 2013). Myogenic-specific ablation or overexpression of Spry1, a member of the Sprouty family of negative regulators of receptor tyrosine kinase signaling (Cabrita and Christofori, 2008), were also employed to modulate FGF signaling during adult myogenesis (Chakkalakal et al., 2012). The Sprouty proteins, however, act as inhibitors of the Ras/MAPK cascade, a pathway downstream of various receptor tyrosine kinases beyond just the FGFRs (Mason, 2007; Cabrita and Christofori, 2008), which can complicate data interpretation.

If FGFR signaling is essential for regulating SC pool size, which in turn may be important for muscle homeostasis, then a better understanding of this topic is needed when considering future therapies for disease- or age-associated muscle wasting. Gaining further understanding of the role of the FGFR system in myogenesis requires models that facilitate direct FGFR ablation, bypassing downstream interventions that may not specifically target individual FGFRs and may affect additional

tyrosine kinase receptor cascades. In the current study we have aimed to gain insight into the role of FGFR1 during adult myogenesis using Fgfr1-ablated mice. As standard Fgfr1-null mice die during gastrulation (Deng et al., 1994; Yamaguchi et al., 1994), investigations of the role of FGFR1 in fetal and adult life have only become possible with the development of conditional Fgfr1-null alleles (Xu et al., 2002; Trokovic et al., 2003). Here, we have ablated Fgfr1 specifically in the myogenic lineage using a genetic approach with a Cre/loxP mouse model that relies on the MyoD^{Cre} allele to mediate excision of the floxed Fgfr1 gene. MyoD is well recognized as a master regulator of the myogenic lineage specification during embryogenesis (Weintraub et al., 1991). While SCs are thought to express MyoD only upon their activation (Yablonka-Reuveni and Rivera, 1994; Cornelison and Wold, 1997; Yablonka-Reuveni et al., 2008), SC progenitors do emerge during embryogenesis from a MyoD-expressing lineage (Kanisicak et al., 2009; Yamamoto et al., 2009). Thereby, MyoD^{Cre}-mediated excision of floxed genes would occur in the embryonic muscle and be stably maintained in the myogenic lineage through adult life. Here we show that myogenic-specific ablation of Fgfr1 does not appear to influence muscle morphology or regeneration following cardiotxin-induced damage in adult mice. Nevertheless, our study provides novel evidence for the obligatory role for FGFR1 in mediating FGF2 mitogenic effect on SCs that is not compensated by FGFR4, which is also highly expressed in SCs.

Materials and Methods

Mice

Experimental procedures were approved by the University of Washington Institutional Animal Care and Use Committee. Mice were typically 4–6 months of age. Knockin heterozygous males MyoD^{Cre} [MyoD1^{tm2.1(icre)Gh} (Kanisicak et al., 2009)] provided by David Goldhamer, backcrossed by us to C57BL/6, were bred with knockin reporter females R26^{mTmG} [Gt(ROSA)26Sor^{tm4(CTB-tdTomato,-EGFP)Luo}/J (Muzumdar et al., 2007)] to generate adult F1 MyoD^{Cre/+}/R26^{mTmG/+} double heterozygous animals. Mice harboring floxed Fgfr1 alleles (Trokovic et al., 2003) were provided by David Ornitz (White et al., 2007). These mice additionally harbored floxed FGFR2 (Yu et al., 2003). Nevertheless, as discussed in the Introduction, FGFR2 has been considered not relevant in adult myogenesis and indeed, as shown in Results, Fgfr2 transcript expression in SCs and their progeny is negligible. The Fgfr1^{f/f}/Fgfr2^{f/f} females were crossed with MyoD^{Cre/+}/R26^{mTmG/+} males and the resulting MyoD^{Cre/+}/R26^{mTmG/+}/Fgfr1^{f/+}/Fgfr2^{f/+} males were backcrossed with Fgfr1^{f/f}/Fgfr2^{f/f} females to produce MyoD^{Cre/+}/R26^{mTmG/+}/Fgfr1^{f/f}/Fgfr2^{f/f} experimental animals harboring muscle-specific (i.e., MyoD-driven) Fgfr deletions. The FGFR1^f allele contains loxP sites flanking exons 8–15 that encompass the transmembrane domain and most of the intracellular region (Trokovic et al., 2003). The FGFR2^f allele contains loxP sites flanking exons 8–10 that encode a portion of the ligand binding Ig-3 domain and the transmembrane domain (Yu et al., 2003).

Primers for genotyping the MyoD^{Cre} (JAX mice stock #014141) and R26^{mTmG} (JAX mice stock #007676) alleles were according to Jackson Lab. Primers for genotyping the floxed Fgfr alleles were according to (Trokovic et al., 2003; White et al., 2007). Myogenic specificity of the MyoD^{Cre}-driven Fgfr deletions was confirmed by the detection of Fgfr delta alleles (Fgfr1^Δ, Fgfr2^Δ) only in skeletal muscles but not in other control organs; PCR primers were according to (Trokovic et al., 2003; White et al., 2007). Likewise, GFP fluorescence was detected only in skeletal muscle myofibers and SCs as we previously published for MyoD^{Cre/+}/R26^{mTmG/+} mice (Stuelsatz et al., 2012, 2014).

Mice carrying a MyoD-null allele (Rudnicki et al., 1992) or α7integrin-null allele (Flintoff-Dye et al., 2005) in a heterozygous or homozygous format were additionally used for comparison when analyzing SC numbers in isolated myofibers from Fgfr1/Fgfr2-ablated mice. Both null strains were utilized in our earlier studies (Yablonka-Reuveni et al., 1999a; Kirillova et al., 2007; Rooney et al., 2009; Stuelsatz et al., 2012) and genotyped according to published procedures (Valdez et al., 2000; Flintoff-Dye et al., 2005). Apart from the MyoD^{+/−} and MyoD^{−/−} mice that were on Balb/C background, all other strains used in this study were on enriched C57BL/6 background.

Cell Sorting by Flow Cytometry

Cells were isolated from hindlimb [limb; pooled tibialis anterior (TA), extensor digitorum longus (EDL) and gastrocnemius] or diaphragm muscles of floxed FGFR and control mice harboring the MyoD^{Cre} and the R26^{mTmG} alleles. The R26^{mTmG} reporter operates on a membrane-localized dual fluorescent system where all cells express Tomato until Cre-mediated excision of the Tomato gene allows for GFP expression in the targeted cell lineage (Muzumdar et al., 2007). Consequently, when the R26^{mTmG} allele is combined with MyoD^{Cre} allele all skeletal muscles and their resident SCs are GFP⁺ (Stuelsatz et al., 2014) due to ancestral MyoD expression in the myogenic lineage (Kanisicak et al., 2009). Using this muscle-specific reporter model, the isolated cells are sorted into myogenic and non-myogenic populations according to GFP vs. Tomato fluorochrome, respectively, combined with antigen-based sorting for maximal purification as we previously described (Stuelsatz et al., 2014). In brief, cell suspensions were released from harvested muscles by collagenase/dispase digestion and were first incubated with 10 μM Hoechst 33342 (Sigma-Aldrich) for 30 min at 37°C to label cell nuclei, followed by incubation with the following fluorescently conjugated antibodies (from eBioscience): anti-Sca1 (APC, clone D7), anti-CD31 (PECy7, clone 390), anti-CD45 (PECy7, clone 30-F11). Cell sorting was then performed using an Influx Cell Sorter (BD Biosciences) equipped with 350, 488, and 638 nm lasers. All sorted cells were collected within the G0-G1 population depleted of CD31⁺ (endothelial) and CD45⁺ (hematopoietic) cells, with myogenic and non-myogenic populations isolated as GFP^{+/Sca1−} and Tomato^{+/Sca1+} cells, respectively. Gates were determined by comparing fluorophore signal intensities between the unstained control and each single antibody/fluorophore control. Data was acquired at 20,000–100,000 events per sample and sorted cells were collected in our culture media described below.

Subsequent analysis and flow cytometry plots were generated using FlowJo (TreeStar). Sorted populations were either used as freshly isolated cells for gene expression studies or first expanded in primary cultures before harvested for DNA/RNA isolation and subsequent PCR/RT-PCR analyses as detailed next.

Primary Cultures of Sorted Myogenic and Non-myogenic Populations

Cells were cultured according to our routine procedures for mouse primary cultures (Danoviz and Yablonka-Reuveni, 2012; Stuelsatz et al., 2014). The basal solution used for all culture medium preparations consisted of Dulbecco's modified Eagle's medium (DMEM, high glucose, with L-glutamine, 110 mg/l sodium pyruvate, and pyridoxine hydrochloride, Hyclone) supplemented with antibiotics (50 U/ml penicillin and 50 mg/ml streptomycin, Gibco-Life Technologies). Sorted cells were cultured in 12-well culture plates pre-coated with Matrigel (BD Biosciences, diluted to a final concentration 1 mg/ml) using our standard DMEM-based medium containing 20% fetal bovine serum (Gibco-Life Technologies), 10% horse serum (Gibco-Life Technologies), and 1% chicken embryo extract [prepared from whole 10-day-old embryos as detailed in Notes #4 and 5 in (Danoviz and Yablonka-Reuveni, 2012)] and were incubated at 37°C, 5% CO₂. Cultures were initiated at a density of 1–2 × 10⁴ cells per well. After the initial plating, growth medium was replaced every 3 days.

Quantitative Gene Expression Analysis of Freshly Sorted Cells

RNA was isolated from freshly sorted myogenic and non-myogenic populations and reverse transcribed according to our published procedure (Day et al., 2010). Sorted cell populations were pelleted (400 × g for 10 min followed by 90 s at 12,000 × g) and suspended in the lysis buffer from the RNeasy Plus Micro kit (Qiagen) used to isolate total RNA. The RNA was then quantified using an Agilent Bioanalyzer and reverse transcribed (at 0.4 ng/μl) into cDNA using the iScript reverse transcriptase (Bio Rad). Gene expression was determined by SYBR Green-based quantitative PCR using 1 μl cDNA per reaction (20 μl final volume) on an ABI 7300 Real Time PCR machine (Life Technologies) as we previously described (Phelps et al., 2013) except that the annealing temperature for Fgfr1 and Fgfr2 primer sets was adjusted at 66°C instead of the standard 63°C used for the remaining primer sets. Raw qPCR cycle threshold values for each individual sample were normalized to eukaryotic translation elongation factor 2 (*Eef2*) reference gene expression as in (Phelps et al., 2013). Each sample was analyzed in triplicate. Genes were considered expressed if cycle threshold values (raw Ct) of less than 33 cycles were detected.

Primer sequences were (fwd/rev): *Pax7*, GCCACAGCTTC TCCAGCTAC/CACTCGGGTTGCTAAGGATG (120 bp, UCSC Genome Browser ID Pax7 uc008vms.1_1_1_2); *Fgfr1*, GCCC TGGAAGAGAGACCAGC/GAACCCCAGAGTTCATGGATGC [244 bp, (Kwiatkowski et al., 2008)]; *Fgfr2*, GCCTCTCGAA CAGTATTCTCCT/ACAGGGTTCATAGGCATGGG [103 bp, PrimerBank ID 2769639a1, (Spandidos et al., 2010)]; *Fgfr3*, GGCTCCTTATTGGACTCGC/TCGGAGGGTACCACTTTC

[219 bp, (Deng et al., 1996)]; *Fgfr4*, TTGGCCCTGTTGAGCAT CTTT/GCCCTTTGTACCACTGACG (189 bp, PrimerBank ID 6679789a1); *Eef2*, TGTCAGTCATGCCATGTG/CATCCT TGCGAGTGTCAGTGA (123 bp, PrimerBank ID 33859482a1). The final concentration of all primers was 500 nM.

Genomic and Transcriptional Analysis of Cultured Cells

Sorted cells cultured for 7 days were rinsed twice with DMEM before adding the lysis buffer from the AllPrep DNA/RNA Mini kit (Qiagen) used for simultaneous purification of genomic DNA and total RNA. Resulting preparations were quantified with a NanoDrop spectrophotometer. Genomic analyses were done by using 5 µl of DNA solution (adjusted to 10 ng/µl) per PCR reaction (25 µl final volume). PCR primers used for *Fgfr1* and *Fgfr2* genomic products (wildtype, flox and Δ) were according to (White et al., 2007). Transcript expression analysis was done by semi-quantitative RT-PCR according to our standard protocol (Day et al., 2007). Briefly, the RNA was reverse transcribed (at 20 ng/µl) into cDNA using the iScript reverse transcriptase (Bio Rad) and 5 µl of cDNA per PCR reaction (25 µl final volume) were used. PCR primers used for transcript expression analysis were previously described by us in (Kwiatkowski et al., 2008; Stuelsatz et al., 2012) and were used here at a final concentration of 400 nM. Expression of *Tbp* (TATA box binding protein) housekeeping control gene served as quality and loading control as in (Stuelsatz et al., 2012). For all PCR reactions, the following cycling parameters: 95°C for 15 min, 22–30 cycles of 94°C for 40 s, 60°C for 50 s, 72° for 1 min, with a final extension step of 72°C for 10 min were used. PCR products were separated on 1.5% agarose gels containing 1:10,000 dilution of SYBR Green I (Molecular Probes). Gels were imaged using Gel Logic 212 Pro (Carestream).

Quantification of SCs on Isolated Myofibers

Single myofibers were isolated from the EDL muscle as we previously described (Day et al., 2010; Keire et al., 2013). For each mouse strain and for each condition tested, myofibers were typically isolated from 3 mice. For analyzing the number of SCs on freshly isolated myofibers, we relied on Pax7 immunostaining following our standard approach using adherent myofibers where each myofiber is dispensed into an individual Matrigel-coated well (Shefer et al., 2006; Day et al., 2007; Keire et al., 2013) prior to fixation and immunostaining. For analyzing SC proliferation/differentiation, myofibers were cultured for 3 days in non-coated wells (24-well trays, 1 myofiber per well) using a DMEM-based medium containing 10% horse serum, an approach that yields non-adhering myofibers and maintains the SCs and their progeny associated with the parent myofibers [adapted from (Zammit et al., 2004)]. For myofibers treated with FGF, FGF2 was supplemented at 5 ng/ml (R&D Systems, recombinant human FGF basic, #234-FSE-025). The cultures were initiated in 0.3 ml and the replenishment of the medium (\pm FGF2) was achieved by adding fresh medium (0.2 ml) on culture day 1 and performing partial medium change (0.25 ml) on culture day 2; this approach ensured that myofibers were not disturbed during medium change.

Myofibers were fixed on day 3 by adding to the medium an equal volume of 4% paraformaldehyde [PFA, prepared as detailed in Note# 14 in (Keire et al., 2013)]. SCs were analyzed by immunostaining using mouse antibodies against Pax7 [Developmental Studies Hybridoma Bank (DSHB), ascites, 1:1000], MyoD (BD Biosciences, 1:800), Myogenin (DSHB, supernatant, 1:5) and counterstaining with DAPI according to our standard protocol for blocking, rinsing and mounting the myofibers (Shefer et al., 2006; Keire et al., 2013), except that extra care had to be taken due to the non-adherent nature of the myofibers.

Muscle Injury and Histology

Mice were anesthetized with isoflurane. For each mouse, the TA muscle from one leg was injected with 25 µl of 20 µM cardiotoxin (Sigma C9759), while the TA from the contralateral leg was injected with 25 µl of 0.9% NaCl as a control. TAs (with EDLs attached, referred later as TA/EDL) were harvested at different time points after injury, embedded in OCT (Tissue-Tek) and flash frozen in isopentane cooled with liquid nitrogen. Transverse sections (10 µm) prepared using a Leica CM1850 cryostat were stained with hematoxylin and eosin [H&E, as described in (Stuelsatz et al., 2015)] or alternatively fixed with 2% PFA for 10 min before being stained with DAPI when analyzed for GFP and Tomato fluorochrome expression.

FGFR4 Immunodetection

FGFR4 immunolabeling was performed on unfixed cryosections or on fixed primary myogenic cultures processed according to our standard protocol (Kwiatkowski et al., 2008; Stuelsatz et al., 2014). Cultures were prepared from Pronase digested muscle and grown on gelatin as in (Danoviz and Yablonka-Reuveni, 2012) before being fixed with ice-cold methanol as we previously published (Yablonka-Reuveni and Rivera, 1997a). In all cases, specimens were prepared from limb muscle of wildtype mice. Rabbit anti-FGFR4 was either from Santa Cruz Biotechnology or produced in our laboratory [(Kwiatkowski et al., 2008), available from Millipore]. FGFR4 immunolabeling of cryosections was done in combination with laminin immunodetection (Stuelsatz et al., 2014) to identify presumptive SCs based on their location underneath the myofiber basal lamina.

Microscopy and Imaging

Cell culture and histological observations were made with an inverted fluorescent microscope (Eclipse TE2000-S, Nikon). Images were acquired using CoolSNAP ES monochrome CCD camera (Photometrics) controlled with MetaVue Imaging System (Universal Imaging Corporation). For acquiring real color images of H&E stained muscle sections, images were taken with a Digital Sight DS-Ri1 color camera controlled by NIS-Elements F software (Nikon). Digitized images were assembled using Adobe Photoshop software. For final images of tissue cross sections showing the whole TA/EDL muscle, several pictures were taken (with a 10 or 20x objective) and merged together, resulting in a high-resolution view of the entire muscle cross-sectional area.

Statistics

Data were analyzed by one-way ANOVA ($p < 0.05$) with Bonferroni–Holm *post hoc* analysis using Excel with Daniel's XL Toolbox Add-In (by Daniel Kraus, Würzburg, Germany).

Results and Discussion

Experimental Approach

To achieve muscle-specific ablation of FGFR1 we have used a Cre/loxP genetic approach relying on the MyoD^{Cre} allele to mediate ablation of the floxed Fgfr1 gene. Regardless of muscle origin, virtually all SCs in adult muscles are derived from progenitors that have expressed the MyoD^{Cre} allele during embryogenesis (Kanisicak et al., 2009; Yamamoto et al., 2009). Hence, as detailed in the Introduction, MyoD^{Cre}-mediated excision of floxed genes would occur in the embryonic muscle and be stably maintained in the myogenic lineage through adult life. Indeed, our use of the R26^{mTmG} mouse (a floxed dual fluorescent reporter system described in Materials and Methods), crossed with the MyoD^{Cre} mouse, has clearly demonstrated specificity of the MyoD^{Cre}-mediated excision (i.e., GFP expression) in all adult muscles in both myofibers (which are formed during embryogenesis by myoblasts fusion) and SCs (Stuelsatz et al., 2012, 2014, 2015). Moreover, this specific expression of GFP in the myogenic lineage of MyoD^{Cre} × R26^{mTmG} mice has provided us with an effective tool for sorting SCs (GFP⁺) from non-myogenic (Tomato⁺) populations (Stuelsatz et al., 2012, 2014, 2015). While we were mostly interested in the present study in the role of FGFR1, the founder mice we had received to establish our colony harbored both floxed Fgfr1 and Fgfr2 alleles. As detailed in the Introduction, Fgfr2 has been considered not relevant in adult myogenesis and indeed, as shown in Figures 1 and 2, its expression level in SCs and their progeny is negligible. However, Fgfr2 could have theoretically been upregulated in the cell culture conditions used in the current study and/or upon Fgfr1 ablation. Hence, in this original investigation of the effect of Fgfr genetic ablation on the myogenic lineage we decided to retain both Fgfr1 and Fgfr2 floxed alleles. Mice carrying these myogenic-specific (MyoD^{Cre}-driven) double homozygous deletions are referred to throughout the manuscript as mR1^{Δ/Δ}/R2^{Δ/Δ}, while control mice, wildtype for Fgfr1 and Fgfr2, or harboring floxed Fgfr1 and Fgfr2 alleles, are referred to as R1^{+/+}/R2^{+/+} or R1^{f/f}/R2^{f/f}, respectively. The mR1^{Δ/Δ}/R2^{Δ/Δ} mice (with or without the R26^{mTmG} allele) were fertile and appeared normal by size and overall morphology (mice were followed up to 16 months of age).

Fgfr Expression in Freshly Isolated SCs

Before embarking on Fgfr ablation, we wished to analyze endogenous Fgfr transcript levels in freshly isolated SCs in comparison with non-myogenic cells. Gene expression analyses were performed on freshly isolated populations sorted from limb and diaphragm muscles of MyoD^{Cre/+}/R26^{mTmG/+} mice (Figure 1, quantitative RT-PCR). For both muscle types analyzed, the Pax7 data validates the myogenic nature of the GFP⁺

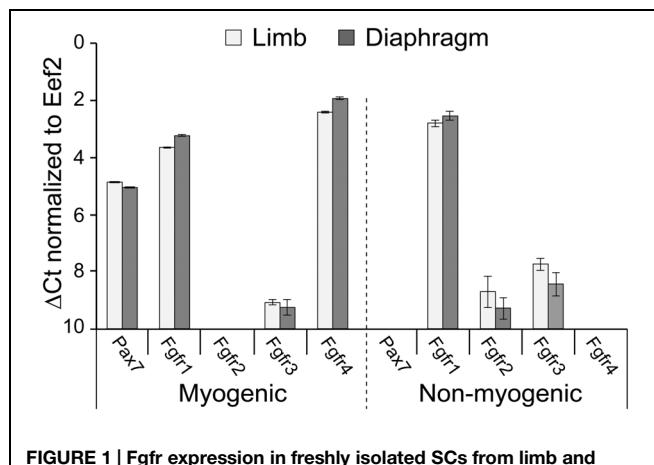


FIGURE 1 | Fgfr expression in freshly isolated SCs from limb and diaphragm muscles of MyoD^{Cre/+}/R26^{mTmG/+} mice. Myogenic and non-myogenic cell populations were sorted by flow cytometry (based on GFP and Tomato fluorescence, respectively, and cell surface antigens) and analyzed by quantitative RT-PCR. Gene expression values were normalized to Eef2 reference gene expression (ΔCt). Average Ct values for Eef2 gene ($\pm SD$) were 23.49 ± 0.09 (limb myogenic), 22.90 ± 0.04 (diaphragm myogenic), 20.84 ± 0.01 (limb non-myogenic), and 20.42 ± 0.01 (diaphragm non-myogenic).

population; i.e., Pax7, the classic marker of SCs, was expressed only by the sorted GFP⁺ population but not by the Tomato⁺ non-myogenic population (Figure 1). As additionally shown in Figure 1, Fgfr1 was expressed at a relatively high level by both the myogenic and non-myogenic populations, while Fgfr4 was expressed only by the myogenic population, in accordance with our previous rat studies (Kastner et al., 2000). Fgfr2 was below detection level in the myogenic population, while some Fgfr2 expression was demonstrated by the non-myogenic population. Fgfr3 was detected at relatively low level in both the myogenic and non-myogenic populations (Figure 1).

MyoD^{Cre} Induces Effective Deletions of the Floxed Fgfr1 and Fgfr2 Alleles in the Myogenic Lineage without Modulating Endogenous Levels of Fgfr3 and Fgfr4

The efficiency of MyoD^{Cre}-driven Fgfr1/Fgfr2 deletions in SCs was evaluated concurrently at the genomic (PCR) and transcript (RT-PCR) levels for both limb and diaphragm muscles (Figure 2). The cells were isolated from mR1^{Δ/Δ}/R2^{Δ/Δ} and control R1^{+/+}/R2^{+/+} mice that also harbored the R26^{mTmG} reporter to facilitate cell sorting of SCs vs. non-myogenic cells and to confirm the purity of the sorted populations in culture according to GFP vs. Tomato reporter color, respectively (Figure 2A). To ensure sufficient material for the analyses, and also to obtain insight into possible modulations in Fgfr gene expression upon proliferation/differentiation vs. freshly isolated cells (Figure 1), the sorted cells were cultured for 7 days in our standard rich-medium conditions, then harvested for simultaneous isolation of DNA and RNA preparations.

Notably, there were no apparent differences in overall morphology of the myogenic cultures from Fgfr1/Fgfr2-ablated (mR1^{Δ/Δ}/R2^{Δ/Δ}) and control (R1^{+/+}/R2^{+/+}) mice, whether

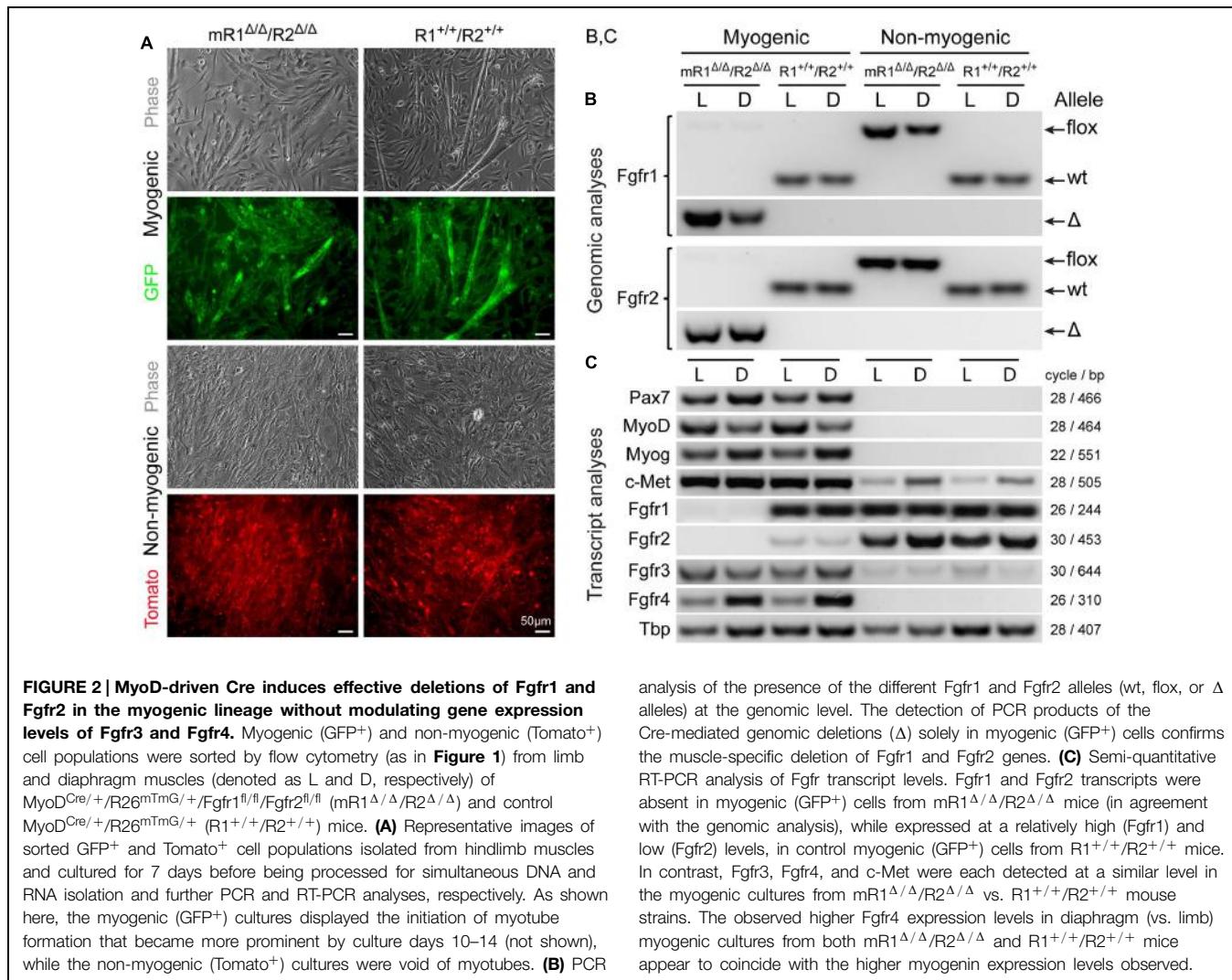


FIGURE 2 | MyoD-driven Cre induces effective deletions of Fgfr1 and Fgfr2 in the myogenic lineage without modulating gene expression levels of Fgfr3 and Fgfr4. Myogenic (GFP⁺) and non-myogenic (Tomato⁺) cell populations were sorted by flow cytometry (as in **Figure 1**) from limb and diaphragm muscles (denoted as L and D, respectively) of MyoD^{Cre/+}/R26^{mTmG/+}/Fgfr1^{fl/fl}/Fgfr2^{fl/fl} (mR1^{Δ/Δ}/R2^{Δ/Δ}) and control MyoD^{Cre/+}/R26^{mTmG/+} (R1^{+/+}/R2^{+/+}) mice. **(A)** Representative images of sorted GFP⁺ and Tomato⁺ cell populations isolated from hindlimb muscles and cultured for 7 days before being processed for simultaneous DNA and RNA isolation and further PCR and RT-PCR analyses, respectively. As shown here, the myogenic (GFP⁺) cultures displayed the initiation of myotube formation that became more prominent by culture days 10–14 (not shown), while the non-myogenic (Tomato⁺) cultures were void of myotubes. **(B)** PCR analysis of the presence of the different Fgfr1 and Fgfr2 alleles (wt, flox, or Δ alleles) at the genomic level. The detection of PCR products of the Cre-mediated genomic deletions (Δ) solely in myogenic (GFP⁺) cells confirms the muscle-specific deletion of Fgfr1 and Fgfr2 genes. **(C)** Semi-quantitative RT-PCR analysis of Fgfr transcript levels. Fgfr1 and Fgfr2 transcripts were absent in myogenic (GFP⁺) cells from mR1^{Δ/Δ}/R2^{Δ/Δ} mice (in agreement with the genomic analysis), while expressed at a relatively high (Fgfr1) and low (Fgfr2) level, in control myogenic (GFP⁺) cells from R1^{+/+}/R2^{+/+} mice. In contrast, Fgfr3, Fgfr4, and c-Met were each detected at a similar level in the myogenic cultures from mR1^{Δ/Δ}/R2^{Δ/Δ} vs. R1^{+/+}/R2^{+/+} mouse strains. The observed higher Fgfr4 expression levels in diaphragm (vs. limb) myogenic cultures from both mR1^{Δ/Δ}/R2^{Δ/Δ} and R1^{+/+}/R2^{+/+} mice appear to coincide with the higher myogenin expression levels observed.

cells were isolated from limb (**Figure 2A**) or diaphragm muscles (data not shown). For both mouse strains, the cultured GFP⁺ cells demonstrated typical myogenic features, fusing into myotubes by day 7 (**Figure 2A**), with myotubes enlarging in number and size in subsequent days (not shown). The non-myogenic cultures (Tomato⁺) from both Fgfr-deleted and control mice harbored typical features of fibroblastic cells as expected, with no myotubes detected even when following the cultures for longer time.

The genomic analysis of the different Fgfr1 and Fgfr2 alleles (wt, flox, or Δ alleles) validated that the mice harbored the anticipated alleles in accordance with mouse genotype and cell type analyzed (**Figure 2B**). The detection of genomic PCR products specific of the MyoD^{Cre}-mediated Fgfr1 and Fgfr2 genomic deletions (Δ allele) solely in myogenic cells confirmed muscle-specific deletions while the concurrent absence of any residual flox allele revealed the high efficiency of the Cre-mediated recombination in the SC lineage. Indeed, as anticipated based on their location within the corresponding Fgfr floxed region, our Fgfr1/Fgfr2 primers did not produce any RT-PCR products when analyzing Fgfr1/Fgfr2 mRNA expression in the myogenic lineage from mR1^{Δ/Δ}/R2^{Δ/Δ} mice (**Figure 2C**). This is in contrast to that seen in non-myogenic cell cultures where both Fgfr1 and Fgfr2 are expressed at a relatively high level for both mouse strains analyzed (**Figure 2C**), demonstrating the specificity of the Fgfr1/Fgfr2 ablation to the myogenic lineage. Fgfr3 and Fgfr4 expression levels in myogenic cells were unaffected when comparing myogenic cells from mR1^{Δ/Δ}/R2^{Δ/Δ} vs. R1^{+/+}/R2^{+/+} muscles. Likewise, the level

of c-Met, the receptor for HGF, also an established mitogen of SCs as detailed in the Introduction, was unaffected following Fgfr1/Fgfr2 deletion (**Figure 2C**). Hence, there is no apparent compensatory upregulation of Fgfr3, Fgfr4, or c-met in the Fgfr1/Fgfr2-ablated myogenic lineage.

The data in **Figure 2C** illustrate additional noteworthy points regarding Fgfr expression in cultures from both limb (L) and diaphragm (D) in the context of the control R1^{+/+}/R2^{+/+} cultures. (i) Fgfr3 appears to be expressed at a higher expression level in the myogenic cultures vs. the non-myogenic cultures and Fgfr4 is clearly expressed only in the myogenic cultures. (ii) When compared to Fgfr expression levels in freshly isolated populations from R1^{+/+}/R2^{+/+} control mice (**Figure 1**), Fgfr1 and Fgfr4 appear to retain the same expression profile in the day 7 cultures (with no Fgfr4 being detected in the non-myogenic cells), but Fgfr2 and Fgfr3 appear to be up-regulated in the cultured non-myogenic and myogenic cells, respectively. Our additional unpublished studies of limb-derived sorted populations have shown that Fgfr2 expression level continues to rise in the non-myogenic population with time in culture, concomitant with adipogenic differentiation that takes place uniquely in this Sca1⁺ sorted population. The latter non-myogenic population has previously been defined by others and us as fibro/adipogenic progenitors (Joe et al., 2010; Stuelsatz et al., 2014).

Muscle Tissue of Adult mR1^{Δ/Δ}/R2^{Δ/Δ} Mice Does Not Show Apparent Signs of Histopathology or Abolishment of Regenerative Activity

Histological examination of muscle tissues from Fgfr1/Fgfr2-ablated mice showed no apparent differences compared to the control (R1^{f/f}/R2^{f/f}) mice. Low and high magnification images of H&E stained cross sections processed from TA/EDL of mR1^{Δ/Δ}/R2^{Δ/Δ} and control R1^{f/f}/R2^{f/f} mice demonstrate for both mouse strains a normal muscle morphology (**Figure 3**). Next, we analyzed muscle regeneration in mR1^{Δ/Δ}/R2^{Δ/Δ} mice (**Figures 4 and 5**) following intramuscular administration of cardiotoxin, which specifically destroys the myofibers but preserves SCs (Harris, 2003). As seen in **Figure 4**, while most of the cardiotoxin-injected muscle tissue did not initiate myofiber formation on day 7 post-injury and still demonstrated large areas of inflammatory cell infiltrations at day 14, by day 21 there was an effective regenerative process throughout the muscle as observed by the characteristic presence of central myonuclei (**Figure 4**). Our unpublished studies with wildtype adult mice have demonstrated formation of nascent regenerative myofibers by day 7 following cardiotoxin injury and an almost complete myofiber recovery by day 14 post-injury. Hence, it appears that mR1^{Δ/Δ}/R2^{Δ/Δ} injured muscle has a lag in muscle regeneration. Nevertheless, our data (**Figures 4 and 5**) clearly indicate a thorough regeneration of the injured muscle by day 21 regardless of Fgfr1/Fgfr2 ablation in the myogenic lineage.

This injury study presented in **Figures 4 and 5** was done in mR1^{Δ/Δ}/R2^{Δ/Δ} mice that also harbored the R26^{mTmG} allele to facilitate direct tracking of myogenic cells/myofibers (GFP⁺) vs. non-myogenic cells (Tomato⁺), and as expected the newly

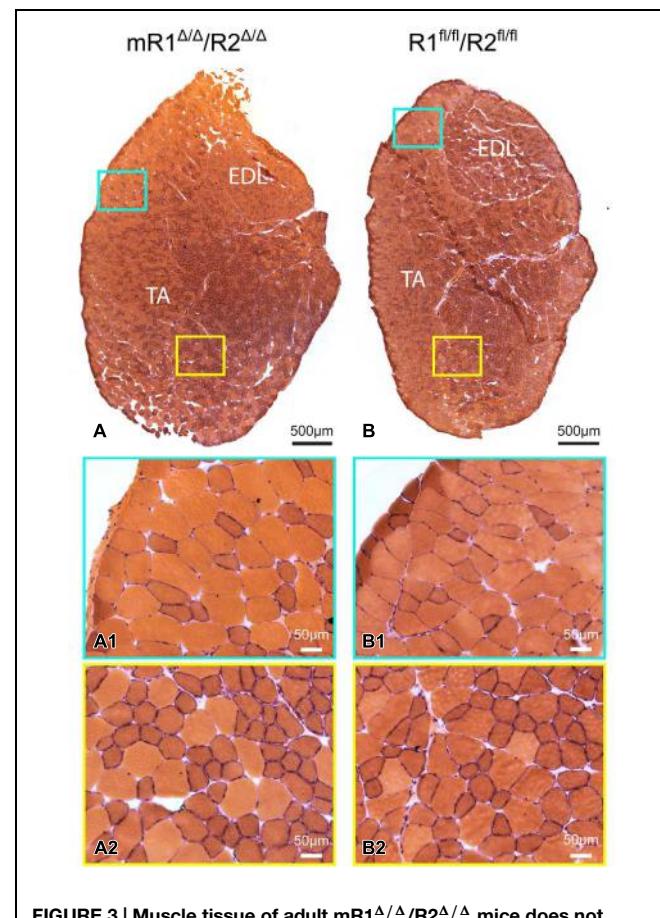


FIGURE 3 | Muscle tissue of adult mR1^{Δ/Δ}/R2^{Δ/Δ} mice does not appear different from that of control muscle from R1^{+/+}/R2^{+/+} mice. Representative images of H&E stained cross sections of TA/EDL from 10-month-old (A) mR1^{Δ/Δ}/R2^{Δ/Δ} and (B) R1^{+/+}/R2^{+/+} mice. For each panel, regions delineated in the low magnification image of the whole TA/EDL (A,B) are shown as higher magnification views (A1–B2) identified with corresponding colored frames. Muscles from both mouse strains harbored typical histology with larger and smaller diameter myofibers with peripheral nuclei.

regenerated myofibers are of MyoD lineage origin (**Figure 5**). The GFP reporter has also permitted the observation of (i) infrequent groups of small-diameter myofibers (**Figure 5A**), and (ii) the tiny intrafusal myofibers (**Figure 5B**) constituting the muscle spindle apparatus that plays a role in proprioception (Walro and Kucera, 1999; Kirkpatrick et al., 2008). Interestingly, the muscle spindle seen in **Figure 5B** is located within a regenerating region characterized by central myonuclei and thus most likely underwent a regeneration process similar to the surrounding myofibers.

SCs in Isolated Myofibers from mR1^{Δ/Δ}/R2^{Δ/Δ} Mice Exhibit Impaired Proliferative Response to FGF2

Based on the outcome of the injury study described above, FGFR1/FGFR2 do not appear to be essential (at least at the histological level) for muscle regeneration following cardiotoxin injury, but it does not necessarily preclude a role for FGF

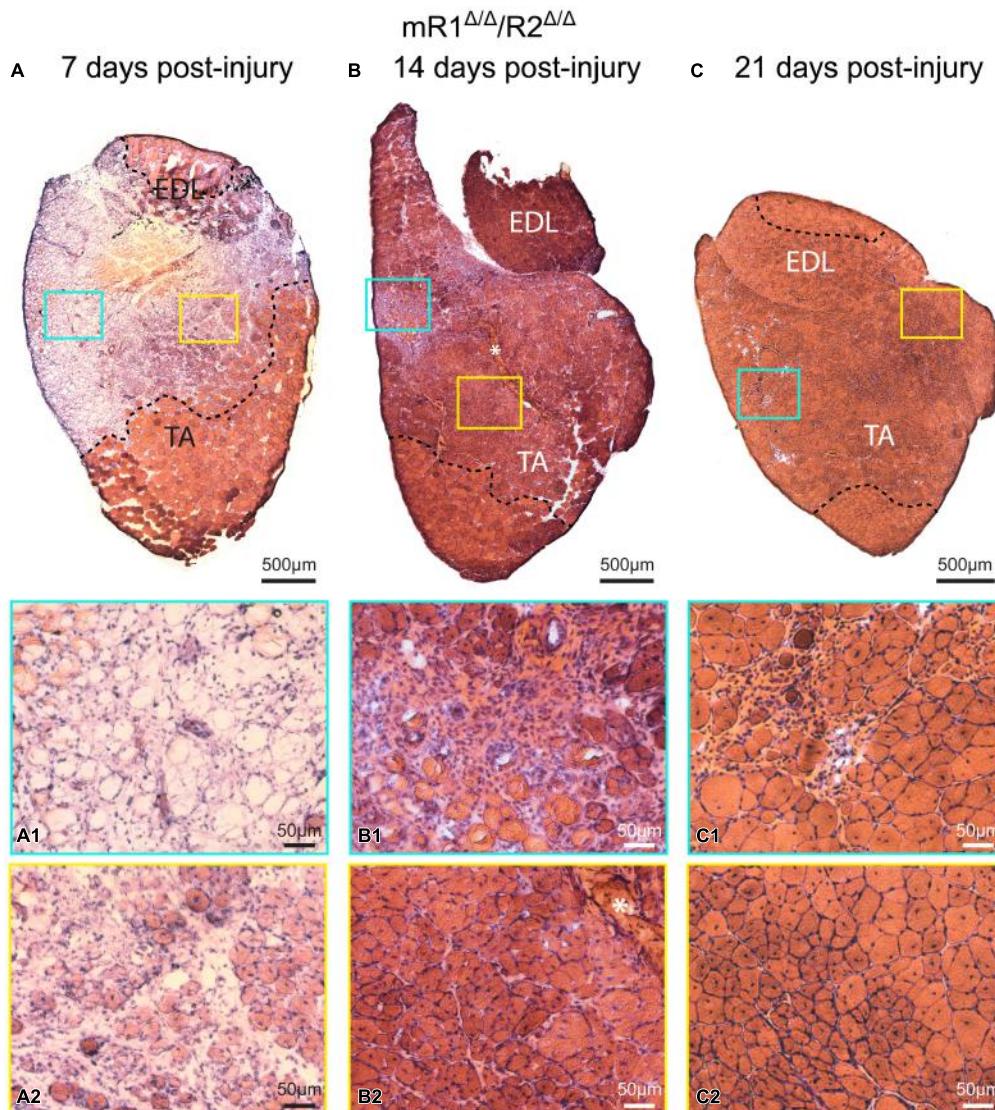


FIGURE 4 | Muscle tissue of adult $mR1^{\Delta/\Delta}/R2^{\Delta/\Delta}$ mice retains regenerative activity. Representative images of H&E stained cross sections of TA/EDL from 4-month-old $mR1^{\Delta/\Delta}/R2^{\Delta/\Delta}$ mice, showing extensive damage at 7 days post cardiotoxin-induced injury, and progressive recovery at 14 and 21 days post-injury. For each panel, regions delineated in the low magnification image of the whole TA/EDL are shown as higher magnification views (**A1–C2**) identified with corresponding colored frames; dotted lines in the low magnification images delineate the outer limits of the region that has been effectively injured. Morphology of control contralateral TAs (NaCl-injected, not shown) appeared similar to that of the uninjured muscle depicted in **Figure 3**. **(A)** As seen on day 7 post-injury, cardiotoxin injection caused massive myofiber degeneration, resulting

in large necrotic regions in which empty remnants of the original myofibers (**A1**) and infiltration of inflammatory cells (**A2**) are detected; regions with small regenerating myofibers with central myonuclei (hallmark of regenerating myofibers) were occasionally observed (**A2**). **(B)** On day 14 post-injury, regenerating myofibers were more abundant (**B2**), but regions showing infiltration of inflammatory cells were still occasionally present (**B1**); asterisk in (**B1**) and (**B2**) indicates the scar left at the needle injection point. **(C)** By day 21 post-injury, most of the original injured region showed successful regeneration based on the presence of larger (relative to day 14) myofibers containing central nuclei and overall tissue morphology (**C2**); infiltration of inflammatory cells was only minimally detected at this stage (**C1**).

signaling system in muscle regeneration. Indeed, multiple growth factors have been implicated in muscle regeneration and might compensate functionally for each other role in the cardiotoxin-induced muscle regeneration model (Charge and Rudnicki, 2004; Shefer and Yablonka-Reuveni, 2008). Hence, to directly investigate the impact of Fgfr ablation on SC number and

performance, we analyzed isolated myofibers maintained in culture conditions where SCs are retained at their native position by the myofiber as the cells undergo proliferation and differentiation (Yablonka-Reuveni and Rivera, 1994; Zammit et al., 2004; Keire et al., 2013). In the current study myofibers were isolated from EDL muscles and were either allowed to adhere to

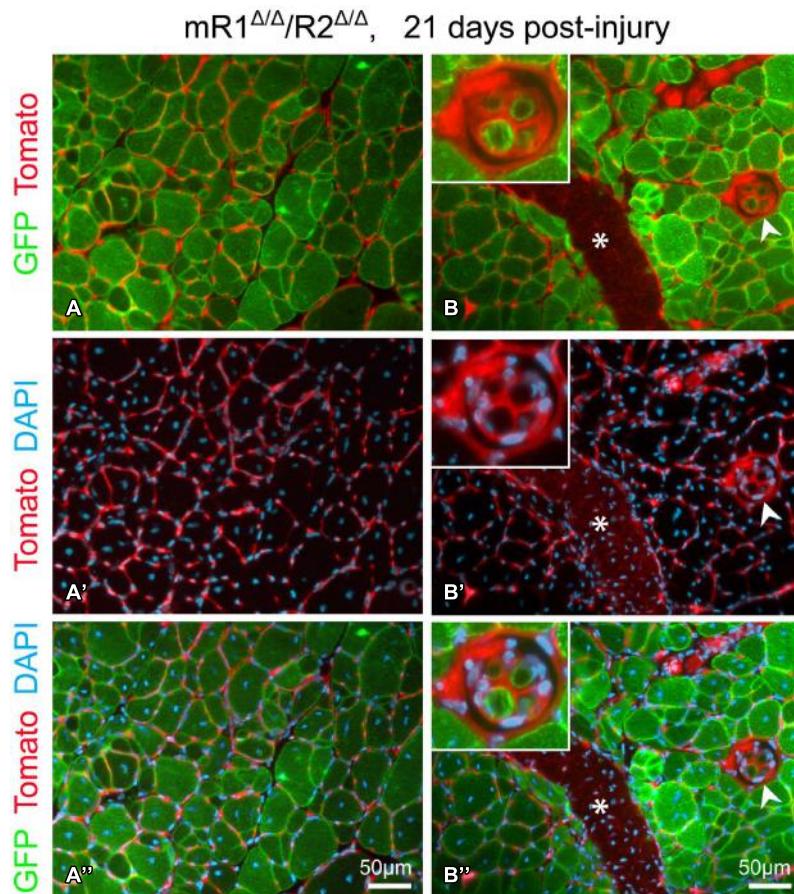


FIGURE 5 | Fluorescent images of cross sections prepared from TA isolated 21 days post-injury from a 4-month-old $mR1^{\Delta/\Delta}/R2^{\Delta/\Delta}$ mouse (also harboring the $R26^{mTmG}$ allele) depicting GFP and Tomato fluorescence, indicative of myogenic and non-myogenic structures, respectively, with DAPI⁺ nuclei. **(A–A'')** The use of the $R26^{mTmG}$ allele together with the MyoD^{Cre} driver (used for recombining the floxed Fgfr1 and Fgfr2 alleles) demonstrates that as expected, the regenerated myofibers identified by their central nuclei, were GFP⁺, hence, of MyoD lineage origin. The capillaries and connective tissue surrounding myofibers are Tomato⁺ (i.e., of non-MyoD⁺ origin). **(B–B'')** In addition to the standard myofibers (extrafusal), a muscle spindle (arrowhead, higher

magnification view in top left insert) can be observed within a regenerating region. While the spindle capsule and the material surrounding each intrafusal myofiber are of a non-MyoD⁺ origin (Tomato⁺), similar to the standard myofibers, the intrafusal myofibers are of MyoD-lineage origin (GFP⁺). Note the distinctive smaller diameter size of the intrafusal myofibers compared to the larger extrafusal myofibers. Asterisk indicates the scar (Tomato⁺) left at the needle injection point. Notably, as shown in panels **(A)** and **(B)**, sites with groups of smaller diameter extrafusal myofibers were observed in addition to the larger diameter myofibers. Morphology of control contralateral TAs (NaCl-injected, not shown) exhibited no differences when compared to uninjured muscle depicted in **Figure 3**.

Matrigel to determine SC numbers on freshly isolated myofibers according to Pax7 immunostaining (**Figure 6A**), or maintained in suspension to investigate SC dynamics (Pax7/MyoD/myogenin immunostaining) in response to FGF2 over 3 days in culture (**Figure 6B**).

The boxplot analysis of freshly isolated EDL myofibers immunostained for Pax7 (**Figure 6A**) suggests that within the four different groups identified as “FGFR-related,” the $mR1^{\Delta/\Delta}/R2^{\Delta/\Delta}$ mice potentially harbor less SCs per myofiber. An ANOVA test indeed revealed a statistically significant difference. Nevertheless, SC number in myofibers of $mR1^{\Delta/\Delta}/R2^{\Delta/\Delta}$ mice does not appear to be overtly affected when each of the FGFR-related groups are compared with mice lacking MyoD or $\alpha 7$ integrin that show a clear increase or

decrease, respectively, in their SC numbers (**Figure 6A**). Overall, the number of SCs per myofiber in each of the FGFR-related groups (and in the MyoD^{+/−} and $\alpha 7$ integrin^{+/−} groups) all fall within the wildtype range of adult male mice (Shefer et al., 2006; Day et al., 2007, 2010). Notably, the increase in SC numbers in MyoD-null mice was previously recognized (Megeney et al., 1996; Yablonka-Reuveni et al., 1999a; Cornelison et al., 2000; Gayraud-Morel et al., 2007), but while $\alpha 7$ integrin has been known to be expressed in the myogenic lineage, including in SCs (Burkin and Kaufman, 1999; Sacco et al., 2008; Rooney et al., 2009; Ieronymakis et al., 2010), we report here the novel finding of significantly reduced SC numbers in the absence of $\alpha 7$ integrin.

To analyze the effect of FGF2 on SC performance, myofibers were maintained for 3 days in suspension in basal medium

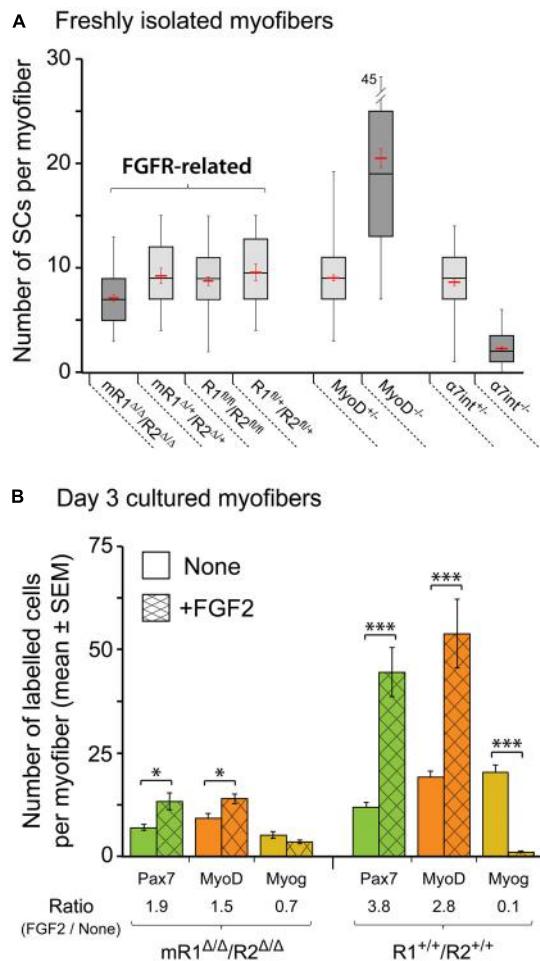


FIGURE 6 | Satellite cells (SCs) in isolated EDL myofibers from mR1^{Δ/Δ}/R2^{Δ/Δ} mice do not display a drastic change in their number but exhibit impaired proliferative response to FGF2. **(A)** Quantification of SCs in freshly isolated myofibers from different mouse strains as listed under the X-axis. SCs were quantified on individual myofibers by Pax7 immunostaining combined with DAPI-staining to highlight both SCs and myonuclei. Data are summarized as boxplots, depicting the quartile distribution and mean \pm SEM (red marks) for the number of SCs per myofiber; the whiskers on each side of the box are taken to the minimum and maximum values. MyoD-null and α1integrin-null data are included for comparison, as these mutations do drastically affect SC numbers. For each strain as listed from left to right under the X-axis, the number of myofibers analyzed was 48, 18, 54, 18, 120, 96, 88, and 95, respectively. **(B)** Single myofibers were maintained in suspension for 3 days with or without FGF2 supplement (5 ng/ml), then fixed and analyzed by immunostaining for the expression of the myogenic markers Pax7, MyoD and myogenin as a means to investigate SC dynamics. For typical Pax7/MyoD/myogenin immunostaining images see our previous mouse myofiber studies (Yablonka-Reuveni et al., 1999a; Shefer et al., 2006; Keire et al., 2013); examples of MyoD staining that depict the proliferative response of SCs to FGF2 supplementation are shown in Figure 7. To quantify the effect of FGF2 on SCs, the ratio in average cell numbers between FGF2-treated and untreated myofibers was determined for each marker (indicated under X-axis legend). Asterisks denote statistically significant differences in the number of labeled cells per myofiber between FGF2-treated and untreated myofibers (single asterisk $p < 0.05$; triple asterisks $p < 0.001$). For each condition as listed from left to right under the X-axis, the number of myofibers analyzed was 19, 21, 18, 21, 16, 17, 17, 12, 16, 12, 15, and 13, respectively.

(DMEM containing 10% horse serum, which is known to contain fewer growth promoting factors than fetal bovine serum) with or without FGF2 supplement. The cultured myofibers were then analyzed by immunostaining using antibodies against Pax7, MyoD and myogenin to quantify SCs and their progeny according to their transcription factor expression status (Figure 6B). The FGF2-mediated increase in Pax7⁺ or MyoD⁺ cells seen by day 3 in control (R1^{+/+}/R2^{+/+}) cultures is drastically affected in myofibers from mR1^{Δ/Δ}/R2^{Δ/Δ} mice (exemplified by MyoD immunostaining in Figures 7A–B'). Indeed, the ratio in average cell numbers between FGF2-treated and untreated myofibers declined by ~50% in the mR1^{Δ/Δ}/R2^{Δ/Δ} mice (1.9 [Pax7] and 1.5 [MyoD]) compared to R1^{+/+}/R2^{+/+} mice (3.8 [Pax7] and 2.8 [MyoD] Figure 6B). There was a slight decline in Pax7⁺, MyoD⁺, and myogenin⁺ cell numbers in untreated (i.e., not exposed to FGF2) mR1^{Δ/Δ}/R2^{Δ/Δ} myofibers. This may be due to the subtle decline in the initial number of SCs noted in freshly isolated myofibers (Figure 6A) and/or due to an impaired response of mR1^{Δ/Δ}/R2^{Δ/Δ} myofibers to the basal levels of FGF2, available in the cell culture serum or contributed by the myofibers (Yablonka-Reuveni and Rivera, 1994; Chakkalakal et al., 2012). The transition to the differentiated, myogenin⁺ state, was suppressed by FGF2 in the R1^{+/+}/R2^{+/+} myofibers (i.e., the ratio of myogenin⁺ cells in FGF2-treated vs. untreated myofibers was 0.1), which is in agreement with the established FGF2 effect on delaying myogenic differentiation (Clegg et al., 1987). Differently, in the mR1^{Δ/Δ}/R2^{Δ/Δ} mice, albeit the number of myogenin⁺ labeled cells appeared slightly reduced in FGF2-treated vs. untreated myofibers, there was no statistical difference between the two groups.

FGFR4 Does Not Appear to Substitute for the Mitogenic Effect of FGFR1 on SC Performance in Isolated Myofibers

Overall, the data in Figure 6B demonstrate an impairment of FGF2-mediated proliferative activity of SCs in isolated myofiber cultures from mice lacking functional FGFR1 (and FGFR2). This impairment suggests that other FGFRs that are possibly expressed by SCs cannot substitute for FGFR1 function. As the expression of Fgfr4 transcripts was indeed detected in freshly isolated SCs and their progeny (Figures 1 and 2), we set out to determine if FGFR4 protein is expressed by SCs. Previously we and others have shown FGFR4 protein in mouse SC progeny using Western blotting of cultured cells (Kwiatkowski et al., 2008; Cassano et al., 2011). Here, we show immunodetection of FGFR4 in limb muscle cross sections (Figures 8A–B'). The observed FGFR4⁺ structures are presumptive SCs based on their location underneath the myofiber basal lamina that is highlighted by laminin immunostaining (Figures 8A–B'). We additionally show here the expression of FGFR4 protein in mouse myogenic primary cultures (Figures 8C,C'). FGFR4 was down regulated in response to FGF2 supplement, therefore it appears to be functional (Figures 8D,D').

The inability of the endogenously expressed FGFR4 to rescue the proliferative effect of FGF2 in isolated myofibers from mR1^{Δ/Δ}/R2^{Δ/Δ} provides further support to our hypothesis that

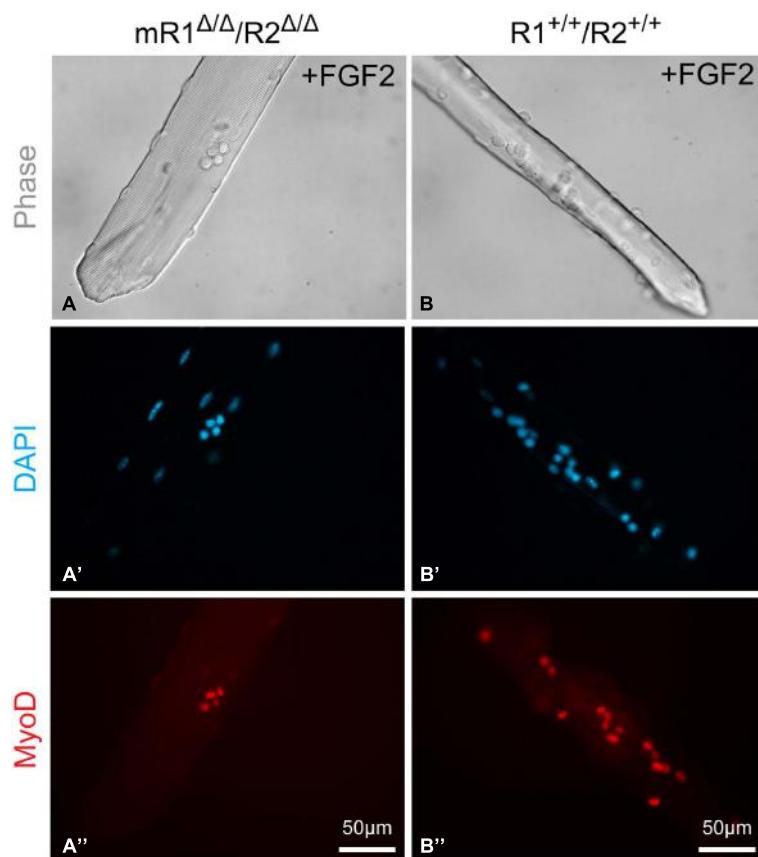


FIGURE 7 | Examples of EDL myofibers isolated from (A–A'') mR1^{Δ/Δ}/R2^{Δ/Δ} or (B–B'') R1^{+/+}/R2^{+/+} mice and cultured in suspension for 3 days with FGF2 supplement and then immunostained for MyoD, which is expressed by proliferating and differentiating SCs. DAPI counterstaining detected both the MyoD⁺ cells

and the myofiber nuclei, but only nuclei at the focal level of the MyoD⁺ cells can be seen in the images shown. The apparent difference in diameter between the two examples of myofibers shown in (A) vs. (B) is arbitrary and does not reflect a strain difference, as clearly demonstrated by the cross section images shown in **Figure 3**.

FGFR4 has a different role from that of FGFR1 during adult myogenesis. Indeed, overexpression studies have indicated that different from the other three FGFRs, FGFR4 appears to be a poor inducer of mitogenesis, whereas a clear mitogenic effect was detected when the intracellular domain of overexpressed FGFR4 was replaced with that of FGFR1 (Ornitz et al., 1996; Zhang et al., 2006). The poor mitogenic effect of FGFR4 could be linked to its much reduced tyrosine kinase phosphorylation compared to the other FGFRs (Kwiatkowski et al., 2008). Our FGFR4 overexpression studies [(Kwiatkowski et al., 2008); R Almuly and Z Yablonka-Reuveni, unpublished] have suggested a role for FGFR4 in suppressing FGFR1 tyrosine kinase activity and downstream signaling via FRS2-Erk1/2 axis (Goetz and Mohammadi, 2013), thereby leading cells to withdraw from the cell cycle. Moreover, an earlier FGFR4 overexpression study using L6E9 rat myoblasts demonstrated a weak mitogenic activity for FGFR4 and a role in inhibition of myogenic differentiation (Shaoul et al., 1995). Hence, FGFR4 might provide fine-tuning among proliferation, differentiation and renewal, counteracting the role of FGFR1 in enhancing myoblast proliferation.

Conclusion

This current study of Fgfr expression profile in freshly isolated SCs and their progeny from adult limb and diaphragm muscles provides new experimental evidence to the commonly held convention that of the four FGFRs, only Fgfr1 and Fgfr4 are of potential relevance to myogenesis. Our earlier work has suggested that these two FGFRs might have different functional roles during adult myogenesis. To begin addressing the possible distinct roles of FGFR1 vs. FGFR4, we employed in the present study a genetic approach relying on the MyoD^{Cre} allele for myogenic-specific ablation of FGFR1 (and FGFR2). Albeit this MyoD^{Cre}-driven ablation occurs early during embryogenesis, muscle development does not seem to be overtly impaired in the absence of functional FGFR1 (and FGFR2) based on the intact muscle histology of the adult mR1^{Δ/Δ}/R2^{Δ/Δ} mice. Furthermore, cardiotoxin-injured muscle of these mR1^{Δ/Δ}/R2^{Δ/Δ} mice showed effective regeneration. However, the SC mitogenic response to FGF2 was drastically repressed in isolated myofiber cultures prepared from the myogenic-specific

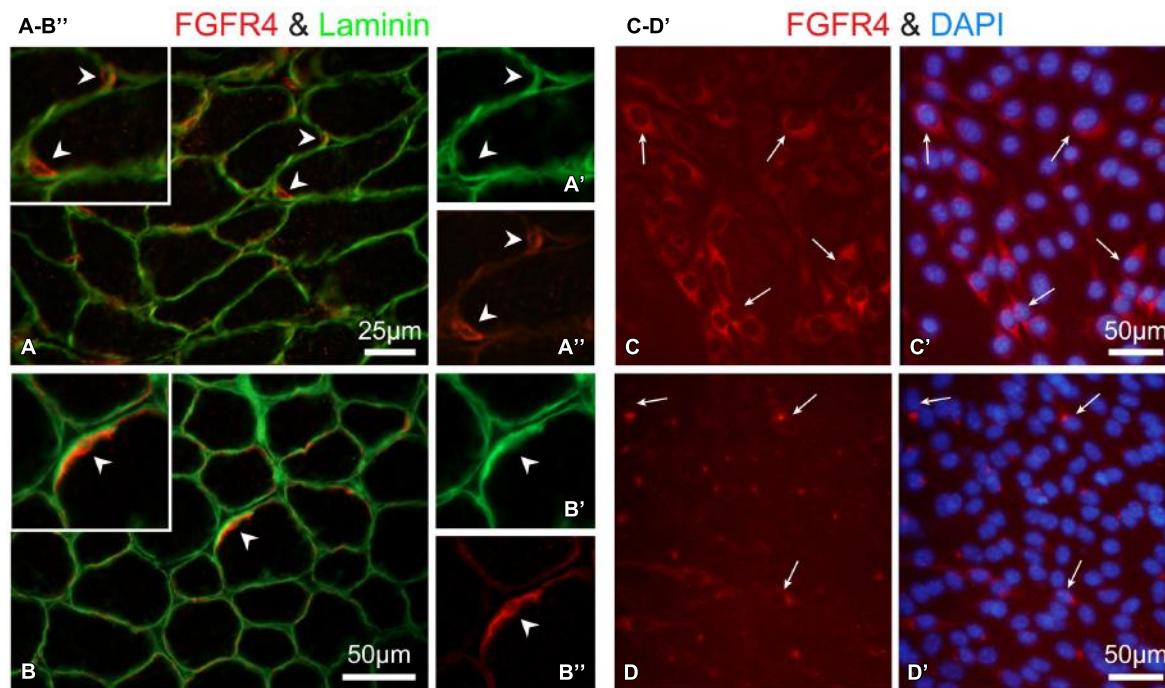


FIGURE 8 | Immuno-detection of FGFR4 protein in muscle tissue and primary myogenic culture from wildtype mice. (A–B'') Detection of FGFR4 in hindlimb muscle sections; positive cells are presumptive SCs based on their location underneath the myofiber basal lamina highlighted by laminin immunostaining. Notably, SC identification using Pax7 immunostaining is precluded as it would require antigen retrieval step which is not compatible with the conditions used here for FGFR4 detection on unfixed cryosections. As expected, SCs (FGFR4^+) were more abundant in (A–A'') the younger aged mouse (12 days old, gastrocnemius muscle) than in (B–B'') the 30-day-old mouse (TA muscle). Corresponding arrowheads denote common locations in the lower and higher

magnification images. **(C–D'')** Detection of FGFR4 in primary myogenic cultures from adult mice; the myogenic nature of the cultured cells was verified with double immunostaining for desmin as in (Yablonka-Reuveni et al., 1999a; data not shown). **(C,C')** FGFR4 protein expression is unique to the myogenic cells while residual non-myogenic cells present in this standard primary culture are negative. **(D,D'')** FGF2 treatment (20 ng/ml in DMEM containing 2% horse serum for 16 hours) of mouse primary myogenic cultures results in the downregulation of FGFR4. Following the overnight treatment with FGF2, FGFR4-immunosignal is restricted to a perinuclear compartment likely reflecting receptor desensitization through its internalization and targeting to endosomes.

Fgfr1/Fgfr2-ablated mice. Collectively, our study indicates that FGFR1 is important for FGF2-mediated proliferation of SCs, while the role of the expressed FGFR4 has yet to be resolved. To further address the role of FGFR1 and FGFR4, we are developing genetic models for myogenic-specific ablation of these receptors in growing and aging mice.

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A muscle stem cell for every muscle: variability of satellite cell biology among different muscle groups

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The human body contains approximately 640 individual skeletal muscles. Despite the fact that all of these muscles are composed of striated muscle tissue, the biology of these muscles and their associated muscle stem cell populations are quite diverse. Skeletal muscles are affected differentially by various muscular dystrophies (MDs), such that certain genetic mutations specifically alter muscle function in only a subset of muscles. Additionally, defective muscle stem cells have been implicated in the pathology of some MDs. The biology of muscle stem cells varies depending on the muscles with which they are associated. Here we review the biology of skeletal muscle stem cell populations of eight different muscle groups. Understanding the biological variation of skeletal muscles and their resident stem cells could provide valuable insight into mechanisms underlying the susceptibility of certain muscles to myopathic disease.

Keywords: satellite cell, muscle, muscular dystrophy, muscle stem cell, hypaxial, epaxial, diaphragm, craniofacial

Introduction

Skeletal muscle is a highly organized tissue that comprises up to 40% of a human's body mass and is required for essential functions such as metabolism, locomotion and breathing (Janssen et al., 2000; MacIntosh et al., 2006). The human body contains approximately 640 unique skeletal muscles, each having distinct functions in human physiology. Multiple factors contribute to skeletal muscle diversity including embryologic origin, myogenic regulatory pathways, and functional/metabolic requirements. Muscle heterogeneity is further underscored by the variable sensitivity of specific subsets of skeletal muscles to numerous distinct genetic mutations that give rise to muscular dystrophies (MD; Hoffman et al., 1987; Monaco et al., 1988; Bione et al., 1994; Brais et al., 1998; Bonne et al., 1999; Nonaka, 1999; Emery, 2002; Robinson et al., 2005; Bonnemann et al., 2014; Vieira et al., 2014). Adult muscle stem cells, called satellite cells, have been implicated in the pathology of some MDs (**Table 1**) and may contribute to the variable muscle sensitivity observed in some dystrophies.

Skeletal muscles are composed of myofibers, large syncytial cells containing hundreds of post-mitotic myonuclei. Juxtaposed between the basal lamina and the myofiber cell membrane, satellite cells reside at the periphery of skeletal myofibers (Mauro, 1961). Recent studies have demonstrated that satellite cells expressing *paired box protein 7* (*Pax7*) are the primary myogenic cell required for muscle regeneration (Lepper et al., 2011; Murphy et al., 2011; Sambasivan et al., 2011). The majority of knowledge concerning satellite cell biology arises from studies examining somite-derived muscles such as the quadriceps, gastrocnemius, tibialis anterior (TA), soleus, extensor digitorum longus, plantaris, biceps, and deltoid muscles, which collectively represent less than 2% of all skeletal muscles. Intriguingly, satellite cells present in other muscle groups, including trunk,

TABLE 1 | Muscular dystrophies (MDs) in which deficits in satellite cell function have been implicated in disease pathology.

Muscular dystrophy	Affected muscles	Mutant gene(s)	Affected protein(s)	Altered SC function	Reference
Duchenne	shoulder, upper limb, diaphragm, and calf	<i>DMD</i>	Dystrophin	Replicative exhaustion; Proliferation; Postnatal myofiber hypotrophy	Blau et al., 1983; Webster and Blau, 1990; Sacco et al., 2010; Jiang et al., 2014; Duddy et al., 2015
Limb girdle:					
LGMD1B	upper limb, shoulder,	<i>LMNA</i>	Lamin A/C	Differentiation	Frock et al., 2006
LGMD2A	chest, hip, and upper leg	<i>CAPN3</i>	Calpain 3	Proliferation to differentiation transition	Rosales et al., 2013
LGMD2C		<i>SGCG</i>	γ -Sarcoglycan	Decreased number; Replicative impairment?	Higuchi et al., 1999
LGMD2D		<i>SGCA</i>	α -Sarcoglycan		
LGMD2E		<i>SGCB</i>	β -Sarcoglycan		
LGMD2F		<i>SGCD</i>	δ -Sarcoglycan		
LGMD2H		<i>TRIM32</i>	tripartite motif-containing 32	Replicative senescence	Kudryashova et al., 2012; Mokhonova et al., 2015
LGMD2O		<i>POMGNT1</i>	Protein O-mannose beta-1,2-N-acetylglucosaminyltransferase	Proliferation	Miyagoe-Suzuki et al., 2009
Emery–Dreifuss:					
EGMD2	shoulder, upper limb, and calf	<i>LMNA</i>	Lamin A/C	Differentiation	Favreau et al., 2004; Frock et al., 2006
EGMD3					
EGMD1		<i>EMD</i>	Emerin	Proliferation; Differentiation	Frock et al., 2006; Meinke et al., 2015
Facioscapulo-humeral					
FSHD1	facial, shoulder, upper arm, foot, and pelvic-girdle	<i>Chrom.4q35 D4Z4 contraction</i>	<i>DUX4</i>	Differentiation; Myoblast toxicity	Kowaljow et al., 2007; Bosnakovski et al., 2008a,b
			<i>DUX4c</i>	Proliferation; Differentiation	Bosnakovski et al., 2008a; Ansseau et al., 2009
			<i>FSHD region gene 1</i>	Proliferation; Differentiation; Fusion	Chen et al., 2011; Feeney et al., 2015
Myotonic dystrophy:					
DM1	eyelid, face, neck, lower arms/legs, diaphragm, intercostals	<i>DMPK</i>	Dystrophia myotonica protein kinase	Decreased satellite cell numbers Proliferation Replicative senescence	Beffy et al., 2010 Thornell et al., 2009 Bigot et al., 2009
DM2	eyelid, face, neck, upper arms/legs, diaphragm, intercostals	<i>ZNF9</i>	Zinc finger protein 9	Differentiation; Replicative senescence	Malatesta et al., 2011; Beaulieu et al., 2012; Renna et al., 2014
Oculopharyngeal	upper eyelid, EOM, pharynx, tongue, upper arms/legs	<i>PABPN1</i>	Poly adenosine binding protein-nuclear one	Proliferation Differentiation	Périé et al., 2006 Apponi et al., 2010
Congenital MD:					
Bethlem Ullrich	upper and lower arms/legs, neck, lumbar paravertebral, intercostals, thigh, gluteus maximus	<i>COL6A1</i> <i>COL6A2</i> <i>COL6A3</i>	Collagen 6A	Self-renewal	Urciuolo et al., 2013; Gattazzo et al., 2014
Rigid spine syndrome related to <i>SEPN1</i>	paravertebral, intercostals, thigh, gluteus maximus	<i>SEPN1</i>	Selenoprotein N	Decreased satellite cell numbers; Proliferation; Exhaustion of satellite cell pool	Castets et al., 2011

diaphragm, larynx, tongue, extraocular, masseter, and pharynx, deviate from the canonical biology of their limb counterparts. Here, we address the muscle-specific variability of satellite cell biology and postulate how this variability could contribute to muscle-specific sensitivities found in MDs.

Limb Muscle Satellite Cells: Establishing the Canon

Skeletal muscles of rodent hindlimbs are commonly used to study satellite cells as these muscles are easy to identify, dissect, collect, and manipulate experimentally. The skeletal muscles

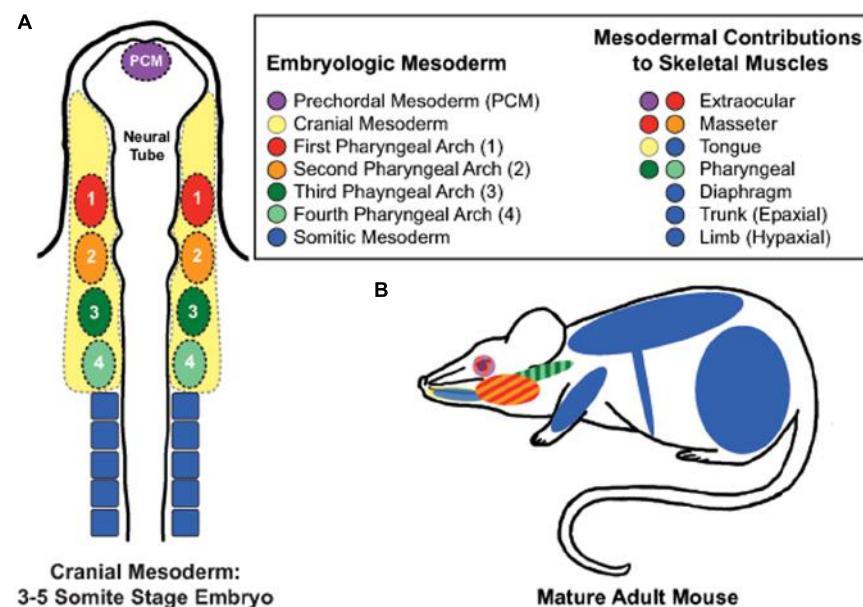


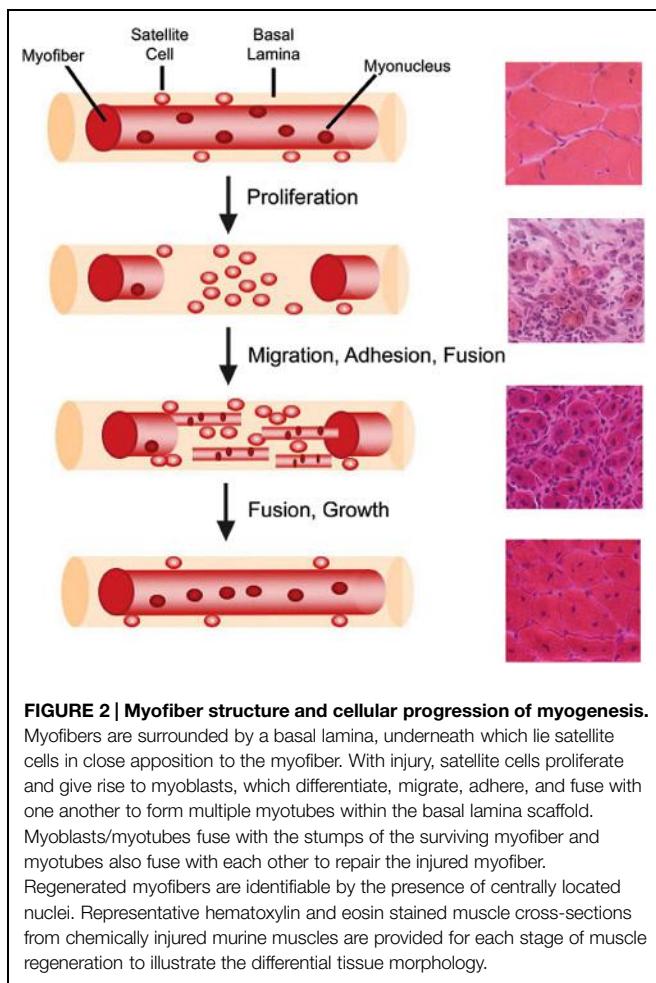
FIGURE 1 | Embryonic mesodermal contributions to adult skeletal muscles. **(A)** Schematic of mesodermal origins in a 3–5 somite stage mouse embryo. **(B)** Skeletal muscles of the trunk, limb, diaphragm, and tongue arise from somitic mesoderm. In contrast, the extraocular muscles (EOMs) arise from prechordal mesoderm and cranial paraxial mesoderm of the first pharyngeal arch; the masseter muscle from the first and second pharyngeal arches of the cranial paraxial mesoderm, and the pharynx from the third and fourth pharyngeal arches of the caudal paraxial mesoderm. Tongue muscles arise from both somitic and cranial mesoderm while developing within the niche of the cranial mesenchyme, which is supplied by all four pharyngeal arches.

of the limbs and abdomen arise from somitic mesoderm and are referred to as hypaxial muscles (Figure 1). They arise developmentally from the ventrolateral dermomyotome of the segmented paraxial mesoderm. *In vivo* and *in vitro* studies examining limb muscles provide fundamental insights into the mechanisms and regulatory pathways involved with skeletal muscle regeneration, muscle growth, and satellite cell biology.

Muscle regeneration is a robust and complex cellular process that restores injured muscle to a state that is morphologically and functionally similar to that of uninjured muscle (Figure 2; Abmayr and Pavlath, 2012). Regeneration of skeletal muscle occurs in two distinct phases: a degenerative phase and a regenerative phase (Rai et al., 2014). The main characteristics of the degenerative phase involve myofiber sarcolemmal damage or myofiber necrosis, followed by an influx of mononucleated inflammatory cells and an increase in fibroblasts (Mathew et al., 2011; Murphy et al., 2011; Rai et al., 2014). Factors released from damaged myofibers initiate an inflammatory response that recruits neutrophils, macrophages, and activates fibro/adipogenic progenitors to facilitate the removal of cellular debris and regulate muscle repair (McLennan, 1996; Lescaudron et al., 1999; Joe et al., 2010; Uezumi et al., 2010; Pallafacchina et al., 2013). The basal lamina remains intact acting as a scaffold for the next phase, muscle regeneration (Schmalbruch, 1976). Several molecular signals, such as growth factors, chemokines, and cytokines, are released which activate satellite cells both locally and systemically within the first 24–48 h following injury (Chang and Rudnicki, 2014; Rodgers et al., 2014). Myoblasts then terminally differentiate becoming post-mitotic myocytes, which

then fuse with other myocytes or myofibers to regenerate or repair damaged myofibers. Thereby, new myonuclei are added to damaged or nascent myofibers (Abmayr and Pavlath, 2012). A subset of myogenic cells repopulate the satellite cell niche, thus maintaining and replenishing the quiescent satellite cell pool for subsequent rounds of regeneration (Collins et al., 2005; Shinin et al., 2006).

The role of satellite cells in postnatal growth has also been studied in limb muscles. In mice, the first 3 weeks of neonatal growth results in a threefold increase in muscle mass during which the satellite cell population undergoes a significant reduction from ~30% of myonuclei per myofiber down to 5%, following fusion with neonatal muscles. Parallel increases in myonuclear numbers and cytoplasmic proteins occur up to postnatal day 21 (White et al., 2010). After postnatal day 21, satellite cells enter into a quiescent cellular state under the regulation of Notch signaling (Fukada et al., 2011), but myofiber size continues to increase without the addition of new myonuclei (White et al., 2010). Recent satellite cell ablation studies have also shown that myonuclear addition from satellite cells is dispensable for hypertrophic growth of limb muscles in the adult (McCarthy et al., 2011). Furthermore, satellite cells do not appear to be required for maintenance of most adult limb muscles. A recent satellite cell ablation study found that loss of >90% of adult limb satellite cells failed to alter muscle size or myofiber type in five different limb muscles with aging (Fry et al., 2015). However, myonuclear addition does occur at a basal level in uninjured postnatal limb muscles and may be required for maintenance of extensor digitorum longus myofiber size with aging (Keefe et al.,



2015). Together, these studies suggest that the initial phase of postnatal muscle growth occurs with the addition of myonuclei from satellite cells, but maintenance of most adult limb muscle size is not dependent on satellite cells.

Regulatory genes involved in satellite cell biology have also been elucidated from studies of limb muscle. In adult skeletal muscle, quiescent satellite cells express Pax7, a transcription factor that specifies the myogenic lineage (Seale et al., 2000). Once activated, satellite cells exit cellular quiescence, enter the cell cycle, and begin progression through the myogenic lineage under the control of myogenic regulatory factors (MRFs), muscle-specific transcription factors of the basic-helix-loop-helix (bHLH) class, including myogenic differentiation protein (MyoD), myogenic factor 5 (Myf5), myogenic regulatory factor 4 (Mrf4), and myogenin (Weintraub et al., 1991; Olson and Klein, 1994; Chang and Rudnicki, 2014). MyoD and Myf5 are expressed during the proliferative phase and regulate myogenic differentiation (Cooper et al., 1999; Valdez et al., 2000), while Mrf4 and myogenin are expressed upon terminal differentiation and exit from the cell cycle (Chang and Rudnicki, 2014).

Increasing evidence suggests that satellite cells within a muscle are heterogeneous (Motohashi and Asakura, 2014). Satellite cells containing high levels of Pax7 demonstrate slower proliferation

rates, lower metabolism, and resistance toward differentiation, indicating a more “stem-like” phenotype compared to satellite cells with lower levels of Pax7 (Rocheteau et al., 2012). Various groups have also discovered distinct satellite cell subpopulations based on differential expression of other proteins including α 7-integrin, β 1-integrin, c-met, CD34, calcitonin receptor, C-X-C chemokine receptor type 4 (CXCR4), M-cadherin, Myf5, neural cell adhesion molecule 1, syndecans 3 and 4, and vascular cell adhesion molecule 1 (Rosen et al., 1992; Cornelison and Wold, 1997; Beauchamp et al., 2000; Blanco-Bose et al., 2001; Cornelison et al., 2001; Tamaki et al., 2002; Sherwood et al., 2004; Fukada et al., 2007; Ikemoto et al., 2007; Kuang et al., 2007; Kafadar et al., 2009). While the mechanisms underlying satellite cell heterogeneity are still being elucidated, growing evidence suggests that satellite cell biology is also variable in a muscle-dependent manner, as discussed below.

The ‘Other’ Somite-derived Muscles: Epaxial, Diaphragm, Internal Larynx, and Tongue Satellite Cells

Epaxial Satellite Cells

Epaxial skeletal muscles include the deep muscles of the back. Similar to hypaxial muscle development, epaxial muscles arise from the dorsomedial dermomyotome of the segmented paraxial mesoderm (Figure 1; Christ and Ordahl, 1995; Burke and Nowicki, 2003). While the development of epaxial muscle is well studied (Munsterberg et al., 1995; Tajbakhsh et al., 1997; Borycki et al., 1999; Gustafsson et al., 2002; McDermott et al., 2005; Borello et al., 2006; L’Honore et al., 2010; Sato et al., 2010), the biological properties of adult epaxial satellite cells remain largely unknown. Mouse models expressing *nLacZ* under the control of the *Pax3* promoter showed coexpression of Pax3 and Pax7 is retained in the majority of adult satellite cells of the deep ventral trunk muscles (Relaix et al., 2006). Pax3 expression was also maintained in cultured epaxial myoblasts, suggesting that Pax3 may contribute to both the quiescent and activated states of epaxial satellite cells (Relaix et al., 2006). How Pax3 contributes to adult epaxial satellite cell biology and the role of Pax3 in epaxial muscle regeneration remains to be determined, as well as other fundamental aspects of epaxial satellite cell biology and myogenesis.

Diaphragm Satellite Cells

The diaphragm muscle is composed of three distinct domains: crural muscle, costal muscle, and a central tendonous domain (Anraku and Shargall, 2009). Recent studies provide evidence that the muscle components arise from the lateral dermomyotome of the cervical somites while the central tendonous connective tissue arises from the pleuroperitoneal folds of lateral plate origin (Figure 1; Noden et al., 1999; Mootooosamy and Dietrich, 2002; Pickering and Jones, 2002; Babiuk et al., 2003; Brown et al., 2005; Merrell et al., 2015). Postnatally, diaphragmatic satellite cells differ from hypaxial muscle in that Pax3 expression is maintained along with Pax7 and Myf5 (Relaix et al., 2006; Day et al., 2007; Stuelsatz et al.,

2012). Recent studies indicate that satellite cell-derived myoblasts of the diaphragm behave differently than those of the hindlimb *in vitro*. Using the Nestin-GFP mouse model (Day et al., 2007) to identify self-renewal of myoblasts, Stuelsatz et al. (2014) found higher percentages of GFP⁺ cells in diaphragm versus limb muscle cultures. *In vitro* clonal expansion assays revealed that diaphragmatic myoblasts proliferated similarly to those of hindlimb muscles (Chen et al., 2011; Stuelsatz et al., 2012), but myogenic differentiation of diaphragm cultures consistently reached maximum fusion indexes earlier than limb cultures (Stuelsatz et al., 2014). However, other studies demonstrated that diaphragmatic satellite cells undergo increased proliferation and decreased differentiation *in vitro* when compared to limb, trunk, and craniofacial muscles (Ippolito et al., 2012). The discrepancies in these studies evidence the need for further examination of diaphragmatic satellite cell biology. *In vivo* studies also indicate some interesting biology associated with diaphragmatic satellite cells. *In vivo* lineage tracing studies examining the contribution of diaphragmatic satellite cells to uninjured diaphragm muscles revealed continued addition of new myonuclei with age, yet myofiber size was not affected with satellite cell ablation (Keefe et al., 2015). One study in rats suggests that heterogeneity exists in the response of diaphragmatic satellite cells to the growth promoting stimuli provided by hemi-diaphragm denervation. By examining satellite cell numbers associated with various fiber types, this study found that only type IIx/b myofibers of the compensating non-denervated hemi-diaphragm showed significant increases in satellite cell numbers in both juvenile and adult mice, while no changes in number were associated with Type I or IIa myofibers (Kawai et al., 2012). Taken together, our current understanding of diaphragmatic satellite cell biology is still rudimentary and warrants further study, both *in vivo* and *in vitro*.

Intrinsic Laryngeal Satellite Cells

The intrinsic muscles of the larynx are critical for respiration, airway protection and vocalization and include the thyroarytenoid, posterior cricoarytenoid (PCA), and lateral cricoarytenoid muscles. The intrinsic laryngeal muscles arise from the occipital somites during development (Figure 1; Noden et al., 1999; Noden and Francis-West, 2006). In thyroarytenoid and PCA muscles, basal levels of myonuclear addition were discovered using BrdU lineage tracing, suggesting that uninjured laryngeal muscle contains a subset of proliferating satellite cells (Goding et al., 2005). *In vitro* studies also found increased proliferation rates associated with Erk1/2 phosphorylation in primary laryngeal muscle cultures compared to hindlimb cultures (Walz et al., 2008). In response to laryngeal denervation, laryngeal satellite cell activation and proliferation occurs *in vivo* within days following denervation (Kumai et al., 2007) with myonuclear addition occurring in all intrinsic laryngeal muscles analyzed (Shinnars et al., 2006). Surprisingly, myogenic cells expressing MyoD and myogenin are still present in thyroarytenoid muscles 2 years post-denervation in humans, suggesting a unique prolonged regenerative potential in laryngeal muscle (Donghui et al., 2010). In spite of this, age-related decreases in laryngeal satellite cell density and impaired

regeneration of thyroarytenoid muscles occur (Malmgren et al., 2000; Lee et al., 2012). Together, these studies provide intriguing insights into intrinsic laryngeal satellite cells, but further studies are needed to elucidate the molecular and regulatory mechanisms underlying their distinct biology.

Tongue Satellite Cells

Tongue muscles arise from mixed mesodermal origins. The intrinsic muscles arise from the first occipital somite, while cranial paraxial mesoderm contributes to the formation of the exterior tongue muscles (Figure 1; Shuler and Dalrymple, 2001; Czajkowski et al., 2014). To date, knowledge of adult tongue muscle satellite cell biology is severely limited. A denervation study of the tongue muscle using ³H-thymidine to label proliferating cells revealed increased numbers of ³H-thymidine labeled myonuclei, which suggests fusion of proliferating satellite cells to the myofibers (McGeachie and Allbrook, 1978). Tongue-derived myoblasts have been isolated, cultured and successfully differentiated into nascent myotubes *in vitro*, but the myogenic characteristics of these cells are yet to be directly studied (Ternaux and Portalier, 1993; LaFramboise et al., 2003).

Craniofacial Satellite Cells: Extraocular, Masseter, and Pharyngeal

Extraocular Satellite Cells

Extraocular muscles (EOMs) are responsible for rotation and movement of the eye and include the superior oblique, inferior oblique, superior rectus, inferior rectus, lateral rectus, and medial rectus muscles. EOM and their associated satellite cells differ from other skeletal muscles in that they arise from the prechordal and cranial paraxial mesoderm of the first pharyngeal arch during development (Figure 1; Couly et al., 1992; Noden and Francis-West, 2006). Early studies examining the effects of aging and dystrophic disease suggested EOM muscles are distinct from their hindlimb counterparts. Aging studies suggested that EOMs were preferentially spared with aging (Porter et al., 1998; Schoser and Pongratz, 2006), while studies examining EOM involvement in Duchenne, Becker, and some limb girdle MDs also showed a preferential sparing of these muscles (Kaminski et al., 1992; Emery, 2002). In addition, satellite cells of EOM have unique gene expression profiles in comparison to quiescent satellite cells of hindlimb muscles (Porter and Baker, 1996; Pacheco-Pinedo et al., 2009). EOM satellite cells also demonstrate distinct biological differences when compared to hindlimb satellite cells. In several species, EOM satellite cells chronically proliferate *in vivo* (McLoon and Wirtschafter, 2002a, 2003; Wirtschafter et al., 2004; Christiansen and McLoon, 2006), which may in part be due to a specific highly proliferative subpopulation (Kallestad et al., 2011). The transcription factor Pitx2 is expressed in postnatal EOM myogenic precursor cells that are of a CD34⁺/Sca1⁻/CD31⁻/CD45⁻ lineage, which is thought to contribute to the proliferative properties of EOM satellite cells (Hebert et al., 2013). In addition, aged EOM satellite cells maintain proliferative and self-renewal abilities out to 24 months of age *in vitro* (Stuelsatz et al., 2014). Furthermore,

global and orbital EOM satellite cells contribute new myonuclei to EOM myofibers in the absence of injury (McLoon and Wirtschafter, 2002a,b, 2003; Wirtschafter et al., 2004; Keefe et al., 2015). Together, these studies highlight satellite cell biology that is distinct from hindlimb satellite cells. Because of their proliferative and self-renewal propensities, EOM satellite cells have been proposed to be ideal candidates for use in cell-based therapies of myopathic disease (McLoon et al., 2007; Kallestad et al., 2011; Stuelsatz et al., 2014). However, in transplantation experiments in which EOM satellite cells were injected into the TA muscle of the hindlimb, EOM satellite cells successfully engrafted into the novel niche, but lost EOM-specific phenotypes such as expression of Myh13 and slow-tonic myosin (Sambasivan et al., 2009). These data suggested that the phenotypes of EOM satellite cells may be controlled by the niche. In support of this hypothesis, a recent study suggested that PW1/peg⁺ interstitial cells (PICs), which are present in higher numbers in EOM compared to the TA, provide a promyogenic environment that contributes to the resistance of both EOM satellite cells and myofibers to dystrophic and age-related disease (Formicola et al., 2014). Together, these data raise some intriguing questions. What roles do intrinsic and extrinsic mechanisms have on EOM satellite cell biology? Can such mechanisms be manipulated to improve the quality of life for individuals suffering from myopathic diseases? Studies are still needed to further elucidate the mechanisms contributing to the unique phenotypes of these satellite cells.

Masseter Satellite Cells

Adult masseter satellite cells arise from the first and second pharyngeal arches of the cranial paraxial mesoderm with contributions from the splanchnic mesoderm and express a unique transcription profile compared to hindlimb satellite cells (Figure 1; Kelly et al., 2004; Noden and Francis-West, 2006; Nathan et al., 2008; Sambasivan et al., 2009). Early *in vivo* studies demonstrated an impaired regenerative ability in masseter muscles compared to hindlimb muscle that was associated with a lower number of satellite cells during regeneration (Pavlath et al., 1998). Masseter satellite cells undergo prolonged periods of proliferation *in vitro* with a concurrent delay of differentiation onset (Ono et al., 2010), which potentially may contribute to the impaired regenerative response observed in acute injury to masseter muscle. In contrast, masseter satellite cells increase in number per myofiber with age while their proliferative capabilities decline *ex vivo* (Ono et al., 2010). What molecular pathways are involved with the age-associated increase in satellite cell numbers in masseter muscles verses the decrease in satellite cell numbers in other skeletal muscles? Little is known regarding the underlying molecular mechanisms driving the phenotypes of masseter satellite cells.

Pharyngeal Satellite Cells

Swallowing depends on the synchronous contraction of seven major muscles lining the nasal, oral, and laryngeal pharynxes to ensure propulsion of food and liquid from the oral cavity into the esophagus (Donner et al., 1985; Rubesin et al., 1987; Ekberg et al., 2009). Pharyngeal muscles arise from the cranial paraxial mesoderm of the third and fourth

pharyngeal arches with contributions from splanchnic mesoderm (Figure 1; Kelly et al., 2004; Noden and Francis-West, 2006; Nathan et al., 2008). Pharyngeal muscles include the stylopharyngeus, palatopharyngeus, salpingopharyngeus, and the superior, middle, and inferior pharyngeal constrictor muscles (Dutta and Basmajian, 1960; Himmelreich, 1973; Donner et al., 1985; Rubesin et al., 1987; Ekberg et al., 2009). The inferior pharyngeal constrictor can be subdivided into the cricopharyngeus and the thyropharyngeus muscles (Donner et al., 1985; Rubesin et al., 1987; Ekberg et al., 2009). Recent *in vivo* and *in vitro* studies indicate that pharyngeal satellite cells have unique biological characteristics compared to hindlimb satellite cells. Pharyngeal muscles contain increased numbers of activated and proliferating satellite cells and contribute new myonuclei to pharyngeal myofibers in the absence of induced injury (Randolph et al., 2015). Additionally, *in vitro* clonal assays suggest a highly proliferative subpopulation of pharyngeal satellite cells could be contributing to the proliferative phenotype (Randolph et al., 2015). *In vivo* ablation studies indicated that satellite cells were required to maintain myonuclear numbers in certain pharyngeal muscles under basal conditions, suggesting that pharyngeal muscles undergo myonuclear turnover and require myonuclear addition from ongoing satellite cell myogenesis (Randolph et al., 2015). Could pathologic mutations enhance myonuclear turnover in pharyngeal muscles to such an extent that pharyngeal satellite cells could no longer adequately supply enough myonuclei to maintain homeostasis? Do disease-causing mutations impair the basal myogenic phenotype of pharyngeal satellite cells, potentially contributing to the pathological sensitivity of pharyngeal muscles observed in some MDs? These questions and many more remain to be addressed. However, several studies have examined pharyngeal satellite cell biology in the context of oculopharyngeal muscular dystrophy and will be discussed below.

Could Variable Satellite Cell Biology Contribute to the Differential Susceptibility of Certain Muscles to Dystrophic Mutations?

Muscular dystrophies are a group of degenerative muscle diseases due to mutations in proteins ranging in function such as sarcolemmal structure (Hoffman et al., 1987), nuclear envelope structure (Bione et al., 1994; Bonne et al., 1999; Mittelbronn et al., 2008), post-translational glycosylation (Bonnemann et al., 2014), and RNA binding (Brais et al., 1998; Kühn et al., 2009). Many mutations have been characterized in both skeletal muscle-specific and ubiquitously expressed genes, yet both manifest in muscular pathology. Intriguingly, each dystrophy affects a specific subset of skeletal muscles within the human body (Emery, 2002), suggesting that biological differences exist between individual muscles that predispose them to specific pathological etiologies.

Satellite cells have been implicated in the pathology of some MDs and may contribute to the variable muscle sensitivity observed in some dystrophies through several mechanisms

(Table 1). For example, in response to chronic myofiber degeneration, satellite cells are subjected to multiple rounds of regeneration, which can “exhaust” the regenerative abilities of the satellite cell population over time (Webster and Blau, 1990; Decary et al., 2000; Morgan and Zammit, 2010; Sacco et al., 2010). Additionally, satellite cell impairment may occur early in the disease process if satellite cells express the mutant genes. Below we discuss the functional deficits in satellite cells observed in Duchenne MD (Blau et al., 1983; Webster and Blau, 1990; Sacco et al., 2010), Emery–Dreifuss MD (Favreau et al., 2004; Frock et al., 2006), facioscapulohumeral MD (Winokur et al., 2003; Barro et al., 2010), myotonic dystrophy (Furling et al., 2001; Thornell et al., 2009), oculopharyngeal MD (Périé et al., 2006), and some congenital MDs as well (Castets et al., 2011; Urciuolo et al., 2013).

Duchenne Muscular Dystrophy

Duchenne muscular dystrophy (DMD) is an early onset childhood X-linked disease associated with the absence of dystrophin (Hoffman et al., 1987), a sarcolemma-associated cytoplasmic protein critical for maintaining sarcolemmal integrity of myofibers (Durbeej and Campbell, 2002). Minimal levels of mechanical stress are needed to impair sarcolemmal integrity in the absence of dystrophin, resulting in recurrent rounds of myofiber damage and repair (Petrof et al., 1993). Patients suffering from DMD experience progressive loss of muscle function, eventually leading to death before the age of 30. The main skeletal muscles affected in DMD are found in the shoulder, upper limbs, hips, thighs, and calves (Emery, 2002). Life-threatening symptoms for many patients involve cardiac and respiratory failure from impairment of the heart and diaphragm muscles, respectively (Nigro et al., 1990; Stedman et al., 1991). Of note, craniofacial muscles, such as the extraocular and internal laryngeal muscles, are mostly spared in DMD with the exception of pharyngeal muscles in advanced stages of the disease (Kaminski et al., 1992; Emery, 2002; Marques et al., 2007; Shinonaga et al., 2008). The mechanism of resistance of EOM to dystrophic changes was recently addressed in irradiation studies using *mdx:utrophin* heterozygous mice. EOMs of these mice failed to develop dystrophic phenotypes even after receiving 18 Gy gamma irradiation. This resistance to dystrophic change was attributed to the presence of multiple EOM myogenic precursor populations that prevented loss of myofiber size, suggesting that the proliferative satellite cell populations of EOM play a role in the muscle sparing of EOM in many dystrophies (McDonald et al., 2014). Satellite cell abnormalities are noted in dystrophin-deficient hindlimb muscles of both mice and humans at early stages of disease. In mouse hindlimb muscles, impaired satellite cell attachment to *mdx* myofibers may contribute to the pre-myonecrosis myofiber hypotrophy found in early postnatal disease (Duddy et al., 2015). Furthermore, premature cell senescence is observed in cultured myoblasts isolated from limb muscles of DMD patients as early as 2 years of age, the age of typical clinical onset (Blau et al., 1983; Webster and Blau, 1990) attributed to both deficiencies in Notch signaling and telomeric shortening following repeated regenerative cycles (Mouly et al., 2005; Sacco et al., 2010; Jiang et al., 2014).

Limb-Girdle Muscular Dystrophy

Limb-girdle muscular dystrophies (LGMD) are associated with mutations of more than 20 different genes in both muscle-specific and ubiquitously expressed genes with a range of molecular functions (Vieira et al., 2014). These include sarcomere proteins (titin), sarcolemmal proteins (sarcolectin), glycosyltransferases (fukutin), nuclear envelope proteins (lamin A/C), and RNA-processing proteins (HNRNPD), to name a few. Intriguingly, despite the vast etiological variation, all mutations elicit dystrophic changes in muscles of the upper limb, shoulder, chest, hip, and upper leg (Broglio et al., 2010; Mitsuhashi and Kang, 2012). Satellite cell involvement has been implicated in some LGMDs. Biopsies from LGMD2A patients demonstrated a decrease in miR-1 and miR206, microRNAs involved in facilitating satellite cell differentiation that correlated with an increased Pax7+ population. Despite the increased Pax7+ population, regeneration was impaired and fibrosis elevated, suggesting an impairment of satellite cell transition from proliferation to differentiation could be contributing to the pathology of LGMD2A (Rosales et al., 2013). In contrast, decreased satellite cell numbers were reported in patients with α -, β -, or γ -sarcolectin mutations (LGMD2D, 2E, and 2C, respectively) when compared to Becker muscular dystrophy patient samples (Higuchi et al., 1999). Additionally, murine POMGnT1-null myoblasts (representative of LGMD2O) demonstrated impaired proliferation *in vitro* (Yoshida et al., 2001; Miyagoe-Suzuki et al., 2009). In a mouse model for LGMD2H, knockout of E3 ubiquitin ligase tripartite motif-containing 32 (TRIM32) resulted in satellite cell senescence both *in vitro* and *in vivo* (Kudryashova et al., 2012; Mokhonova et al., 2015). Interestingly, pharmacologic induction of follistatin expression in satellite cells using the deacetylase inhibitor, trichostatin A, has proved beneficial in restoring myofiber size in α -sarcolectin-deficient LGMD *in vivo* (Minetti et al., 2006). Of note, *in vitro* treatment of α -sarcolectin-deficient murine satellite cells with trichostatin A resulted in hypernucleated myotubes, suggesting a pharmacologic enhancement of myoblast differentiation/fusion (Minetti et al., 2006). It remains to be seen if other pharmacologic approaches that alter satellite cell function might also benefit LGMD patients with other mutations.

Emery–Dreifuss Muscular Dystrophy

Emery–Dreifuss muscular dystrophy results in progressive weakness of the shoulder, upper limb, and calf muscles of patients. The most common forms of this dystrophy are caused by mutations in the ubiquitously expressed nuclear envelope proteins emerin, lamin A, or lamin C (Helbling-Leclerc et al., 2002). Why skeletal and cardiac muscles are preferentially affected in this disease, is still unclear. Patients with Emery–Dreifuss MD can have severe cardiac pathology occurring as early as 30 years of age (Vohanka et al., 2001; Emery, 2002; Broglio et al., 2010). *In vitro* studies using primary muscle cultures from *Lmna*^{-/-} knockout mice, overexpression of mutant lamin A^{R453W}, or RNAi knockdown of *emerin* demonstrated defects in myoblast differentiation (Favreau et al., 2004; Frock et al., 2006). In a recent study, *in vitro* culture of patient-derived myoblasts lacking emerin demonstrated enhanced proliferation

with spontaneous differentiation, compared to control myoblasts, thus suggesting that satellite cell impairment could play a role in Emery–Dreifuss MD (Meinke et al., 2015).

Facioscapulohumeral Muscular Dystrophy

Facioscapulohumeral muscular dystrophy (FSHD) is named for the muscles mainly affected in the disease, facial, shoulder, and upper arm muscles, but foot and pelvic-girdle muscles can also be affected (Tawil and Van Der Maarel, 2006). Of the dystrophies affecting craniofacial muscles, FSHD carries the best prognosis for long-term survival, as it is a slowly progressive disease that rarely affects the heart or the ability to breathe (Tawil and Van Der Maarel, 2006). The causative deletion for FSHD type 1 (FSHD1) occurs in the subtelomeric region of chromosome 4, which can induce the expression of genes such as *FSHD region gene 1 (FGR1)*, *FGR2*, *ANT1*, *DUX4*, and *DUX4c* (Gabellini et al., 2002; Dixit et al., 2007; Ansseau et al., 2009; Bodega et al., 2009; Snider et al., 2010). The pathogenic contributions of these genes to FSHD are still being dissected. However, evidence for satellite cell involvement in FSHD1 is growing. *DUX4* expression in cultured myoblasts inhibited myogenic differentiation by repression of *Myf5* and *MyoD* (Bosnakovski et al., 2008a,b, 2009), while overexpression of *DUX4* was toxic to myoblasts *in vitro* (Kowaljow et al., 2007). In contrast, overexpression of *DUX4c* stimulated myoblast proliferation but inhibited differentiation *in vitro* (Bosnakovski et al., 2008a; Ansseau et al., 2009). Additionally, *FGR1* overexpression impaired myoblast proliferation as well as myoblast fusion (Chen et al., 2011; Feeney et al., 2015). Primary myoblasts collected from affected thigh muscles of a transgenic mouse overexpressing *FGR1* produced smaller clonal colonies than myoblasts derived from the unaffected diaphragm muscle (Chen et al., 2011). Taken together, these results indicate that satellite cells could play a direct role in FSHD1 pathology. The mechanism(s) underlying the muscle specificity of these altered myogenic phenotypes remains to be determined.

Myotonic Dystrophy

Myotonic dystrophy (DM) is a complex, multisystemic group of dystrophies that genetically arise from untranslated repeat nucleotide expansions of two separate genes, *dystrophia myotonica protein kinase (DMPK)* and *zinc finger protein 9 (ZNF9)* (Day and Ranum, 2005). A (CTG)_{80–4000} repeat in the 3' untranslated region of *DMPK* is present in patients with myotonic dystrophy type 1 (DM1). The expanded regions of *DMPK* transcripts result in altered RNA biogenesis and processing of multiple transcripts, in part, by the sequestration of the splicing factor muscle blind (MBNL1) and stabilization of CUG-binding protein 1 (CUGBP1; Mastroyannopoulos et al., 2010). In myotonic dystrophy type 2 (DM2), up to 75–11,000 repeat expansions of (TG)_n(TCTG)_n(CCTG)_n reside in intron 1 of *ZNF9* (Day and Ranum, 2005), dysregulating alternative slicing as well as protein production by sequestration of the 20S proteasome (Salisbury et al., 2009). While DM1 and DM2 result from distinct genetic mutations, the biological consequences are similar as myotonia, muscular dystrophy, muscle pain, cataracts, cardiac arrhythmias, insulin insensitivity, and diabetes, hypogammaglobulinemia, and

testicular failure occur in both (Schoser and Timchenko, 2010). DM affects muscles of the eyelid, face, neck, lower arms, and legs, diaphragm, and intercostal muscles (Batten and Gibb, 1909; Zifko et al., 1996). However, DM1 is associated with muscle weakness and atrophy in the lower limb muscles, while in DM2 the disease is more predominant in the upper limbs (Tieleman et al., 2012). Life-threatening conditions involving cardiac disease, respiratory failure, and difficulties in swallowing can occur (Zifko et al., 1996; Tieleman et al., 2009, 2012). Satellite cell number, proliferation and differentiation are differentially altered in DM1 patients. Decreased satellite cell numbers may result from induced autophagic processes in DM1 myoblasts (Beffy et al., 2010). Cultured myoblasts, obtained from affected lower limb muscles, proliferated less compared to unaffected upper limb muscle cultures derived from the same patients (Thornell et al., 2009). Enhanced expression of prostaglandin E2 by DM1 myoblasts inhibited differentiation and fusion in an autocrine manner (Beaulieu et al., 2012). Additionally, the p16-pathway induced premature senescence in DM1 myoblasts (Bigot et al., 2009). Satellite cells have also been implicated in DM2 pathology. For example, satellite cells underwent premature senescence in DM2 patients, but in a non-p16 dependent manner through a telomere-driven pathway (Malatesta et al., 2011; Renna et al., 2014). Impairment of satellite cells could be a major pathologic determinant in myotonic dystrophies.

Oculopharyngeal Muscular Dystrophy

Oculopharyngeal muscular dystrophy (OPMD) is an autosomal dominant disease, which typically affects people older than 50 years of age (Abu-Baker and Rouleau, 2007; Messaad and Rouleau, 2009). An aberrant expansion of alanines in the N-terminus of poly adenosine binding protein nuclear one (PABPN1) is the underlying cause of this presently incurable disease (Brais et al., 1998; Abu-Baker and Rouleau, 2007; Messaad and Rouleau, 2009). PABPN1 is a ubiquitously expressed protein that plays key roles in RNA biogenesis (Banerjee et al., 2013). The endogenous protein contains a 10-alanine repeat at its N-terminus, but expansions resulting in 12–18 alanines are reported in OPMD patients (Brais et al., 1998; Abu-Baker and Rouleau, 2007; Messaad and Rouleau, 2009; Jouan et al., 2014). Muscle weakness and dystrophy occur preferentially in craniofacial skeletal muscles including the upper eye-lid, pharynx, EOMs, and tongue with weakness in upper limb muscles developing later in the disease (Emery, 2002; Abu-Baker and Rouleau, 2007; Messaad and Rouleau, 2009).

The major life-threatening difficulty for OPMD patients is the resultant dysphagia, or impairments in swallowing (Périé et al., 2006). Pharyngeal muscles of the nasal, oral, and laryngeal pharynxes are essential components of the swallow reflex (Miller, 2002; Ertekin and Aydogdu, 2003; Miller, 2008), which prevents aspiration of food and water into the trachea and lungs and the formation of life-threatening pneumonia (Martin et al., 1994; Prasse and Kikano, 2009). Of note, decreased proliferation of pharyngeal satellite cells isolated from OPMD patients was observed *in vitro* (Périé et al., 2006). In transgenic mice that overexpress wild-type *PABPN1* specifically in skeletal muscle, increased numbers of myofibers with central nuclei suggested

a positive effect of wild-type PABPN1 on satellite cell fusion *in vivo* (Randolph et al., 2014). Together, the above studies suggest that PABPN1 plays a critical role in pharyngeal satellite cell myogenesis and mutations in this protein may contribute to satellite cell impairment in OPMD patients.

Results from recent clinical trials provide preliminary evidence for the use of satellite cell transplantation as a therapeutic treatment for dysphagic OPMD patients. Phase I/IIa clinical trials were performed with dysphagic OPMD patients in which myoblasts obtained from unaffected skeletal muscles were amplified in culture and transplanted into cricopharyngeal muscles following surgical treatment of the cricopharyngeal muscle. Patients receiving injections of larger numbers of myoblasts into the cricopharyngeal area demonstrated significant improvement in swallowing over a 2-years period (Perie et al., 2014), thus providing experimental support for the use of satellite cell-based therapies for OPMD patients.

Congenital Muscular Dystrophies

Congenital muscular dystrophies (CMD) represent a large group of congenital onset muscle diseases. While this group of muscle diseases has been widely studied, satellite cell involvement has only been implicated in the pathology of two forms to date: collagen VI-related myopathies (COL6-RD) and selenoprotein N-related myopathies (SEPN1-RM). COL6-RD include Ullrich MD and Bethlem myopathy and arise from mutations in COL6A1, COL6A2, or COL6A3 (Bonnemann et al., 2014). Muscle weakness occurs in distal limb, neck flexor muscles, lumbar, intercostals, and diaphragm, as well as proximal limb muscles such as the quadriceps, biceps and triceps (Haq et al., 1999; Camacho Vanegas et al., 2001; Quijano-Roy et al., 2012). When human biopsy samples were examined for changes in Pax7⁺ satellite cell numbers in normal versus Ullrich MD, no changes were observed (Paco et al., 2012). However, in *Col6a1* knockout mice, satellite cell self-renewal was impaired following multiple bouts of induced injury while satellite cell numbers were maintained in *Col6a1*^{-/-} mice when treated with cyclosporin A (Urciuolo et al., 2013; Gattazzo et al., 2014). Whether these results are recapitulated in satellite cells from Ullrich MD patients has yet to be determined. Rigid spine syndrome related to SEPN1 is a CMD within the SEPN1-related myopathies. Affected muscles include the thigh, gluteus maximus, paravertebral, intercostal, and sternocleidomastoid muscles (Quijano-Roy et al., 2012). Studies examining satellite cells in *Sepn1*^{-/-} mice revealed decreased satellite cell numbers, impaired self-renewal, enhanced

satellite cell proliferation, and exhaustion of the satellite cell pool following one round of regeneration (Castets et al., 2011). These studies suggest a potential role for satellite cells in the pathology of some CMDs. Further studies are needed to determine if satellite cells would be beneficial therapeutic targets for CMD patients.

Summary

Skeletal muscles are a highly diverse and dynamic group of tissues. As discussed, many factors contribute to skeletal muscle diversity including embryologic origin, gene expression, and functional/metabolic requirements. Such diversity likely contributes to the pathologic sensitivities of different skeletal muscles to aging and disease. Unfortunately, little is known about the effects of age or disease on non-limb muscles as a whole or what factors predispose them to the effects of pathologic conditions. Additionally, satellite cells could serve as pathologic determinants in some dystrophies; however, our knowledge of non-limb satellite cells and their role in muscle biology is severely lacking. Recognizing and elucidating the distinct differences in satellite cell biology between different skeletal muscles could be the key to unraveling the conundrum of muscle specificity between the various MDs. This review highlights the potential benefit of exploring satellite cell biology of non-limb skeletal muscles for the development of novel therapeutic approaches for patients suffering from MDs.

Author Contributions

MR: Conception and design, financial support, collection, and assembly of data, manuscript writing, final approval of manuscript.

GP: Conception and design, financial support, administrative support, collection, and assembly of data, manuscript writing, final approval of manuscript.

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The extraocular muscle stem cell niche is resistant to ageing and disease

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Specific muscles are spared in many degenerative myopathies. Most notably, the extraocular muscles (EOMs) do not show clinical signs of late stage myopathies including the accumulation of fibrosis and fat. It has been proposed that an altered stem cell niche underlies the resistance of EOMs in these pathologies, however, to date, no reports have provided a detailed characterization of the EOM stem cell niche. PW1/Peg3 is expressed in progenitor cells in all adult tissues including satellite cells and a subset of interstitial non-satellite cell progenitors in muscle. These PW1-positive interstitial cells (PICs) include a fibroadipogenic progenitor population (FAP) that give rise to fat and fibrosis in late stage myopathies. PICs/FAPs are mobilized following injury and FAPs exert a promyogenic role upon myoblasts *in vitro* but require the presence of a minimal population of satellite cells *in vivo*. We and others recently described that FAPs express promyogenic factors while satellite cells express antimyogenic factors suggesting that PICs/FAPs act as support niche cells in skeletal muscle through paracrine interactions. We analyzed the EOM stem cell niche in young adult and aged wild-type mice and found that the balance between PICs and satellite cells within the EOM stem cell niche is maintained throughout life. Moreover, in the adult *mdx* mouse model for Duchenne muscular dystrophy (DMD), the EOM stem cell niche is unperturbed compared to normal mice, in contrast to *Tibialis Anterior* (TA) muscle, which displays signs of ongoing degeneration/regeneration. Regenerating *mdx* TA shows increased levels of both PICs and satellite cells, comparable to normal unaffected EOMs. We propose that the increase in PICs that we observe in normal EOMs contributes to preserving the integrity of the myofibers and satellite cells. Our data suggest that molecular cues regulating muscle regeneration are intrinsic properties of EOMs.

Keywords: extraocular muscles, muscle stem cell niche, Duchenne muscular dystrophy, PICs, satellite cells

INTRODUCTION

Duchenne muscular dystrophy (DMD) is the most common X-linked recessive disease in humans, affecting 1 in 3500 males and causing premature death in the second decade of life following cardiac and pulmonary failure (Tabebordbar et al., 2013). Duchenne muscular dystrophy is due to mutations in the dystrophin gene, which encodes a structural protein linking the cytoskeleton of the myofiber to the surrounding basal lamina (Ervasti and Campbell, 1991; Chaturvedi et al., 2001). The absence of functional dystrophin leads to degeneration of the myofibers, which results in repeated rounds of degeneration and regeneration (Wallace and McNally, 2009). Despite the regenerative response, muscle tissue gradually becomes replaced by fibrotic and fat tissue followed by loss of muscle function (Wallace and McNally, 2009; Tabebordbar et al., 2013). Satellite cells are the principal myogenic progenitor population in skeletal muscle that give rise to new myofibers (Relaix and Zammit, 2012). Satellite cells actively proliferate during prenatal life and progressively enter a quiescent state after birth (Bismuth and Relaix, 2010). In the adult, satellite cells remain quiescent and

can be identified based upon their location under the basal lamina of the myofibers as well as by the expression of *Pax7* (Sambasivan and Tajbakhsh, 2007; Pallafacchina et al., 2010; Pannérec et al., 2013). While quiescent in the adult, satellite cells re-enter the cell cycle in response to injury to give rise to new myofibers as well as restore the satellite cell pool (Bismuth and Relaix, 2010; Yin et al., 2013). Muscle tissue also possesses multiple interstitial cell populations that regulate satellite cell function (Pannérec et al., 2012; Relaix and Zammit, 2012). The fibroadipogenic progenitors (FAPs) that reside in the interstitium are required for proper regeneration (Pannérec et al., 2012; Yin et al., 2013). Fibroadipogenic progenitors become activated in response to injury and promote satellite cell differentiation *in vitro* (Joe et al., 2010; Uezumi et al., 2010). However, when satellite cells are depleted or functionally impaired, FAPs differentiate into adipocytes and contribute to fibrosis (Joe et al., 2010; Uezumi et al., 2010, 2011). We reported previously that the cell stress-mediator gene, *PW1/Peg3*, is expressed in multiple progenitor populations in adult tissues, including skeletal muscle (Mitchell et al., 2010; Besson et al., 2011). In skeletal muscle

PW1/Peg3 expression is observed in satellite cells and a subset of interstitial cells referred to as positive interstitial cells (PICs; Mitchell et al., 2010; Pannérec et al., 2013). We demonstrated that PICs include the entirety of the FAPs and that this subpopulation expresses follistatin (FST) and insulin-like growth factor-1 (IGF-1) that account, in part, for their promyogenic activity (Pannérec et al., 2013; Formicola et al., under review and see Mozzetta et al., 2013). The close proximity of the PICs to the satellite cells suggests that they act in part as progenitor niche cells (Mitchell et al., 2010; Formicola et al., under review). Taken together, both the satellite cells and a subset of interstitial cells are required for proper muscle regeneration and impairment of either cell type can lead to loss of muscle tissue and increased fat and fibrosis which is typical of mid-to late-stage degenerative muscle diseases (Serrano et al., 2011; Shadrach and Wagers, 2011; Tabebordbar et al., 2013; Yin et al., 2013).

All skeletal muscle tissue is composed of the same basic cellular components, however specific groups of muscles display either increased sensitivity or resistance to muscle diseases. While the limb and diaphragm muscles are severely affected in most muscle myopathies, the extraocular muscles (EOMs) do not undergo a significant loss of function and maintain their tissue integrity in many myopathies including DMD and amyotrophic lateral sclerosis, whereas they are selectively targeted by some myopathies that do not affect other skeletal muscles such as the oculopharyngeal muscular dystrophy and myasthenia gravis (Kaminski et al., 1992; Khurana et al., 1995; Porter et al., 1995; Davies et al., 2006; Abu-Baker and Rouleau, 2007; Soltys et al., 2008). The EOMs consist of six muscles surrounding the ocular globe responsible for eye movements (Bohsack et al., 2011). These muscles have a distinct embryological origin compared to other skeletal muscles of the body and display unique properties including the expression of a specific myosin heavy chain isoform (Porter et al., 1995, 2001, 2006; Porter, 2002). Moreover, EOM satellite cells show a different genetic requirement during embryonic development as well as postnatal differentiation (Sambasivan et al., 2009, 2011). Following engraftment into limb muscles, EOM satellite cells efficiently differentiate into myofibers and form myofibers that do not express EOM-specific myosin isoforms indicating that local niche-derived or nerve-specific signals are important in specifying muscle phenotype (Sambasivan et al., 2009). Several studies have described the presence of activated satellite cells in uninjured normal adult EOMs from different species (rabbit, mouse, monkey) (McLoon and Wirtschafter, 2002a,b, 2003; McLoon et al., 2004) as well as more satellite cells per myofiber as compared to limb muscles (McLoon et al., 2007), leading to the hypothesis that EOMs undergo continuous myonuclei addition providing a cellular basis for continued tissue remodeling throughout life. Other studies comparing adult EOMs and limb muscles revealed different transcriptomes and proteomes (Porter et al., 2001, 2003, 2006; Khanna et al., 2003, 2004; Pacheco-Pinedo et al., 2009; Lewis and Ohlendieck, 2010). Interestingly, members regulating the transforming growth factor beta (TGF β) and IGF-1 signaling pathways are differentially expressed between EOMs and limb muscles suggesting that the EOM progenitor cells are exposed to a more promyogenic

environment (Porter et al., 2003; Pacheco-Pinedo et al., 2009). We have shown previously that PW1/Peg3 is expressed by muscle niche cells (Besson et al., 2011; Pannérec et al., 2012, 2013) that express several of these promyogenic factors in limb muscles and that the niche has a profound influence on regenerative capacity (Mozzetta et al., 2013; Formicola et al., under review). Since the EOM is resistant to multiple myopathies, it is possible that the EOM niche differs substantially from other muscle groups.

In this study, we compared EOMs to limb muscles in normal adult and aged mice as well *mdx* mutant mice. While EOMs have the same number of satellite cells per fiber as compared to limb muscles, we note that the number of PICs is markedly higher. Limb muscle derived PICs secrete both IGF-1 and FST (Formicola et al., under review), and here we observed a higher level of these growth factors in EOMs. Furthermore, while both EOMs and limb muscles display a decline in satellite cell number with age, PICs are maintained in EOMs at a similar ratio with satellite cells at all ages whereas they are markedly decreased in limb muscles with age. Moreover, PICs are maintained at higher numbers in *mdx* limb muscles as compared to wild-type counterparts and these high numbers are comparable to the ones observed in wild-type EOMs. Taken together, these data reveal that the PIC population is uniquely regulated in EOMs and suggest that the maintenance of a high number of PICs provides a more promyogenic environment. This unique stem cell niche may contribute to EOM resistance to multiple muscle degenerative diseases and age-related functional decline through the maintenance of tissue plasticity throughout life.

METHODS

MICE

Animal models used were: 7 week-old and 18 month-old C57Bl6J mice, 7 week-old and 18 month-old C57Bl6J PW1IRESnLacZ transgenic reporter mice (PW1^{nLacZ}) (Besson et al., 2011), 7 week-old C57Bl10 and *mdx* (Bulfield et al., 1984) mice. All work with mice was carried out in adherence to French government guidelines.

HISTOLOGICAL ANALYSES

Tibialis Anterior (TA) muscles were removed, mounted in tragacanth gum (Sigma Aldrich) and snap frozen in liquid nitrogen-cooled isopentane (Sigma Aldrich) as previously described (Mitchell et al., 2010). For EOM dissection, the skin of the head was removed to expose the eye. An incision of the basal part of the eyelids was performed and the globe was gently pulled out of the ocular cavity. A perpendicular cut in proximity of the skull inside the cavity was performed to release the globe with the EOMs attached *in situ*. Eyelids were removed from the globe, which was then mounted in tragacanth gum (Sigma Aldrich) and snap frozen in liquid nitrogen-cooled isopentane (Sigma Aldrich) as previously described (Mitchell et al., 2010). Muscles were cryosectioned (5–7 μ m) before processing.

To stain nuclei and muscle fibers, cryosections were stained with hematoxylin and eosin (H&E) (Sigma Aldrich). For PW1^{nLacZ} mice, cryosections were stained with Xgal as previously

reported (Besson et al., 2011) and Nuclear Fast Red solution (Sigma Aldrich) according to manufacturer's instructions.

For immunofluorescence, EOMs and TA cryosections were fixed in 4% (w/v) paraformaldehyde and processed for immunostaining as described previously (Mitchell et al., 2010). Primary antibodies used were: PW1 (Relaxia et al., 1996) (rabbit, 1:3000), Pax7 (mouse, Developmental Studies Hybridoma Bank, 1:10), MyoD (mouse, BD Biosciences, 1:100), Ki67 (mouse, BD Biosciences, 1:100), Ki67 (rabbit, Abcam, 1:100), PH3 (rabbit, Abcam, 1:100), laminin (rabbit, Sigma, 1:100). Antibody binding was revealed using species-specific secondary antibodies coupled to Alexa Fluor 488 (Life Technologies), Cy3 or Cy5 (Jackson Immunoresearch). Nuclei were counterstained with DAPI (Sigma Aldrich).

RNA EXTRACTION AND qPCR

Extraocular muscles from six different C57Bl6J mice were pooled into a single sample. Tibialis Anterior from three different C57Bl6J mice were analyzed separately. RNA extracts were prepared using RNeasy Fibrous Midi Kit (Qiagen) according to manufacturer's instructions, and reverse transcribed using the SuperScript First-Strand Synthesis System (Life Technologies). Quantitative polymerase chain reaction was performed using SYBR® green (Thermo Fisher Scientific) under the following cycling conditions: 95°C for 5 min followed by 50 cycles of amplification (95°C for 15 s, 61°C for 15 s and 72°C for 20 s), then 95°C for 5 s followed by a final incubation at 65°C for 1 min. Each sample was analyzed in triplicate. Primers sequences used were: FST, FWD 5'-CCCCAACACTGCATCCCTTGTAAA-3' and REV 5'-TCCAGGTGATGTTGAAACAGTC-3'; IGF-1, FWD 5'-T GCTCTTCAGTCGTGTG-3' and REV 5'-ACATCTCCAGTCTC CTCAG-3'; myostatin (MST), FWD 5'-GGCTCAAACAGCCTG AATCCAA-3' and REV 5'-CCAGTCCCACATCCAAAGGCTTCA AA-3'; 18S: FWD 5'-CGGCTACCACATCCAAGGAA-3' and REV 5'-TATACGCTATTGGAGCTGGAA-3'. Levels of FST, IGF-1 and MST expression were normalized using 18S gene expression.

STATISTICAL ANALYSIS

All statistics were performed using an unpaired Student's *t*-test in the StatView software. Values represent the mean \pm s.e.m.
p* < 0.05, *p* < 0.01 and ****p* < 0.001.

RESULTS

EOM STEM CELL NICHE IS CONSERVED THROUGHOUT POSTNATAL LIFE

It has been reported previously that *PW1/Peg3* RNA levels are higher in normal EOMs as compared to limb muscles (Porter et al., 2003), suggesting either an increase of *PW1* gene expression or an increase of the total number of *PW1*-expressing cells in EOMs. We therefore analyzed the EOM and limb muscle progenitor cell niche and compared our results with those obtained previously for limb muscles during postnatal stages (Mitchell et al., 2010; Pannérec et al., 2013). We have shown previously that in limb muscles PICs and satellite cells undergo a progressive decline within the first 3 weeks after birth, however these two cell types maintain a 1:1 ratio in homeostatic conditions in young and adult mice (Mitchell et al., 2010; Formicola et al., under review). We analyzed 7 week-old wild-type EOMs and TA muscles and

found a 2-fold higher number of PICs in EOMs as compared to TA, whereas the number of satellite cells per muscle fiber was the same in the two sets of muscles (Figures 1A,B). This higher number of PICs per muscle fiber results in a PICs/satellite cells ratio of 2:1 in EOMs as compared to 1:1 found in the TA (Figure 1B; Mitchell et al., 2010; Formicola et al., under review). We (Mozzetta et al., 2013; Formicola et al., under review) described recently that PICs express promyogenic factors, such as FST and IGF-1, which are able to counteract the antimyogenic effect exerted by MST and other TGFβ superfamily members, including activins (Amthor et al., 2004; Latres et al., 2005; Lee et al., 2010). We wondered whether the larger PIC population observed in EOMs could provide a higher level of promyogenic factors in the EOM. Our analyses of whole EOM extracts revealed that the EOMs display levels of expression of *Fst* and *Igf-1* that are respectively 11 and 2 folds higher as compared to the TA, whereas differences in *Mst* levels are less pronounced (1.5 fold higher level of expression in EOMs as compared to TA; Figure 1C). Using *PW1^{nLaz}* reporter mouse (Besson et al., 2011) we then compared EOMs and TA from 7 week-old and 18 month-old mice. We noted that while TA shows a marked decrease in *PW1*-expressing cells with age, in EOMs the amount of *PW1*-expressing cells were less affected (Figure 1D). A more detailed analysis revealed that although both muscle groups show a decrease in PICs and satellite cell number with age, PICs/satellite cells ratio remains unchanged in aged EOMs as compared to young EOMs (Figure 1E). Specifically, PICs from limb muscles undergo a marked decline as compared to satellite cells, as aged TA exhibits a 0.3:1 ratio between PICs and satellite cells (Figure 1E), supporting the notion that factors regulating progenitor cells within the niche are different in EOMs as compared to limb muscles. In addition, we noted a rare population of interstitial cells that expressed Pax7 in 7 week-old EOMs, completely or partially surrounded by the basal lamina (Figure 1F), whereas we did not observe any Pax7^{pos} interstitial cell in age-matched TA (Figures 1F,G), suggesting that a subpopulation of satellite-like cells has a different anatomical location in EOMs as compared to limb muscles. Moreover, we noted that half of the Pax7^{pos} interstitial cells observed in adult EOMs co-express *PW1* (Figure 1G), raising the possibility that a subset of PICs is committed to the myogenic lineage. We note that Pax7^{pos} interstitial cells can also be detected in aged limb muscles as well as aged EOMs (Figure 1G). A previous report showed that a subset of aged limb muscle satellite cells is more prone to exit quiescence due to high homeostatic FGF-2 expression (Chakkalakal et al., 2012). Interestingly, FGF-2 levels have been reported to be elevated in human EOMs as compared to limb muscles (Fischer et al., 2005). Whether these Pax7^{pos} interstitial cells are a subpopulation of FGF-2-responsive PICs that enter the myogenic lineage through expression of Pax7 or they are a subset of satellite cells responding to FGF-2 with a different anatomical location is an issue that remains to be resolved.

In order to know if these differences in EOMs are related to a different activation status of progenitor cells in EOMs as compared to TA, we checked for cell cycle markers (as Ki67 and PH3) as well as myogenic activation marker MyoD. We failed to detect expression of Ki67 and PH3 as well as MyoD in PICs or satellite cells from EOMs indicating that PICs and satellite cells are not

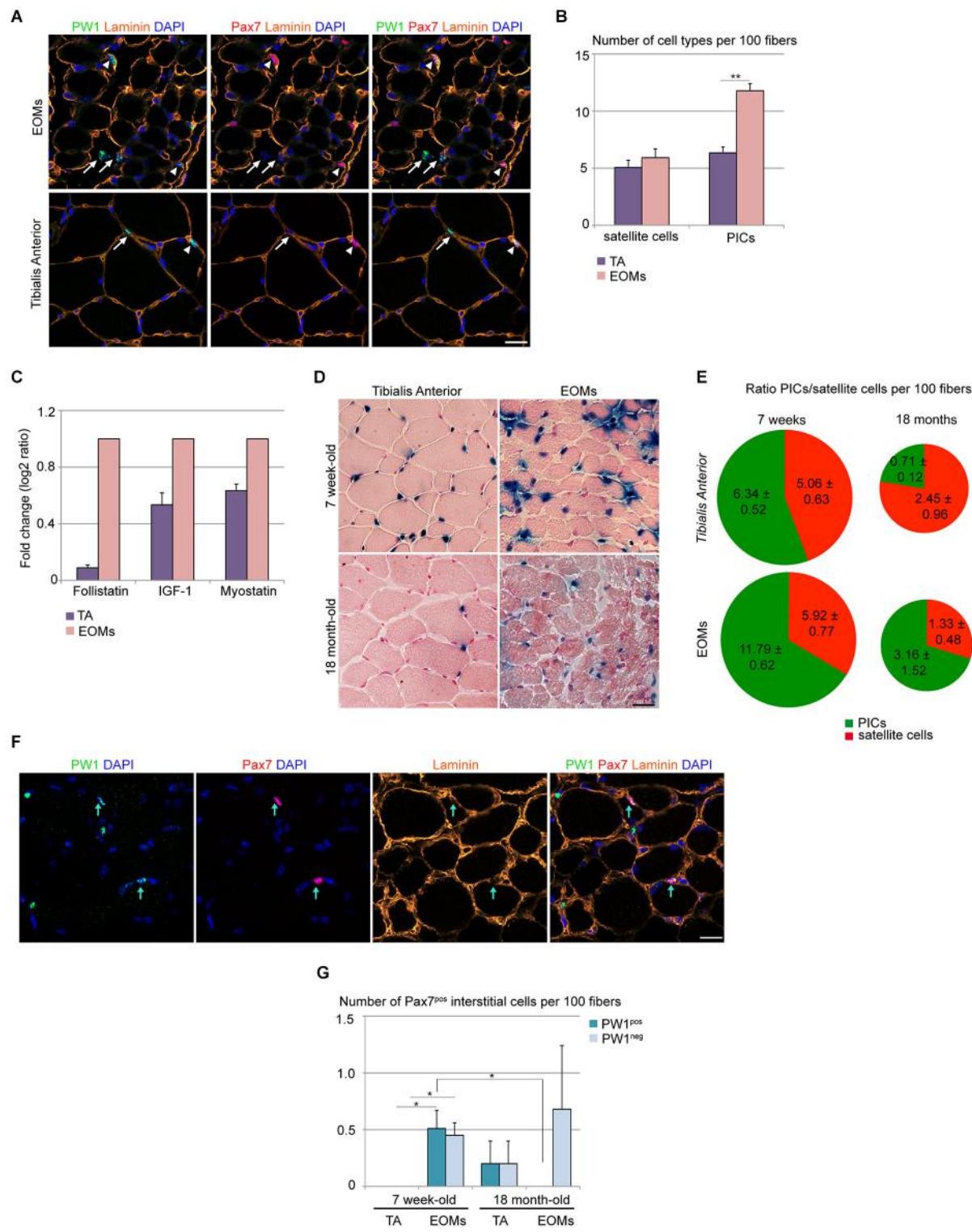


FIGURE 1 | Wild-type EOM stem cell niche is intrinsically different from limb muscles. (A) Cross-sections of 7-week old EOMs (upper panels) and TA (lower panels) from C57Bl6 mice stained for PW1 (green) and the satellite cell marker Pax7 (red). Laminin staining (orange) shows the basal lamina. Nuclei were counterstained by DAPI (blue). Arrows indicate PICs, arrowheads indicate satellite cells. Scale bar, 50 μ m. **(B)** Number of satellite cells and PICs

per 100 fibers in 7-week old EOMs and TA cross-sections as stained in **(A)** revealed a bigger amount of PICs but not satellite cells in EOMs compared to TA. **(C)** Fold change of FST, IGF-1 and myostatin expression levels from qPCR analysis on total RNA extracts from EOMs and TA from 7 week-old wild-type mice revealed an strong increase in FST expression in EOMs. For TA muscles,

(Continued)

FIGURE 1 | Continued

three different animals ($n = 3$) were considered separately; for EOMs, six different animals ($n = 6$) were pooled into one sample. Each sample was analyzed in duplicate. Error bar indicates s.e.m. calculated for number of samples. **(D)** Xgal staining on cross-sections of 7-week old (upper panels) and 18 month-old (lower panels) EOMs and TA from PW1^{nLacZ} mice. Nuclei and myofibers were counterstained with Nuclear Fast Red™ Solution. Scale bar, 40 μm . **(E)** Ratio between PICs (green) and satellite cells (red) per 100 fibers in 7-week old and 18-month old TA and EOMs cross-sections demonstrated that EOM but not TA stem cell niche is retained throughout life. **(F)** Cross-sections of 7-week old EOMs stained as in **(A)**. We observe Pax7^{pos} cells totally or partially surrounded by the basal lamina and often co-expressing PW1. Arrows indicate double-labeled PW1^{pos}Pax7^{pos} interstitial cells. **(G)** Number of Pax7^{pos} interstitial cells (PW1^{pos} and PW1^{neg} subsets) per 100 fibers in 7 week-old and 18 month-old EOMs and TA cross-sections stained as in **panel A**. For all graphs, values represent the mean number of cells \pm s.e.m. For **(B,E)**, PICs were determined as interstitial PW1^{pos}Pax7^{neg} cells, satellite cells were determined as Pax7^{pos} cells underneath the basal lamina. Statistical significance was calculated from at least three animals of each condition. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$.

activated in EOMs (data not shown) and consistent with observations in TA and other skeletal muscles (Pallafacchina et al., 2010) but in contrast with previous reports in EOMs (McLoon and Wirtschafter, 2002a,b, 2003; McLoon et al., 2004). This likely reflects the different animal species and ages considered (rabbits, mice, rats) as well as the different techniques used to reveal satellite cell activation and proliferation (i.e., single myofiber reconstruction vs. histological analysis of transversal sections) (McLoon and Wirtschafter, 2002a,b; McLoon et al., 2004). Moreover, in those previous reports, no direct co-labeling of MyoD with satellite cell markers was performed and cell proliferation status was assessed through BrdU incorporation (McLoon and Wirtschafter, 2002a,b; McLoon et al., 2004). Nonetheless, the presence of both MyoD^{pos} cells and BrdU-labeled cells were rare events (McLoon and Wirtschafter, 2002a,b; McLoon et al., 2004). We noted that PW1^{pos}Pax7^{pos} interstitial cells decline with age in EOMs, however we still detected PW1^{neg}Pax7^{pos} interstitial cells (**Figure 1G**). The role and biological significance of these two subsets of the Pax7^{pos} interstitial cell population based on PW1 expression remains to be determined. Taken together, our observations strongly support the notion that the EOM stem cell niche composition is intrinsically different from that seen in limb muscles.

Mdx MICE DISPLAY AN UNALTERED EOM STEM CELL NICHE

Mdx skeletal muscles undergo continuous cycles of degeneration and regeneration due to a defect in the dystrophin gene, making it a valuable model for the study of DMD (Burghes et al., 1987; Partridge, 2013; Tabebordbar et al., 2013). While the mice and humans vary in the degree of disease severity, both share the feature that the EOMs are not affected (Kaminski et al., 1992; Porter et al., 1995, 2003). We analyzed the EOM and TA stem cell niches in 7 week-old wild-type and *mdx* mice. EOM cross-sections from *mdx* mice did not display histological signs of ongoing regeneration and looked as their wild-type counterparts, whereas TA cross-sections showed widespread regions with centrally nucleated fibers, as previously reported (Wallace and McNally, 2009;

Figures 2A,B). We observed a single and highly restricted region containing a few centrally nucleated fibers in one EOM in only one out of three mice examined. Furthermore, we observed an increase in the number of interstitial cells in *mdx* TA as compared to wild-type TA, but no changes in EOMs (**Figure 2C**), confirming that *mdx* EOMs were unperturbed. Both satellite cells and PICs were increased in *mdx* TA as compared to its wild-type counterpart (**Figures 2D,E**), whereas *mdx* EOMs displayed an unchanged content of PICs and satellite cells as compared to wild-type (**Figures 2D,E**). Taken together, these data support the hypothesis that EOM stem cell niche is unperturbed in *mdx* as compared to wild-type. Interestingly, the number of PICs in *mdx* TA is comparable to wild-type EOMs (**Figure 2E**). Based on previous observations strongly indicating a role of PICs/FAPs as support niche cells, our data suggest that mechanisms normally occurring during muscle regeneration to promote progenitor cell survival, activation and differentiation, are intrinsic to the EOM stem cell niche.

DISCUSSION

Duchenne muscular dystrophy is the most common form of muscular dystrophy in humans affecting boys, leading to a loss of skeletal muscle mass and function and premature death following heart and respiratory failure (Wallace and McNally, 2009; Shadrach and Wagers, 2011; Tabebordbar et al., 2013). In late-stage DMD, muscle fibers are replaced by fibrotic and fat tissue, due to a massive deregulation of signaling pathways within the muscle tissue and a promotion of fibrosis (Wallace and McNally, 2009; Serrano et al., 2011; Tabebordbar et al., 2013). Several observations indicate that FAPs are the main source of fibrotic extracellular matrix deposition and adipocytes in this context (Joe et al., 2010; Uezumi et al., 2010, 2011; Pannérec et al., 2013). Nonetheless, FAPs are important regulators of the muscle regeneration process (Joe et al., 2010; Uezumi et al., 2010), suggesting a dual role for these cells in governing muscle homeostasis as well as the importance of the microenvironment in directing their behavior. The observations that human as well as mouse EOMs are resistant to several dystrophies including DMD as compared to other sets of skeletal muscles of the body (Porter et al., 1995; Porter, 2002) suggest that muscle-type specific endogenous mechanisms operate in the stem cell niche leading to a more efficient regenerative response or protection against fiber atrophy or degeneration. We recently proposed that PICs, which include the FAP population, act as support niche cells for satellite cells in postnatal skeletal muscle by secreting promyogenic factors (Pannérec et al., 2013; Formicola et al., under review). Our data confirm previous observations showing that the EOM stem cell niche is intrinsically different as compared to limb muscles (Porter et al., 2001, 2003; Porter, 2002; Khanna et al., 2003). Adult EOMs display a larger PIC population and an increased expression of the promyogenic factors FST and IGF-1, in contrast to limb muscles where PICs and satellite cells are in equal amounts (1:1) during postnatal growth and adulthood (Mitchell et al., 2010; Formicola et al., under review). Interestingly, despite a decline in both PICs and satellite cells in aged muscle, the ratio between these two progenitor cell types is tightly conserved in EOMs whereas the PIC compartment undergoes a marked decline

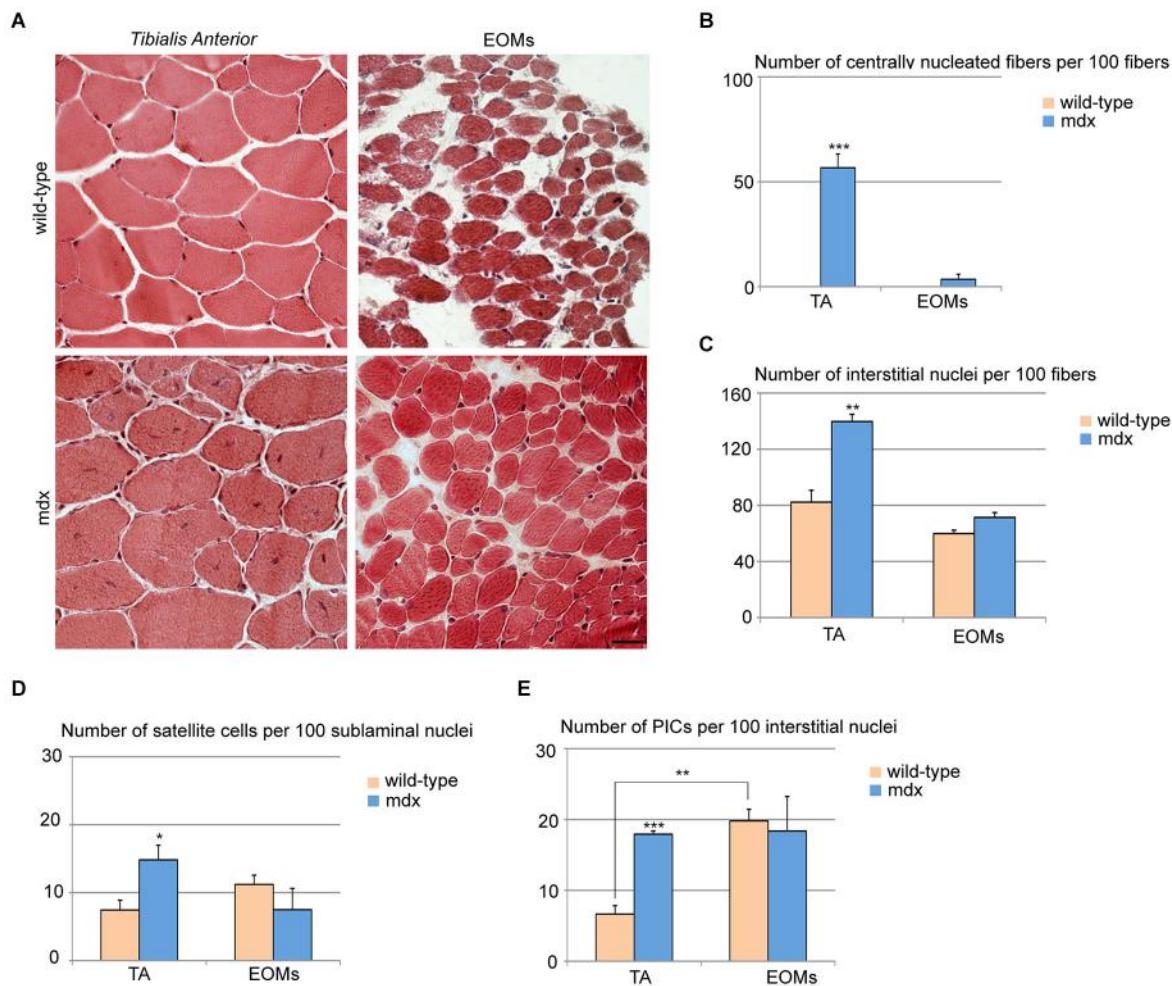


FIGURE 2 | Extraocular muscles from *mdx* and wild-type mice exhibit a similar muscle stem cell niche. (A) Cross-sections of 7-week old TA (upper panels) and EOMs (lower panels) from *mdx* and age-matched wild-type mice stained with hematoxylin and eosin showed large regenerating areas in TA but not EOMs from *mdx* mice. **(B,C)** Number of centrally nucleated fibers **(B)** and interstitial nuclei **(C)** per 100 fibers indicated the presence of an ongoing regeneration process in *mdx* TA but not EOMs. **(D,E)** Number of satellite cells per 100 sublaminar

nuclei **(D)** and PICs per 100 interstitial nuclei **(E)** revealed an activation of both progenitor cell types in TA but not EOMs from *mdx* mice, as compared to their wild-type counterparts. Positive interstitial cells were determined as interstitial PW1^{pos}Pax7^{neg} cells, satellite cells were determined as Pax7^{pos} cells underneath the basal lamina. For all graphs, values represent the mean number \pm s.e.m. Statistical significance was calculated from at least three animals per each condition. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

with age in limb muscles. We propose that the EOM provides a more promyogenic environment as compared to limb muscle that is more resistant to diseases throughout life. We note that a recent study described the presence of fibroadipogenic Sca1^{pos} progenitor cells in the periocular connective tissue that have a Myf5^{pos}Pax3^{pos} origin (Stuelsatz et al., 2014b). While we previously demonstrated that limb-isolated PICs are not derived from Pax3^{pos} progenitor cells (Mitchell et al., 2010), a possible different developmental origin of EOM PICs should not be discarded and could account for the different features exhibited by the muscle stem cell niche in normal as well as *mdx* and aged EOMs as compared to the limb muscles. Moreover, our observations of aged muscles are in contrast with hypotheses that an increase in FAP number in aged muscles accounts for an increase in fibrosis

during sarcopenia (Shadrach and Wagers, 2011; García-Prat et al., 2013) and strongly support the idea that the balance of interactions between the niche components is the major determinant of muscle homeostasis rather than single cell population levels. Indeed, we found that EOM stem cell niche from *mdx* mice do not display any particular difference as compared to wild-type mice and *mdx* EOMs are not affected by the disease. It is interesting to note that the regeneration process in *mdx* limb muscles induces an increase of satellite cells and PICs up to a level comparable to the one observed in wild-type EOMs. A previous report has described increased *Fst* and *Mst* gene expression levels in limb muscles from young *mdx* mice as compared to wild-type counterparts (Abe et al., 2009) and we observed in this study a similar trend in wild-type EOMs as compared to wild-type

limb muscles. We described recently that satellite cells secrete MST whereas PICs secrete FST (Mozzetta et al., 2013; Formicola et al., under review). The observations and data presented in this study support the hypothesis that changes in the stem cell niche composition and their signaling during muscle regeneration are intrinsic properties of EOMs at the steady state. Indeed, another study (Porter et al., 2003) showed that wild-type EOMs and *mdx* limb muscles highly express specific genes involved in muscle regeneration and fibrosis, such as interleukin-10 receptor beta (IL10R β), connective tissue growth factor (Ctgf), follistatin-like 1 (Fstl-1) and TGF β -induced protein (TGF β i; Porter et al., 2003; Serrano et al., 2011; Deng et al., 2012). Interestingly, data from our previously published microarray comparing PICs and satellite cells reveal that these genes are upregulated in PICs (Pannérec et al., 2013). Moreover, a recent study revealed that satellite cells isolated from *mdx* EOMs as well as normal adult and aged EOMs exhibit a robust growth and self-renewal capacity *in vitro* and high engraftment performance as compared to their limb and diaphragm counterparts (Stuelsatz et al., 2014a). We propose that the EOMs are spared in degenerative diseases because they have a high number of PICs. This increase provides an environment rich in promyogenic and hypertrophic factors, which protect both the fibers and the satellite cells, preventing the loss of regenerative capacity observed in late stages of muscular dystrophies.

AUTHORS' CONTRIBUTIONS

Luigi Formicola performed experiments. All authors designed experiments, analyzed and interpreted data and prepared the manuscript.

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The quasi-parallel lives of satellite cells and atrophying muscle

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Skeletal muscle atrophy or wasting accompanies various chronic illnesses and the aging process, thereby reducing muscle function. One of the most important components contributing to effective muscle repair in postnatal organisms, the satellite cells (SCs), have recently become the focus of several studies examining factors participating in the atrophic process. We critically examine here the experimental evidence linking SC function with muscle loss in connection with various diseases as well as aging, and in the subsequent recovery process. Several recent reports have investigated the changes in SCs in terms of their differentiation and proliferative capacity in response to various atrophic stimuli. In this regard, we review the molecular changes within SCs that contribute to their dysfunctional status in atrophy, with the intention of shedding light on novel potential pharmacological targets to counteract the loss of muscle mass.

Keywords: skeletal muscle, stem cells, satellite cells, atrophy, cachexia

Introduction

Skeletal muscle atrophy is characterized by a loss of muscle mass and force, that occurs in response to a variety of pathological and physiological stimuli such as aging, cancer, chronic kidney disease (CKD), chronic obstructive pulmonary disease (COPD), chronic heart failure (CHF), diabetes, AIDS infection, sepsis, burns, muscle disuse, loss of muscle innervation, malnutrition, steroid-induced catabolic stimulation, and different congenital neuromuscular diseases (Bonaldo and Sandri, 2013; Egeman and Glass, 2014; Cohen et al., 2015). Loss in muscle mass is characterized by a decrease in cross-sectional area of muscle fibers, that is primarily the outcome of a preferential increase in intracellular protein degradation over protein synthesis (Bonaldo and Sandri, 2013; Egeman and Glass, 2014; Cohen et al., 2015). In most muscle wasting conditions, the loss of muscle tissue is not homogeneous, displaying differential effects on specific muscle groups and impacting distinct fiber types (Ciciliot et al., 2013). In specific forms of atrophy, such as age-related atrophy (sarcopenia), the reduction in fiber size is also accompanied by a reduction in the number of fibers, further highlighting the heterogeneity of the atrophying process occurring in different muscle wasting conditions (Lexell et al., 1988).

Muscle fibers are post-mitotic syncytia formed by the fusion of several hundreds of myogenic progenitors during pre- and post-natal development (Biressi et al., 2007). A majority of the myonuclei are added to developing fibers during post-natal growth, when there is a dramatic increase in muscle mass (Zhang et al., 1998). Not all myogenic progenitors terminally differentiate into muscle fibers during development, but a fraction of them remains in the adult muscles as a pool of undifferentiated myogenic stem cells. Different cellular types that possess the ability to differentiate into muscle fibers have been identified (Cossu and Biressi, 2005; Peault et al., 2007). Nevertheless, a population of stem cells, called “satellite cells” (SCs),

due to the peculiar anatomical location between the fiber sarcolemma and the basal lamina surrounding the fiber, is believed to contribute in a major way to post-natal growth and muscle repair upon injury (Schultz, 1996; Lepper et al., 2011; Murphy et al., 2011; Sambasivan et al., 2011). In healthy adult muscles, SCs are largely quiescent, but can be activated by appropriate stimuli in the form of injury or exercise, resulting in regeneration and possibly further growth (hypertrophy) of the muscle tissue (Zammit et al., 2006; Schiaffino and Partridge, 2008). Several studies have investigated the role played by SCs in muscle hypertrophy and conclusions drawn from these studies are equivocal (Blaauw and Reggiani, 2014). A key role for SCs in the hypertrophic response is supported by observations that SCs are consistently activated in hypertrophic models and that their elimination by irradiation severely blunts overload-induced muscle hypertrophy (Rosenblatt et al., 1994). On the contrary, various observations using mutant mice to genetically ablate SCs and induce hypertrophy indicate that SC functionality does not constitute an absolute requirement in initiating muscle hypertrophy, suggesting that the prerequisite nature of their involvement might be limited to later stages in stabilizing the full hypertrophic response on a long-term basis (Amthor et al., 2009; Blaauw et al., 2009; McCarthy et al., 2011; Lee et al., 2012; Fry et al., 2014).

Whereas our knowledge of SC behavior in muscle hypertrophy is rapidly increasing, the role played by SCs during muscle atrophy remains largely enigmatic. Recent reports have generated an active debate in the scientific community on the active participation of SCs in the atrophic response, or whether muscle fiber alterations exclusively account for atrophy. In this manuscript, we review the experimental evidence supporting either view.

Several studies have investigated the effects of atrophic stimuli on muscle fibers and have identified key signaling pathways operating within the fiber that contribute to a decrease in fiber cross-sectional area. These studies highlighted the importance of protein homeostasis in regulating muscle mass, and identified the ubiquitin-proteasome and the autophagy-lysosome machineries as the two most important protolithic systems controlling protein turnover in muscle fibers. Several recent reviews describe the advances in understanding signaling mechanisms controlling the activity of these two systems in skeletal muscle fibers (Sandri, 2013; Schiaffino et al., 2013). In this review, we focus on the signaling pathways triggered in SCs in response to various atrophic stimuli. Moreover, we review the studies that address whether these molecular changes can be suppressed or reversed to induce SCs to increase the mass of atrophied muscles.

Does SC Activity Affect the Loss of Muscle Mass?

Muscle homeostasis depends on a fine balance between catabolism and anabolism. A large body of evidence indicates that alterations in the control of protein turnover modify this balance and play an important role in essentially all forms of muscle atrophy (Sandri, 2008). Nevertheless, the mass of every

tissue, including skeletal muscle is not only dependent on protein turnover, but also on cellular turnover (Sartorelli and Fulco, 2004). In keeping with this, muscle fibers are lost in certain forms of muscle wasting (Lexell et al., 1988). Moreover, muscle fibers are multinucleated structures, and it has been proposed that there are distinct "myonuclear domains", whereby each myonucleus governs the surrounding cytoplasm by producing enough protein to support only a limited portion of the fiber (Mitchell and Pavlath, 2004). Although some studies report a reduction in muscle fiber size without a change in myonuclear number (Wada et al., 2002; Gundersen and Bruusgaard, 2008), several reports have documented a decrease in the number of myonuclei accompanying different forms of atrophy, supporting the hypothesis that myonuclear turnover and a reduction in muscle mass are causally related (Darr and Schultz, 1989; Schmalbruch et al., 1991; Allen et al., 1995, 1996; Day et al., 1995; Hikida et al., 1997). More importantly, this raises the prospect that alterations in myonuclear turnover can stabilize the reduction in muscle mass, at least under certain atrophic conditions.

As myonuclei are postmitotic, the need to replenish lost myonuclei to maintain a constant myonuclear number must come from myogenic progenitors that are able to fuse with the fibers. SCs, which have been reported to contribute to a large fraction of the fiber myonuclei during regeneration and growth, represent a natural candidate for this role (Moss and Leblond, 1971). In keeping with this idea, ablation of SCs in a paired box protein (Pax7)^{DTR} knock-in mouse model revealed a 20–40% loss of muscle mass 2 weeks after intramuscular injection of diphtheria toxin, an effect that persisted for 7 weeks after SC elimination and was exacerbated with strenuous resistance exercise (Sambasivan et al., 2011). Another study demonstrating an essential role for miRNAs in SC quiescence observed mild muscle atrophy within 6 months in uninjured mice that expressed a SC-specific conditional knockout (KO) of the gene *Dicer* (Cheung et al., 2012). These reports raise the tantalizing possibility that despite a low turnover rate in healthy muscle (Spalding et al., 2005), SCs could have an impact on homeostatic control of muscle mass. In contrast, a study using a genetic approach that allows for long-term depletion of SCs upon tamoxifen intraperitoneal injection in sedentary mice challenges this view (Fry et al., 2015). Despite the low regenerative capacity, these mice do not present signs of atrophy in hind limb muscles 1 month after tamoxifen administration and do not display exacerbated atrophy in 2-year-old mice. Therefore, these findings suggest that skeletal muscles do not necessarily require stem cell participation for tissue maintenance, at least under non-stressful conditions (Fry et al., 2015). Intriguingly, in a recent study, SCs were genetically labeled in adult mice and their fusion to myofibers in the absence of injury was followed throughout the lifespan of the mice. These experiments showed a contribution of SCs to myofibers in all muscles considered, although the extent and timing of their involvement differed in distinct muscles (Keefe et al., 2015). Importantly, the ablation of SCs using a genetic approach similar to that used by Fry et al. (2015) also showed a muscle group-specific response. In corroboration with the Fry et al. (2015) data, limb muscles were

not significantly affected 6 months after SC depletion (Keefe et al., 2015). However, the diaphragm and extra ocular muscles displayed smaller fibers after 6 months of depletion of SCs, a decrease that was not exacerbated at later time-points (Keefe et al., 2015). Although a possible explanation for the different results derived from the individual studies could depend on the different nature of the animal models and experimental settings employed, the different types of muscles analyzed, or on the potential stress derived from the intramuscular injection of diphtheria toxin (Sambasivan et al., 2011), further investigations appears to be necessary to clarify the role of SCs in muscle homeostasis.

The discussion on the role played by SCs in the homeostatic maintenance of mass in healthy muscles has broader implications for the study of SC involvement in counteracting muscle mass loss under atrophying stimuli. The long-term SC depletion study conducted by Fry and colleagues, whereby the absence of SCs did not exacerbate sarcopenia in 2-year-old mice, is suggestive of the notion that SCs do not exert a compensatory action to counteract atrophy with age (Fry et al., 2015). Despite the compelling observations made in this study, the conclusions could possibly be limited by the incomplete depletion of SCs obtained in aged mice (an average of ~83%; Fry et al., 2015). Notably, elimination of ~85% of SCs by freeze- or cardiotoxin-mediated injury still results in muscle regeneration (Gayraud-Morel et al., 2007). Therefore, it is particularly relevant that the study from Keefe and colleagues using a mouse strain which allowed for the ablation of >95% of the SCs, obtained results that were similar to those reported by Fry and colleagues. When mice depleted of SCs were analyzed at 20 months of age, the contribution of SCs to myofiber maintenance appeared to be minimal in most hind limb muscles, with the striking exception of extensor digitorum longus (EDL) muscles that displayed a ~15% decline in fiber size (Keefe et al., 2015). Although an age-related reduction in fiber cross-sectional area is apparent at 20 months of age in most muscles, it is mainly after 2 years that a dramatic loss of muscle mass occurs in mice, which correlates with the appearance of an irreversible pre-senescent state in SCs that prevents activation and expansion (Sousa-Victor et al., 2014). The appearance of severe sarcopenia in older mice opens up the possibility for a primary requirement for SC contribution at later stages. Therefore, it would be interesting to extend the ablation studies to mice older than 2 years of age.

Intriguingly, several reports indirectly suggest that SCs may play a role in ameliorating sarcopenia, and that sarcopenia could be at least in part, a consequence of defective SC function. Several changes affecting the SC compartment have been observed in aged muscles (Alway et al., 2014). Strikingly, stem cell function and consequently regenerative potential are severely affected by aging in different tissues, including skeletal muscle (Liu and Rando, 2011). Alterations in the muscle and systemic environment occur during the aging process, thereby contributing to reversible and irreversible changes in SCs (Brack and Rando, 2007; Jang et al., 2011; Sousa-Victor et al., 2015). Moreover, despite the lack of a clear consensus, a reduction in SC number has been reported

during aging, and it has been correlated with a reduction in fiber myonuclear content (Brack et al., 2005). Presence of centrally-nucleated fibers and an up-regulation of Myogenic differentiation (MyoD), Myogenin and embryonic Myosin Heavy Chain expression, which are generally considered as hallmarks of fiber regeneration, have also been documented in aging muscle (Edström and Ulfhake, 2005). Notably, a loss of myonuclei with aging in *MyoD* KO mice was exacerbated compared to wild-type mice. Since SCs display defective differentiation in the absence of MyoD expression, this observation has been interpreted as evidence in favor of a role for SCs in replenishing fiber myonuclei during aging (Brack et al., 2005).

An up-regulation of MyoD expression has also been documented in other forms of atrophy, and is particularly well established in denervation models of atrophy (Legerlotz and Smith, 2008). Upon denervation, SCs proliferate transiently, but do not progress through differentiation and form small immature (embryonic Myosin Heavy Chain^{+ve}) fibers, indicative of an unsuccessful attempt to restore muscle mass and function (Viguie et al., 1997; Borisov et al., 2005; Doppler et al., 2008). As in aging muscle, the increase in MyoD expression observed in denervated muscle could be interpreted as a result of SC activity. Nevertheless, it is notable that muscle fibers also express MyoD at low levels, even in the absence of ongoing regeneration (Hughes et al., 1993). It is therefore possible that the increase in MyoD expression in muscle observed after denervation, and possibly in other models of atrophy as well, could constitute a SC-independent response of the fibers to atrophic stimuli (Koishi et al., 1995). Indeed it has been proposed that the induction of MyoD could represent an attempt by muscle fibers to regain sensitivity to neural activity. In keeping with this idea, MyoD has been shown to regulate the expression of Acetylcholine receptor (AChR), the expression of which is also increased upon denervation (Legerlotz and Smith, 2008). On similar lines, Myogenin is also expressed in fibers in the absence of a regenerative response (Hughes et al., 1993), and can act as an essential mediator of neurogenic atrophy by regulating the expression of Murf1 and Atrogin-1 within the fiber, thereby promoting muscle proteolysis (Moresi et al., 2010).

Alterations in the myogenic program have also been described in cachexia, a complex metabolic syndrome characterized by a loss of muscle mass, which is initiated by underlying illnesses of different nature, such as cancer, CHF, COPD, CKD, burns, chronic infection and sepsis (Evans et al., 2008; Fearon et al., 2012). Changes in the expression of myogenic factors and impaired differentiation have been reported in cachectic muscles (Coletti et al., 2005; Langen et al., 2006; Schwarzkopf et al., 2006; Penna et al., 2010; Zhang et al., 2010a; Wu et al., 2013). In tumor-bearing mice that recapitulated clinical features of cancer-induced cachexia, as well as in muscle biopsies from patients with pancreatic cancer, a significant expansion of cells expressing high levels of the SC-marker Pax7 was observed (Penna et al., 2010; He et al., 2013). Intriguingly, the majority of the Pax7^{+ve} cells were noted

in the interstitium and a fraction of them were reported to express mesenchymal (*Pdgfr- α* , *Sca1*) and pericyte (*NG2*) markers, which are not expressed in the SC lineage (He et al., 2013). These data reveal that cancer-induced atrophy triggers myogenic commitment in multiple stem cell progenitors, in addition to the SC population (He et al., 2013). In stark contrast with other atrophy models (see above), *MyoD* and *Myogenin* expression are not significantly induced in tumor-bearing mice compared to controls, and an impaired myogenic program prevents these cells from efficiently fusing with existing myofibers and therefore ameliorating the wasting condition (He et al., 2013). *Pax7* has been reported to inhibit differentiation by suppressing *MyoD* and *Myogenin* expression (Olguin and Olwin, 2004). Positive or negative modulation of *Pax7* expression in cachectic mice impact muscle mass in a negative or positive manner, respectively. Moreover, depletion of *Pax7⁺ve* cells further exacerbated muscle loss, directly indicating that stem cells are able to counteract muscle atrophy in cachectic mice. Nevertheless, the persistent expression of *Pax7* stalls the myogenic program and limits the functionality of muscle stem cells (He et al., 2013). In line with the idea that SC functionality can compensate for the effects of atrophic stimuli, *mdx* mouse models of Duchenne muscular dystrophy (DMD) showed a correlation between muscle mass and alterations in regenerative potential. Specifically, during early stages of pathology characterized by effective regeneration, a compensatory hypertrophy has been observed. However, at later stages when regeneration becomes defective, there is a dramatic decrease in muscle mass (Mouisel et al., 2010).

Alterations in SC function, including defective fusion properties, have also been observed in the hind limb suspension model of disuse atrophy (Mitchell and Pavlath, 2004). Conversely, changes in SC numbers are not a consistent finding in disuse atrophy, reflecting the complexity of events occurring in this context (Brooks and Myburgh, 2014). Notably, the decrease in muscle mass accompanying different pathophysiological conditions displays variable changes in SC numbers depending on the type and the severity of the atrophic stimulus (Table 1).

Also the time-scale of the analysis appears to play an important role in SC quantification, as highlighted in denervation experiments, in which an initial phase of proliferation is followed by a decrease in numbers of SCs (Viguie et al., 1997). The inconsistent reports on changes in SC numbers, together with the observations that alterations in SC function appear to be a common feature in most atrophic conditions, raises the prospect that rather than absolute numbers, it is the functionality of the SCs that is more relevant in their ability to counteract atrophy. Further studies will be required to confirm this view, and to conclusively impart a non-redundant role for SCs and other muscle resident stem cells in specific atrophic processes.

Molecular Alterations in SCs During Atrophy

It is increasingly becoming clear that distinct signaling pathways mediate a common outcome of a loss of muscle mass observed in instances of atrophy, sarcopenia, or cachexia (Glass, 2005; Fanzani et al., 2012). While the involvement of these molecular mechanisms has been described in great detail in myofibers in response to atrophic stimuli (Bonaldo and Sandri, 2013; Schiaffino et al., 2013), in this section we review alterations in these pathways occurring in SCs accompanying fiber atrophy. Moreover, we focus on studies reporting functional changes in SCs resulting from alterations in these pathways, which possibly contribute to the loss of muscle mass. Importantly, these pathways extensively modulate one another and coordinate overlapping responses not only in the muscle fiber, but also in the SC compartment (Figure 1). Below, we discuss five major signaling pathways traditionally associated with skeletal muscle atrophy: (a) insulin-like growth factor (IGF)-Akt-FoxO signaling; (b) Transforming Growth Factor Beta (TGF β) superfamily signaling; (c) Glucocorticoids and androgen signaling; (d) nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) signaling; (e) Sirtuin 1 (Sirt1) signaling and mitochondrial dysfunction. Additionally, we review recent

TABLE 1 | Satellite cell alterations in different atrophic conditions.

Condition	Changes in number	Changes in function	Notes	References
Aging	↓ =	Yes	↓ proliferation ↓ myogenic differentiation	Snijders et al. (2009) and Alway et al. (2014)
Cachexia	↑	Yes	↑ activation/proliferation ↓ differentiation/fusion	Penna et al. (2010) and He et al. (2013)
Denervation	↑ *	Yes	↑ activation/proliferation ↓ fusion	Viguie et al. (1997), Borisov et al. (2005) and Doppler et al. (2008)
Hind limb suspension	↓ = ↑	Yes	↓ proliferation ↓ differentiation	Mitchell and Pavlath (2004) Brooks and Myburgh (2014)
Glucocorticoids	n.d.	Yes	↓ proliferation ↓ differentiation	Dong et al. (2013)
DMD	↑	Yes	↓ proliferation ↓ myogenic differentiation/fusion	Blau et al. (1983) and Delaporte et al. (1984) Jasmin et al. (1984), Melone et al. (1999) and Biressi et al. (2014)

↑, Increase; ↓, decrease; =, unchanged; n.d., not defined; * initial increase, but reduced in the long-term (Viguie et al., 1997).

studies involving (f) Notch signaling, a pathway well known to influence SC functionality for a role in regulating muscle mass.

IGF-Akt-FoxO Signaling

A central pathway regulating fiber size is the IGF-Akt pathway (Rommel et al., 2001). Activation of this pathway triggers the activation of mammalian target of Rapamycin (mTOR) that results in the phosphorylation of its targets p70 S6K and 4E-BP1 (Bodine et al., 2001; Pallafacchina et al., 2002; Wullschleger et al., 2006). An additional consequence of these growth-promoting stimuli is the inhibition of the FoxO family of transcription factors that are key in initiating the atrophy program (Sandri et al., 2004). Consistent with this finding, components of the FoxO pathway were found to be increased in skeletal muscle during sepsis, cancer cachexia, and treatment following lipopolysaccharide (LPS), glucocorticoid, and cytokines (Liu et al., 2007; Schmitt et al., 2007; Crossland et al., 2008; Moylan et al., 2008). Additionally, the ability of the FoxO transcription factors to regulate muscle mass during disuse atrophy and following glucocorticoid treatment via the regulation of atrophy-related genes has been well documented (Kamei et al., 2004; Lecker et al., 2004; Sandri et al., 2004; Senf et al., 2010).

Only recently have studies begun to address the influence of IGF1 signaling in the behavior of SCs accompanying the atrophic response in fibers. For instance, in CKD-induced muscle atrophy, SCs displayed a reduction in phosphorylated

Akt levels indicative of impaired IGF-1 signaling, and a decrease in activation and myogenic progression (Zhang et al., 2010a). Moreover, both CKD and *IGF-1 receptor* KO mice developed fibrosis in regenerating muscles, suggesting a decline in SC functionality (Zhang et al., 2010a). Similarly, old dystrophic *mdx* mice also displayed reduced Akt phosphorylation along with defective regeneration and atrophy (Mouisel et al., 2010). In studies aimed at addressing the role of the FoxO's in SCs, overexpression of FoxO3 in myogenic progenitors decreased their proliferation (Rathbone et al., 2008). Similarly, abolishing FoxO activity either by injecting dominant negative (DN) FoxO-expressing plasmid into murine muscles or deleting the *Foxo3* gene specifically in SCs, resulted in an increase in the proliferation of SCs (Reed et al., 2012; Gopinath et al., 2014). Interestingly, the former study reported a hypertrophic response following global suppression of FoxO activity, which acts as a protective mechanism to suppress atrophy following sepsis and cancer (Reed et al., 2012). However, while FoxO functionality may support atrophy when expressed in the fiber, the expression of a specific isoform, FoxO3, in SCs seems to be a prerequisite for maintaining the regenerative capacity of muscle and might therefore act to protect the muscle from atrophying stimuli (Gopinath et al., 2014). These paradoxical observations suggest that FoxO activity promotes different programs in SCs vs. fibers, and underscores the importance of identifying the biochemical processes that promote atrophy in combination with a SC-focused approach.

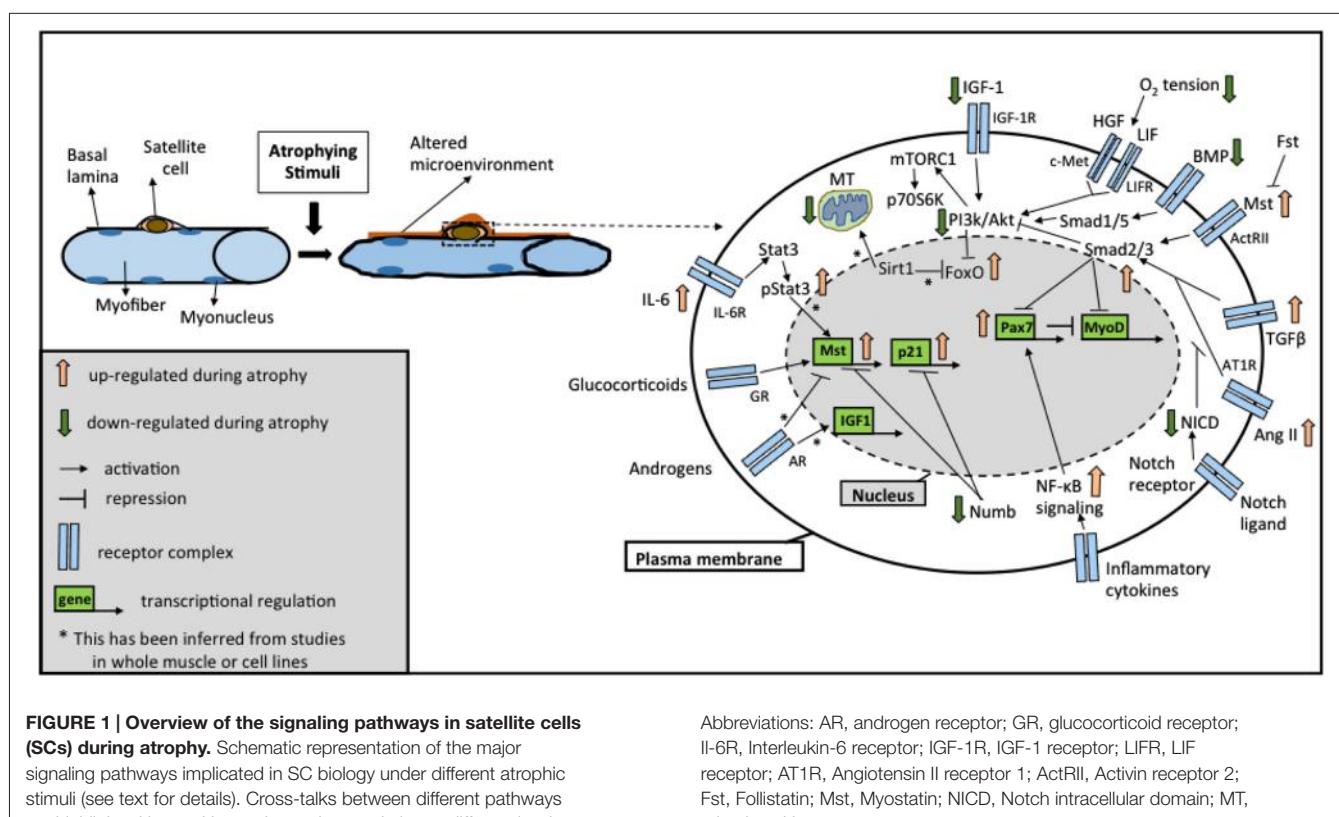


FIGURE 1 | Overview of the signaling pathways in satellite cells

(SCs) during atrophy. Schematic representation of the major signaling pathways implicated in SC biology under different atrophic stimuli (see text for details). Cross-talks between different pathways are highlighted by positive and negative regulation at different levels.

Abbreviations: AR, androgen receptor; GR, glucocorticoid receptor; IL-6R, Interleukin-6 receptor; IGF-1R, IGF-1 receptor; LIFR, LIF receptor; AT1R, Angiotensin II receptor 1; ActRII, Activin receptor 2; Fst, Follistatin; Mst, Myostatin; NICD, Notch intracellular domain; MT, mitochondria.

TGF β Superfamily Signaling

Members of the TGF β superfamily are potent regulators of muscle mass (Goodman and Hornberger, 2014). Canonical TGF β signaling operates through the Smad signaling cascade. Specifically, the TGF- β s, Activins, and some members of the growth differentiation factor (GDF) subfamilies activate a Smad2/3-dependent signaling cascade, whereas members of the bone morphogenetic protein (BMP) subfamily primarily induce a Smad1/5/8-dependent signaling mechanism (Massagué, 1998). An increasing amount of evidence has implicated Smad2/3 in possessing a catabolic function, while Smads 1/5/8 have an anabolic function (Goodman and Hornberger, 2014). Members of the TGF β superfamily play an important role in controlling the proliferation and differentiation of myogenic cells, suggesting that the effects exerted on muscle mass are in part achieved by controlling SC function (Kollias and McDermott, 2008).

Myostatin (GDF8), is a member of the TGF β superfamily and a potent inhibitor of muscle growth, such that various mammalian species bearing *Myostatin-null* mutations display a hypertrophic response (Kambadur et al., 1997; Lee and McPherron, 2001; Schuelke et al., 2004). Myostatin binding to Activin receptor 2 (ActRII) and Activin receptor-like kinase 4 and 5 (ALK 4/5) activates the Smad2/3-dependent signaling cascade and blocks the Akt-mTOR growth-promoting pathway (Rommel et al., 2001). Systemic administration of Myostatin in adult mice was found to induce profound muscle loss (Zimmers et al., 2002). Moreover, enhanced levels of Myostatin have been observed in a variety of muscle wasting conditions (Gonzalez-Cadavid et al., 1998; Lalani et al., 2000; Yarasheski et al., 2002; McKay et al., 2012). Importantly, Myostatin was suggested to play a role in negatively regulating SC activity by inhibiting myogenic progression (Langley et al., 2002; McCroskery et al., 2003; McFarlane et al., 2008; Trendelenburg et al., 2009). Consistent with the notion that modulation of Myostatin expression is bound to have a significant impact on muscle mass and SC behavior, it was found that inhibition of the ActRIIB pathway stimulated SC proliferative potential and reversed muscle wasting in cancer-bearing mice (Zhou et al., 2010). Moreover, altered levels of Myostatin and its receptor resulted in impaired SC proliferation and differentiation in muscle wasting accompanying liver cirrhosis in a portacaval anastomosis (PCA) rat model (Dasarathy et al., 2004). While it has been suggested that a loss in body weight of the PCA rats compared to sham controls is an overall consequence of specific fiber type atrophy (type 2) and impaired SC functionality, what remains to be determined is a quantitative assessment of SC dysfunction alone that contributed to the atrophy observed in this model.

Several studies have provided insights into mechanisms operating upstream and downstream of Myostatin, thereby identifying diverse signaling pathways that have a shared outcome of a loss of muscle mass. Indeed, it has been shown that Numb, a mediator of asymmetric cell division suppresses Myostatin expression, such that *Numb*-deficient SCs display impaired proliferation characterized by high levels of p21 and Myostatin (George et al., 2013). A decrease in muscle mass was apparent after developmental deletion of *Numb* (George

et al., 2013). Given these results, it would then be interesting to investigate whether SC-specific *Numb* conditional KO animals display a reduction in muscle mass, and if the loss is exacerbated in the background of liver cirrhosis, cancer cachexia or other muscle wasting conditions. Intriguingly, lower levels of Numb expression have been reported in muscle biopsies from older men (60–75 years old) than in muscles from younger men (18–25 years old; Carey et al., 2007).

Recent reports have implicated a role for Stat3, a downstream effector of Interleukin 6 (IL-6) in muscle wasting (Muñoz-Cánores et al., 2013; Zhang et al., 2013). IL-6, an inflammatory cytokine is itself known to be involved in initiating muscle wasting when present systemically and for sustained periods of time (Strassmann et al., 1992; Haddad et al., 2005). Elevated levels of activated Stat3 (p-Stat3) were shown to initiate Myostatin-mediated muscle wasting and inflammation in patients with CKD or diabetes via increase in C/EBP δ levels (Zhang et al., 2013). Consistent with this, muscle-specific deletion of *Stat3* and inhibition of CCAAT/enhancer-binding protein gamma (C/EBP δ) expression countered the loss of muscle mass in CKD (Zhang et al., 2013). While this study did not address the status of the Stat3 pathway and the consequences of its modulation within SCs, another study demonstrated that conditional ablation of Stat3 specifically in SCs increased their expansion during regeneration, but compromised their myogenic differentiation and prevented their contribution to regenerating myofibers (Tierney et al., 2014). Intriguingly, transient inhibition of Stat3 function by pharmacological treatment led to an expansion of SCs at a higher rate, and maintained their ability to differentiate into fibers, thus enhancing tissue repair in both aged and dystrophic muscle (Price et al., 2014; Tierney et al., 2014).

Recent findings have proposed a key role for BMPs in controlling muscle mass. Unlike Myostatin signaling, BMP acts through Smad1/5/8, eliciting a hypertrophic response in muscle and involving the Akt pathway, the inhibition of which by Rapamycin attenuates at least partially the BMP-mediated response (Sartori et al., 2013; Winbanks et al., 2013). Inhibition of BMP signaling causes muscle atrophy, abolishes the hypertrophic phenotype of *Myostatin-null* mice, and exacerbates the effects of denervation and fasting (Sartori et al., 2013; Winbanks et al., 2013). The atrophic response observed after fiber-specific *Smad4* conditional ablation suggests that BMPs regulate muscle mass by directly acting on muscle fibers (Sartori et al., 2013). Indeed, the degradation of muscle fiber proteins stimulated by the ubiquitin ligase MUSA1 has been implicated in this process (Sartori et al., 2013; Winbanks et al., 2013). Importantly, the inhibition of BMP signaling not only counteracts the increase in muscle mass in *Myostatin-null* mice, but also blunts the hypertrophic response induced by Follistatin (Winbanks et al., 2013). Follistatin is a powerful regulator of muscle mass, which exerts its function mainly by inhibiting the action of Myostatin and Activins, both of which are implicated as negative regulators of muscle growth (Link and Nishi, 1997; Souza et al., 2008; Gilson et al., 2009; Lee et al., 2010). Strikingly, the presence of SCs seems to be required for a full Follistatin-dependent hypertrophy, as muscle irradiation which abolishes the proliferative capacity of SCs blunted the effects of Follistatin overexpression on muscle mass

(Gilson et al., 2009). A caveat in this set of experiments is that the use of electroporation to introduce Follistatin-expressing plasmids into muscles is accompanied by a regenerative response that almost certainly involves SC participation (Skuk et al., 2013). Nevertheless, since Follistatin-induced hypertrophy is mitigated by both the inhibition of BMP signaling as well as by blocking cell proliferation, it is conceivable that BMP-mediated hypertrophy is dependent on SC activity to a significant extent. Indeed, BMP signaling strongly inhibits the myogenic differentiation program in myogenic precursor cells, and is able to induce features typical of the osteoblast lineage in myogenic cell lines under specific *in vitro* conditions (Murray et al., 1993; Katagiri et al., 1994). While there is a lack of evidence of the ability of SCs to commit to the osteogenic program *in vivo*, several observations indicate that BMP signaling plays an important role during muscle regeneration by controlling myogenic progenitor differentiation and positively modulating their proliferative expansion (Lounev et al., 2009; Clever et al., 2010; Friedrichs et al., 2011; Ono et al., 2011). Interfering with normal BMP signaling *in vivo* leads to smaller regenerated fibers after muscle injury and to smaller muscles during fetal development (Wang et al., 2010; Ono et al., 2011). Together, these observations suggest that BMP signaling occupies a central position in a complex network of signals that control SC biology and muscle mass, besides being implicated in the etiology of atrophy. These observations also support the idea of an active cross-talk between different branches of the TGF β signaling pathway in the control of muscle mass.

An involvement of SCs in the control of muscle mass is particularly relevant in diseases that continually demand a damage-repair response from the tissue, such as muscular dystrophies. Muscular dystrophies form a group of heterogeneous genetic diseases, often characterized by progressive muscle atrophy (Shin et al., 2013). Little is known about the molecular changes occurring in SCs within the dystrophic environment that prevents them from restoring the growth of the atrophied muscles. Several members of the TGF β superfamily, including Myostatin, TGF β 1, and TGF β 2 have been implicated in the progression of different forms of muscular dystrophy (Yamazaki et al., 1994; Bogdanovich et al., 2002; Wagner et al., 2002; Andreetta et al., 2006; Onofre-Oliveira et al., 2012; Biressi et al., 2014). Enhanced TGF β signaling appears to be responsible for the aberrant myogenic program observed in the muscles of *mdx* mice (Biressi et al., 2014). TGF β signaling appears to alter SC function also in other diseases, such as Emery-Dreyfuss muscular dystrophy (AD-EDMD), in which mutations in the *Lamin A/C* genes cause muscle atrophy and necrosis (Sewry et al., 2001). An analysis of SC activity in the *Lmna*^{-/-} mice revealed that the proliferation rates and kinetics of activation of the SC progeny derived from *Lmna*^{-/-} muscles were slower and delayed compared to wild type muscles (Cohen et al., 2013). More importantly, *Lmna*^{-/-} myoblasts displayed elevated levels of Smad2/3 that did not localize to the nucleus in response to TGF β stimulation. As a result, there was an increase in cell death in myotube cultures, an event that could contribute to wasting (Cohen et al., 2013). Additionally, defective SC behavior, fiber atrophy, and enhanced TGF β signaling have also been observed in mouse models of *Caveolin*

3-deficient limb-girdle muscular dystrophy (Ohsawa et al., 2012). Importantly, administration of an inhibitor of TGF β type I receptor not only ameliorates muscle atrophy, but also restores the number and differentiation potential of SCs, indicating that both TGF β -dependent reduction in SCs and impaired myoblast differentiation contribute to the cellular mechanism underlying *Caveolin* 3-deficient muscle atrophy (Ohsawa et al., 2012).

A key modulator of TGF β signaling in muscle and an effector molecule of the renin-angiotensin system, Angiotensin II (Ang II), has been implicated in muscle degeneration and accumulation of fibrotic tissue in several congenital and acquired muscle disorders (Cohn et al., 2007). In keeping with this, down-regulation of TGF β signaling by Ang II inhibitors ameliorates muscle wasting in different myopathies (MacDonald and Cohn, 2012). Ang II is also involved in the etiology of cachexia, such that patients with CKD and CHF often have elevated Ang II levels, and treatment with an angiotensin converting enzyme inhibitor can reduce weight loss (Anker et al., 2003). Consistent with this notion, earlier studies showed that the infusion of Ang II in rodents decreased muscle weights by increasing protein degradation, disrupting IGF-1 signaling and inducing apoptosis (Brink et al., 1996). In SCs, Ang II was demonstrated to impair SC proliferation and differentiation by signaling events mediated by one of its receptors, Angiotensin 1 (AT1R), during the course of muscle regeneration (Yoshida et al., 2013). Indeed, by inhibiting AT1R activity in CHF, a condition in which high Ang II levels are present, the observed reduction in SC numbers and muscle weight could be blocked (Yoshida et al., 2013). Intriguingly, an opposite function was ascribed to Ang II Type 2 receptor (AT2R) that is expressed in differentiating SC progeny and is known to promote regeneration (Yoshida et al., 2014). These observations highlight the importance of defining the mechanisms operating downstream of atrophic signals, in order to develop therapeutic approaches that are specific and effective.

Glucocorticoid and Androgen Signaling

Glucocorticoids, either in their synthetic or physiological form, cortisol (in human) and corticosterone (in rodents), have emerged as potent negative regulators of muscle mass (Braun and Marks, 2015). Elevated levels of circulating glucocorticoids have been linked to different atrophic conditions including sepsis, diabetes, and cancer (Braun and Marks, 2015). Glucocorticoids act by binding to glucocorticoid receptors, a family of nuclear receptors, and exerting their function using different mechanisms, including stimulating the expression of muscle-specific E3 ubiquitin ligases in muscle fibers and modulating the function of Akt, Myostatin, and multiple other signaling pathways involved in the control of muscle mass (Braun and Marks, 2015). By stimulating Myostatin expression, glucocorticoids can also decrease SC proliferation and differentiation (Dong et al., 2013).

Importantly, the effects of glucocorticoid administration on muscle mass are counteracted by androgens in both patients and animal models (Creutzberg et al., 2003; Eason et al., 2003). Androgens are efficient positive modulators of muscle mass and function by binding to the androgen receptor, also a member of the nuclear receptor superfamily (Dubois et al.,

2012). The androgen receptor is expressed not only in muscle fibers, but also in SCs, in particular in the *levator ani* muscle, a muscle that is dramatically affected in animal models with a conditional ablation of androgen receptor expression in SCs and fibers (Swift-Gallant and Monks, 2013; Dubois et al., 2014). Several reports suggest that androgens are able to modulate gene expression and function in SCs and myoblast cell lines (Chen et al., 2005). Testosterone, the principal circulating androgen is decreased in the serum of older men, possibly contributing to the SC dysfunction observed in this condition (Yialamas and Hayes, 2003). Intriguingly, the activation of SCs observed in denervated *levator ani* muscle does not occur in castrated rats, and this correlates with a reduction in myonuclear number accompanying the atrophy observed in denervated muscles of castrated rats (Nnodim, 1999). These observations suggest that at least in specific muscles and under specific atrophic conditions, the control of SC activity by androgens may contribute to fiber homeostasis.

NF- κ B Signaling

A large body of evidence has implicated the NF- κ B transcription factors as being major modulators of muscle mass (Li et al., 2008; Peterson et al., 2011). When inactive, NF- κ B is maintained in the cytoplasm by a class of proteins called I κ B. In response to inflammatory cytokines, such as TNF α , I κ B is degraded leading to the nuclear translocation of NF- κ B and activation of NF- κ B-dependent transcription (Li et al., 2008; Peterson et al., 2011). An increase in NF- κ B signaling has been reported in the atrophic response associated with different disease conditions. NF- κ B signaling has been shown to mediate atrophy by increasing the expression of components of the ubiquitin-proteasome system, and by promoting the atrophic effects of inflammation-related proteins (Li et al., 2008; Peterson et al., 2011). More importantly, an increasing number of observations suggest that NF- κ B also regulates SC activity in different atrophic conditions. Specifically, the presence of systemic inflammation accompanying clinical features associated with cachexia led to the discovery of a cytokine-induced up-regulation of NF- κ B signaling in the suppression of myogenic gene expression in cell culture (Guttridge et al., 2000). Moreover, a detailed examination of events within muscles from tumor-bearing mice revealed that the induction of NF- κ B activity by cachectic serum contributed to Pax7 dysregulation in muscle-resident progenitors, and was followed by a significant decline in muscle mass (He et al., 2013). Although these observations do not distinguish between increases in NF- κ B levels in SCs vs. fibers, there is a clear indication that together with functioning as a potent modulator of muscle regeneration and myogenic differentiation, NF- κ B signaling promotes atrophy, an effect which is achieved in part by altering the behavior of SCs (Langen et al., 2001; Dogra et al., 2006; Mourkioti et al., 2006; Wang et al., 2007; Bakkar et al., 2008).

Sirt1 and Mitochondrial Dysfunction

Mitochondrial function and metabolism is crucial for SC activation, proliferation, and for efficient muscle regeneration

(Jash and Adhya, 2012; Rodgers et al., 2014). A reduction in mitochondrial mass, increased damage to mitochondrial DNA, and increased levels of reactive oxygen species (ROS) produced by the existing mitochondria were observed with age (Minet and Gaster, 2012; Wang et al., 2013). Sirt1 is a potent regulator of mitochondrial metabolism, displaying altered expression in tumor-bearing animals and contributing to reduced regeneration in muscle wasting (Toledo et al., 2011). Intriguingly, an increase in Sirt1function is one of the many outcomes of caloric restriction, that also include an increase in SC proliferation, an increase in mitochondrial abundance and an enhancement of the regenerative capacities of muscles (Lee et al., 1998; Cohen et al., 2004; Cerletti et al., 2012; McKiernan et al., 2012). These observations support a possible role for Sirt1 in reducing muscle loss that occurs with aging. Indeed, Sirt1 overexpression has been reported to block fasting and denervation-induced fiber atrophy by reducing FoxO activity (Lee and Goldberg, 2013). Moreover, Sirt1 controls the transcription of peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC1 α), which in turn induces mitochondrial biogenesis and regulates peroxisome proliferator-activated receptors gamma (PPAR δ), a positive regulator of SC proliferation (Amat et al., 2009; Angione et al., 2011). Consistent with this observation, over-expression of Sirt1 increases the proliferation of myogenic progenitors (Rathbone et al., 2009). Moreover, as a consequence of ablating Sirt1 in SCs, myogenic progenitors undergo premature differentiation, thereby negatively affecting muscle regeneration and growth (Ryall et al., 2015). Although additional mechanistic studies are required, this body of evidence suggests that the Sirt1-PGC1 α axis could ameliorate the loss of muscle mass at least in part by improving mitochondrial function and the regenerative potential of SCs.

Notch Signaling

The importance of Notch signaling during muscle development and regeneration has been well established (Luo et al., 2005; Vasyutina et al., 2007a; Mourikis and Tajbakhsh, 2014). Notch signaling plays an important role in maintaining quiescence, proliferation, homing and self-renewal capabilities of SCs (Conboy and Rando, 2002; Bjornson et al., 2012; Bröhl et al., 2012; Mourikis et al., 2012b; Wen et al., 2012; Gopinath et al., 2014). Notch signaling is essential to maintain muscle progenitors during fetal development, to promote their expansion, and to generate SCs (Vasyutina et al., 2007b; Mourikis et al., 2012a). Indeed, developmental inactivation of the Notch transcriptional complex by the selective ablation of the Notch regulators, recombination signal binding protein J (RBPF-J) or mastermind-like (MAML1), specifically in the myogenic compartment resulted in the reduction of muscle mass (Vasyutina et al., 2007b; Lin et al., 2013).

Aging muscles consist of SCs with decreased regenerative potential, which in turn is an outcome of reduced Notch signaling, and can be restored by exposure to a young systemic environment (Conboy et al., 2003, 2005). Intriguingly, it was also reported that the consequences of Vitamin D depletion in aged rats exacerbated the muscle loss associated with aging

and was accompanied by reduced Notch activity in these muscles (Domingues-Faria et al., 2014). At this point it remains to be established whether Vitamin D exerts its effects on skeletal muscle through the inhibition of the Notch signaling pathway. Additionally, it remains to be demonstrated whether enhancing Notch function in the Vitamin D-depleted aged rats display possible beneficial effects on muscle mass, in addition to the involvement of SCs in this context. Intriguingly, the observation that Notch activation was able to abrogate the inhibitory effects of the cachectic factor Ang II on proliferation of myogenic progenitors in culture raises the possibility of using positive modulators of Notch signaling to boost SC proliferation and counteract muscle loss (Yoshida et al., 2013).

However, recent literature suggests that the outcome of manipulating Notch activity has been met with incongruous results. Using a Notch reporter mouse, it was demonstrated that SCs isolated from *mdx* mice displayed reduced activation of Notch signaling, and that constitutive Notch activation could at least in part rescue the self-renewal deficit observed in *mdx* SCs, without ameliorating muscle pathology associated with these dystrophic mice (Jiang et al., 2014). On the other hand, a recent study observed that acute manipulation of Notch signaling by the injection of activators or inhibitors did not affect muscle mass or maximal force in *mdx* mice, as well as mice that were double-deficient for *Utrophin* and *Dystrophin* (Church et al., 2014). These observations suggest that while SCs display a stage-specific requirement for Notch signaling, dystrophic muscles present a complex environment with SCs at different stages of progression and consequently exhibit different responses to Notch manipulation that may not contribute to a net increase in muscle weight. These studies underscore the importance of not only investigating specific signaling pathways in the context of specific forms of atrophy, but also addressing the efficacy of manipulating a particular signaling mechanism for therapeutic purposes.

Reversal of Atrophy: A Role for SCs?

The primary objective of obtaining a comprehensive understanding of the mechanistic processes underlying atrophy in a manner that discerns molecular changes in SCs from those in fibers, is to address whether manipulating these signaling mechanisms can enhance the participation of SCs in restoring muscle mass. In this section, we review those studies in which SC activity has been modulated to expand their functionality in muscle wasting accompanying chronic diseases. Broadly, the focus of these interventions has been signaling pathways that modulate SC proliferation, differentiation, senescence and survival.

In muscle wasting accompanying COPD, patients experience decreased oxygen saturation level (hypoxemia) that can elicit hypoxic responses in tissues (Wüst and Degens, 2007). This includes an impairment in anabolic pathways, decrease in food intake by the induction of leptin, and muscle disuse that are directly responsible for a loss in muscle mass (Wüst and Degens,

2007). In studies aimed to increase the protein synthesis pathway in SCs under conditions of hypoxia-induced atrophy, a regimen of alternating treatments with hepatocyte growth factor (HGF) and leukemia inhibitory factor (LIF) not only increased SC proliferation, but also increased the cross sectional area of fibers and total muscle weight (Hauerslev et al., 2014). Corroborating these results in normoxic mice, this growth factor treatment promoted SC proliferation and increased the weight of the *tibialis anterior* muscle in mice deleted for Myostatin expression (Hauerslev et al., 2014). These results further highlight the potential of exploring Myostatin regulation in SCs for therapeutic purposes in combatting atrophy.

Strategies aimed at enhancing SC proliferation to increase their participation in muscle mass restoration have been particularly useful in limb-girdle muscular dystrophy type 1B (LGMD1B) and AD-EDMD forms of dystrophies. Under these conditions, increased levels of lamina-associated polypeptide alpha (*Lap2α*), a protein that interacts with Lamin A/C and phosphorylated Rb has been implicated in reducing SC proliferation (Mancini et al., 1994; Ozaki et al., 1994). Since the *Lap2α*^{-/-} cells are hyperproliferative due to a defect in cell cycle exit, the authors created a *Lmna*^{-/-} *Lap2α*^{-/-} double KO mice to enhance SC proliferation, thereby resulting in an increase in the fusion index and overall muscle size of the double KOs (Cohen et al., 2013). This study not only offers valuable insights into the etiology of laminopathies but also provides alternate strategies using SCs for therapeutic intervention.

Contrary to the atrophic conditions discussed above, the increase in SC numbers in the interstitium in cancer-induced cachexia prompted the use of mutant mouse models to inactivate Pax7 expression in tumor-bearing mice (He et al., 2013). Not only did this cause a reversal of wasting by promoting cell differentiation and fusion with injured fibers, but also demonstrated that impaired myogenic progression by sustained Pax7 expression was the primary cause for muscle wasting in these mice, and offered the attractive possibility of gene therapy approaches to modulate Pax7 expression (He et al., 2013). In particular, the identification of regulators that repress Pax7 expression to limit its window of action and allow for myogenic progression during the course of normal regeneration can be potentially exploited for therapeutic purposes. Indeed, the activation of the Polycomb Repressive Complex 2 (PRC2) by p38 α kinase results in the formation of repressive chromatin on the *Pax7* locus, thereby providing an additional interventional target that could be explored in a tumor-promoting milieu (Palacios et al., 2010).

In disuse atrophy, the focus of various interventional studies have been on regulators that enhance SC functionality and increase muscle growth, especially in the subsequent recovery phase. In a recent study, E3 ubiquitin ligase tripartite motif-containing 32 (TRIM32) was demonstrated to be essential for the selective regrowth of Type 2 fast fibers after hind limb suspension-induced atrophy (Kudryashova et al., 2012). TRIM32-deficient myoblasts displayed impaired differentiation, and elevated levels of senescence-associated β-galactosidase (β-gal; Kudryashova et al., 2012). Premature senescence of SCs was also demonstrated to be the underlying cause for the pathogenic

features associated with limb-girdle muscular dystrophy 2H (LGMD2H), that arise from mutations in *TRIM32* (Saccone et al., 2008; Cossée et al., 2009). These studies suggested that unlike other E3 ubiquitin ligases that promote atrophy, *TRIM32* might possess a unique function of preventing premature senescence in SCs, thereby enhancing muscle growth. Although these speculations support the idea of an involvement of SCs, more investigations are required to explore the mechanisms by which *TRIM32* could promote regrowth after atrophy. Indeed, the extent to which SCs are required in the process of recovery from hind limb suspension is still unclear. Ablation studies indicate that muscle mass recovery after hind limb suspension could also occur in the absence of SCs (Jackson et al., 2012). Nevertheless, during the recovery phase of soleus muscle mass upon its reloading, the decrease in myonuclear content derived from hind limb unloading was restored to control levels, suggesting that myogenic precursor cells can proliferate and fuse with myofibers during the recovery process (Mitchell and Pavlath, 2001). Moreover, after an initial phase of muscle regrowth, inhibiting the proliferation of muscle precursor cells by irradiation prevented a full recovery (Mitchell and Pavlath, 2001). As such, it would be crucial to quantify the relative contributions of SC-mediated and myofiber-mediated processes, in addition to investigating molecular events that distinguish between SC-intrinsic and fiber-intrinsic processes during muscle recovery.

IGF-1 is another factor that has been shown to modulate SC activity and has been reported to be beneficial in promoting regrowth after muscle unloading. In a study aimed to investigate the effects of IGF-1 overexpression on the recovery of muscle size during ambulation after cast immobilization, it was observed that viral-mediated IGF-1 transfer to skeletal muscle enhanced regeneration (Stevens-Lapsley et al., 2010). Intriguingly, IGF-1 overexpression did not protect against cast immobilization-induced muscle atrophy, indicating that there are different mechanisms regulating muscle mass during unloading and reloading (Stevens-Lapsley et al., 2010). Delivery of IGF-1 into muscle and muscle-specific overexpression of IGF-1 were also beneficial in ameliorating sarcopenia and stimulating recovery in immobilized old muscles (Barton-Davis et al., 1998; Chakravarthy et al., 2000; Musarò et al., 2001). Moreover, increased levels of IGF-1 in muscle has been shown to control SC activity and reduce muscle wasting in different genetic disorders, including muscular dystrophy and amyotrophic lateral sclerosis (ALS; Barberi et al., 2009).

A crucial aspect underlying the enhancement of SC functionality for cellular therapy is the investigation of mechanisms that specifically promote cell survival without affecting proliferation, in order to avoid the risk of cancer-promoting effects. A growth factor-derived engineered protein, Magic-Factor 1 (or Met-Activating Genetically Improved Chimeric Factor 1), has been developed to elicit the activation of the Akt survival pathway, but not the mitogenic ERK pathway. Magic-Factor 1 decreased the expression of Myostatin and apoptotic markers in myogenic cells *in vitro*. Consistent with these observations, Magic-Factor 1 promoted survival and differentiation (Cassano et al., 2008). Moreover, transgenic mice expressing muscle-specific Magic-factor 1 displayed

hypertrophic fast twitch fibers with increased endurance to exercise, in addition to partially rescuing the degeneration observed in α -sarcoglycan KO mice (Cassano et al., 2008). Thus, tissue-specific engineered proteins hold potent clinical applications for ameliorating muscle wasting associated with dystrophies.

In addition to modulating specific signaling pathways, exercise and electrical stimulation appear to be promising therapeutic approaches in countering atrophy. Exercise has been proven to be effective in reducing muscle loss and in mobilizing SCs in aging muscles (Snijders et al., 2009). Concomitant to the increase in SC content, an up-regulation of myogenic regulatory factors and a reduction in Myostatin expression have been observed (Snijders et al., 2009). The beneficial effects of exercise have also been reported in disuse atrophy occurring with immobilization, and correlate with an increase in IGF-1 and Myogenin, and a decrease in Myostatin levels (Adams et al., 2007). Recent reports have demonstrated that the decrease in muscle size and SC number occurring during hind limb unloading could also be attenuated by electrical stimulation (Zhang et al., 2010b; Guo et al., 2012; Dirks et al., 2014). Although a causal relationship between SC activation and an attenuation in atrophy remain to be fully established, it seems that electrical stimulation can modify SC activity and prove beneficial to other forms of atrophy such as sarcopenia (Kern et al., 2014).

Another promising approach to ameliorate loss of muscle mass is cell therapy. Transplantation of SCs and other cellular types capable of myogenic differentiation have been shown to improve muscle phenotypes associated with different primary genetic diseases, especially in muscular dystrophies. In most of these studies, the rational of the cell therapy approach involves using cells to carry therapeutic genes into myofibers (Partridge and Davies, 1995). After transplantation, healthy donor-derived or patient-derived corrected cells fuse with existing myofibers and correct for the absence of expression from the mutated gene. The efficacy of this approach lies in the ability of the transplanted cells to not only differentiate into myofibers, but also to replenish the reservoir of stem cells and thereby sustain the repair process. Several studies have been exploring the potential of other stem cells besides SCs for therapeutic purposes (Peault et al., 2007). A complete and exhaustive discussion of these studies is beyond the scope of the present report and the readers are directed to comprehensive reviews that extensively describe cell transplantation approaches in primary genetic myopathies (Skuk and Tremblay, 2003; Price et al., 2007; Quattrocelli et al., 2010; Tedesco et al., 2010; Meng et al., 2011; Meregalli et al., 2013; Sirabella et al., 2013). We focus in this paragraph on acquired muscle wasting conditions. Recent observations suggest that a cell transplantation approach could be useful in reducing muscle loss after hind limb suspension and denervation (Plowman et al., 2014; Kim et al., 2015). Importantly, it has been reported that transplantation of SCs attached to their myofiber coupled with muscle injury could prevent the loss of muscle mass associated with aging (Hall et al., 2010). Intriguingly, transplantation in the absence of injury did not result in a similar increase in muscle mass (Hall et al., 2010). The interpretation of this observation is confounded by

the high rate of lethality of donor cells generally associated with transplantation (Beauchamp et al., 1999). Nevertheless it supports the hypothesis that the authors of this study propose that unknown factors produced during injury can signal to fiber-associated SCs, and promote their long-term engraftment and hypertrophic function (Hall et al., 2010). In these set of experiments, fiber-associated SCs were transplanted into muscles of young mice and an increase in muscle mass was observed in 2-year-old mice (Hall et al., 2010). However, it remains to be tested whether heterologous transplantation of young fiber-associated SCs into injured muscles of old mice would also be beneficial in ameliorating sarcopenia. A large body of evidence indicates that during aging, local and systemic environments undergo profound changes that bear a negative impact on muscle precursor cell activity and regenerative potential (Gopinath and Rando, 2008), thus proving to be a major hurdle in transplantation approaches in aged muscles. Intriguingly, a recent study demonstrated that inhibiting p38 α and p38 β signaling transiently in myogenic progenitors isolated from aged mice, in conjunction with culture on soft hydrogel substrates rejuvenates their regenerative potential and increases muscle strength upon transplantation into damaged muscles of aged mice (Cosgrove et al., 2014). Although it is not clearly understood as to how these mechanisms enhance the regenerative capacity of old SCs, these observations suggest that the detrimental effects of an aging muscle microenvironment can be circumvented. Changes in the local and systemic environments are not a feature of aging alone, but are also profoundly altered in other chronic muscle wasting conditions, such as cancer cachexia (He et al., 2013). Future studies will be required to evaluate the efficacy of cell transplantation approaches in ameliorating muscle loss under various atrophic stimuli.

Concluding Remarks

In conclusion, it is becoming increasingly evident that understanding the basic molecular mechanisms underlying various forms of atrophy is crucial to develop defined parameters and describe distinct features that categorize different forms of atrophy. It is also possible that different forms of atrophy could co-exist, such as disuse atrophy that accompanies aging coupled to bedridden conditions, as a result of chronic debilitating illnesses. Moreover, aging is a risk factor for many pathologies impacting muscle mass (Evans, 1995). Such multiple cause-related atrophies warrant the use of combinatorial therapies that selectively target the signaling pathway or pathways involved in specific atrophic processes. It has also become significantly

clear that SC-focused studies expand the approaches that can be used to counter muscle loss. In general, despite a large body of evidence in favor of a role for SCs at least in some forms of atrophy, there have been reports that have restricted and thereby defined the window of SC action, or have evaluated SC functionality quantitatively rather than qualitatively. As such, these studies may have contributed to the “quasi-status” of SC involvement in atrophy. This, in addition to a knowledge of the molecular and cellular changes in SCs accompanying atrophy offer a powerful tool in being able to manipulate this compartment pharmacologically to increase muscle mass. In this context, SC-specific gene ablation studies in mice would provide a promising avenue to uncover novel signaling networks and thereby adopt a more focused approach towards tackling muscle wasting diseases in humans. These studies are currently being limited by difficulties in achieving a complete ablation of genes in a SC-specific manner, and the development of highly efficient strategies will be key in obtaining an accurate assessment of the requirement of the candidate genes during atrophy. A corollary to this approach is a comprehensive understanding of the systemic factors and the molecular milieu constituting the SC niche in the context of various atrophic conditions. In providing the means to modulate and eventually enhance SC function, this area of study renders itself to alternate therapeutic strategies such as nutritional interventions, other than exercise or pharmacological-based solutions (Alway et al., 2014). This is especially relevant in aging individuals with unrelated muscle wasting pathologies, wherein there are considerable limitations during the course of treatment. This shift in focus of examining molecular events during atrophy from fiber to SCs can potentially further be exploited even in scenarios that have reported limited involvement of the SC compartment in restoring muscle mass. A critical discussion of the cellular and molecular events operating in the atrophying muscles and SCs will contribute to the field for further studies investigating novel approaches to ameliorate muscle wasting diseases.

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Muscle wasting in myotonic dystrophies: a model of premature aging

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Myotonic dystrophy type 1 (DM1 or Steinert's disease) and type 2 (DM2) are multisystem disorders of genetic origin. Progressive muscular weakness, atrophy and myotonia are the most prominent neuromuscular features of these diseases, while other clinical manifestations such as cardiomyopathy, insulin resistance and cataracts are also common. From a clinical perspective, most DM symptoms are interpreted as a result of an accelerated aging (cataracts, muscular weakness and atrophy, cognitive decline, metabolic dysfunction, etc.), including an increased risk of developing tumors. From this point of view, DM1 could be described as a progeroid syndrome since a notable age-dependent dysfunction of all systems occurs. The underlying molecular disorder in DM1 consists of the existence of a pathological (CTG) triplet expansion in the 3' untranslated region (UTR) of the *Dystrophin Myotonia Protein Kinase (DMPK)* gene, whereas (CCTG)n repeats in the first intron of the *Cellular Nucleic acid Binding Protein/Zinc Finger Protein 9 (CNBP/ZNF9)* gene cause DM2. The expansions are transcribed into (CUG)n and (CCUG)n-containing RNA, respectively, which form secondary structures and sequester RNA-binding proteins, such as the splicing factor muscleblind-like protein (MBNL), forming nuclear aggregates known as foci. Other splicing factors, such as CUGBP, are also disrupted, leading to a spliceopathy of a large number of downstream genes linked to the clinical features of these diseases. Skeletal muscle regeneration relies on muscle progenitor cells, known as satellite cells, which are activated after muscle damage, and which proliferate and differentiate to muscle cells, thus regenerating the damaged tissue. Satellite cell dysfunction seems to be a common feature of both age-dependent muscle degeneration (sarcopenia) and muscle wasting in DM and other muscle degenerative diseases. This review aims to describe the cellular, molecular and macrostructural processes involved in the muscular degeneration seen in DM patients, highlighting the similarities found with muscle aging.

Keywords: myotonic dystrophy, aging, muscle wasting, satellite cells, sarcopenia

Introduction

Myotonic dystrophy type 1 (DM1), also known as Steinert's disease (OMIM: 160900), is a dominantly inherited multisystem disease. DM1 is the most common form of adult-onset muscular dystrophy; it affects one out of 8000 people worldwide, with an even greater prevalence in some specific areas such as Quebec (Canada) and the Basque Country (Spain) (López de Munain et al., 1993; Mathieu and Prévost, 2012). The DM1 phenotype shows an extremely wide variability among affected patients, with some being asymptomatic while others have severe congenital forms. Patients are classified into four categories regarding the age of onset of symptoms: late-onset, adult-onset, childhood-onset and congenital forms (Harper, 2001; Arsenault et al., 2006).

The disease is caused by an unstable expansion of a trinucleotide (CTG) repeat motif located in the 3' untranslated region (UTR) of the *Dystrophin Myotonia Protein Kinase (DMPK)* gene (Brook et al., 1992; **Figure 1**). Unaffected individuals carry less than 50 triplet repeats, whereas expansions ranging between 50 and 4000 CTG repeats have been found in affected individuals. Importantly, the length of CTG expansion is associated with the age of onset of the disease and its severity (Martorell et al., 1998).

DM1 patients experience a progressive dysfunction of multiple organs and tissues, including skeletal, cardiac and smooth muscles, the endocrine system, eyes, gonads, the central nervous system (CNS), and an increased risk of developing neoplasias (López de Munain et al., 1993; Harper et al., 2002; Gadalla et al., 2013). Thus, DM1 somewhat resembles a progeroid syndrome, defined as an accelerated aging and dysfunction of several systems.

A second form of myotonic dystrophy exists, DM type 2 (OMIM: 602668), initially named proximal myotonic myopathy due to the greater weakness of proximal as compared to distal muscles (Ricker et al., 1994). DM2 patients also develop a multisystem dysfunction but they generally experience a milder phenotype as compared to DM1 (**Table 1**). Consistent with this, congenital and childhood-onset forms of DM2 are absent, and the disease phenotype ranges from early adult-onset severe forms to very late-onset mild forms (Day et al., 2003). The prevalence of this disorder has yet to be clearly defined but it is estimated to be similar to DM1 in Northern European Countries (Udd and Krahe, 2012). However, very late-onset

TABLE 1 | Summary of main clinical features that differ between both DM forms.

Features	DM1	DM2
Age of onset	At any age	Adulthood
Congenital forms	Yes	No
Gene expansion	DMPK, (CTG)n	CNBP, (CCTG)n
Predominantly affected muscles	Distal	Proximal
Predominantly affected fibers	Type 1	Type 2

forms of this disease might often go undiagnosed due to its mild phenotype, which can be concealed by other age-related dysfunctions. Unlike what occurs in DM1, aging-like symptoms in DM2 might not be so evident. Regarding the muscle-specific phenotype, it is unknown why distal muscles are predominantly affected in DM1 patients whereas DM2 patients show a more proximal affection.

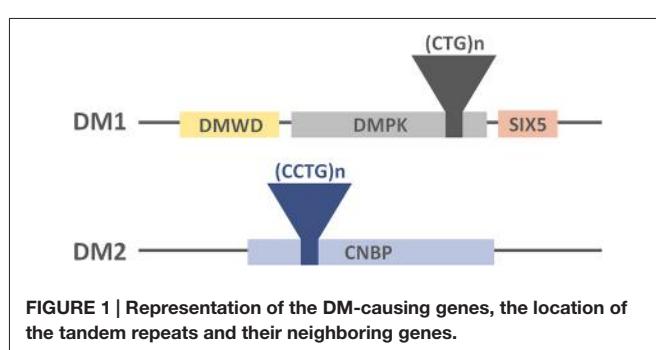
As is the case with the genetic origin of DM1, DM2 is caused by a tetranucleotide (CCTG) expansion in intron 1 of the *Nucleic Acid-binding Protein (CNBP or ZNF9)* gene (Liquori et al., 2001; **Figure 1**). Healthy individuals carry less than 30 tetranucleotide repeats, whereas repetition lengths between 55 and 11000 have been found in affected patients (Liquori et al., 2001).

Skeletal muscle, one of the most severely affected tissues in these diseases, may age prematurely in DM patients, mimicking sarcopenia, known as the age-related loss of muscle mass and function (Evans and Campbell, 1993; Fielding et al., 2011). This syndrome can refer to loss of muscle mass alone or in conjunction with fatty substitution (Fielding et al., 2011). Although the causes of sarcopenia have not as yet been accurately determined, several age-related factors, such as muscle disuse, nutritional deficiencies, hormonal changes and insulin resistance could notably contribute to its onset.

Multisystem Dysfunction in DM

The previously described genomic tandem repeats lead to the progressive degeneration of several tissues and organs, which is more prominent in DM1 and milder in DM2 patients (**Figure 2**).

DM patients may suffer a broad variety of symptoms affecting the three muscle types: cardiac, skeletal and smooth muscles. Cardiac failure is common in DM1 patients, often manifested as arrhythmias and conduction defects (Antonini et al., 2000; Mammarella et al., 2000; Pelargonio et al., 2002; Groh et al., 2008; Cudia et al., 2009; Petri et al., 2012). Although congestive heart failure is a rare complication, subclinical systolic dysfunction as shown by echocardiographic or magnetic resonance imaging is frequent (De Ambroggi et al., 1995; Tokgozoglu et al., 1995; Bhakta et al., 2004; Hermans et al., 2011; Petri et al., 2012). The frequency of heart failure correlates with age, male gender, length of the tandem repeat sequence and the degree of neuromuscular disability (Tokgozoglu et al., 1995; Antonini et al., 2000; Groh et al., 2008; Cudia et al., 2009; Kaminsky and Pruna, 2012). Symptoms involving the smooth muscle, such as dysphagia, constipation, intestinal



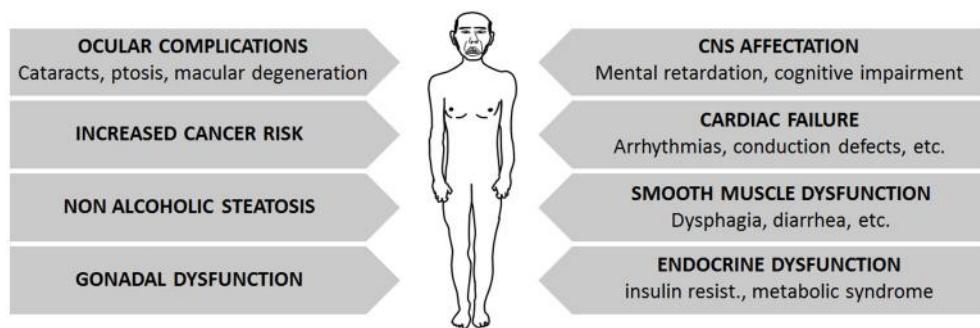


FIGURE 2 | Summary of main symptoms affecting DM patients, which constitute the multisystem affection found on them.

pseudo-obstruction and diarrhea, are also relatively frequent in DM1 patients (Bujanda et al., 1997; Bellini et al., 2006; Ercolin et al., 2013). As for skeletal muscle involvement, both DM forms share common muscle histopathologic features, with a markedly increased variation of fiber diameter and prominent central nucleation, which is a feature of constantly regenerating muscles with immature fibers. Other signs, such as the presence of basophilic regenerating fibers, branched fibers, and adipose and fibrotic tissue can also be found to varying degrees, depending on the extent of muscle degeneration and the severity of the disease. Although DM1 affects mainly distal muscles and DM2 affects proximal muscles, only a few DM type-specific features have been described. Among these, ring fibers (peripheral myofibers that surround other fibers) and sarcoplasmic masses are more frequently seen in DM1 muscles. Nuclear clumps (condensed chromatin structures in the myonuclei, indicating cell death) are found in DM2 patients even when muscular weakness is not clinically evident, whereas they arise later in DM1 patients, mainly in end-stage muscles (Vihola et al., 2010). Moreover, it must be noted that DM1 muscles show a prominent loss of type 1 fibers, whereas type 2 fibers are predominantly affected in DM2 patients (Vihola et al., 2003; Schoser et al., 2004; Bassez et al., 2008; Pisani et al., 2008). Interestingly, the histology of DM muscles resembles that of aged muscles, with fiber size variability, centrally located nuclei with chromatin clumps and fiber atrophy. Muscle regeneration also seems to be decreased in both conditions, probably due to satellite cell dysfunction, which may fail to activate and/or differentiate to muscle upon myogenic stimuli (Huichalaf et al., 2010; Malatesta, 2012; Malatesta et al., 2014).

As part of the multisystem involvement, many DM1 patients show insulin resistance due to the aberrant splicing of the insulin receptor (IR) mRNA, which is highly expressed in skeletal muscle. Consequently, patients show a reduced responsiveness to insulin as compared to healthy individuals (Morrone et al., 1997; Savkur et al., 2001).

The CNS is also negatively affected in DM1 patients. The large majority of congenital and childhood-onset DM1 patients suffer mental retardation, whereas patients with the adult-onset forms may show varying degrees of cognitive

dysfunction, where a positive correlation is observed between triplet expansion length and patients' age. Cognitive dysfunction is characterized by a dysexecutive syndrome with predominant frontoparietal involvement (Sistiaga et al., 2010). Moreover, DM1 patients go through behavioral-personality changes (e.g., reduced initiative, inactivity, apathetic temperament and paranoid personality traits) and excessive daytime sleepiness. There is tentative data supporting an age-dependent decline of cognitive functioning in DM1 patients (Modoni et al., 2008), possibly associated with the degeneration of the diffuse (predominantly temporo-insular) subcortical white matter, and a reduction of the cerebral blood flow in frontal areas (Romeo et al., 2010).

DM1 patients also present hepatic involvement. Indeed, 66% of patients show abnormal hepatic enzyme levels and non-alcoholic steatosis (Achiron et al., 1998). Ocular complications, including ptosis, weakness of the ocular muscle and cataracts are also common in DM1 patients, and other less frequent features, such as retinal changes or macular degeneration, may also be present in these patients (Kimizuka et al., 1993; Krishnan and Lochhead, 2010).

Finally, DM patients may also suffer fertility dysfunction. Approximately two thirds of affected males have reduced sperm quality as a result of testicular atrophy (Pan et al., 2002). Affected female fertility is less well documented (Verpoest et al., 2008), but the length of triplet expansion does not seem to be correlated with this aspect of the disease. Importantly, the age of the pregnant patient and parity significantly affect the live birth delivery rate (Verpoest et al., 2010).

In order to decipher why some specific tissues are more severely affected than others in these diseases, it must be highlighted that some tissues and cell types possess a higher tendency to extend these tandem repeat sequences. This leads to the existence of cells with different repeat lengths within an organism, known as *somatic mosaicism*. The longest tandem repeats have been found in severely affected tissues; indeed, skeletal muscle cells possess considerably longer repeat sequences as compared to other cell types (Anvret et al., 1993; Thornton et al., 1994). Importantly, cells with longer tandem repeats tend to accumulate more repetitions than cells with shorter repeat sequences (Monckton et al., 1995), thus aggravating

the degenerative state of predominantly affected tissues in these patients, such as the CNS and cardiac and skeletal tissues.

In addition to somatic cells, germline cells are also prone to genomic instability and thus accumulate tandem repeats in DM. This leads to *anticipation*, which refers to the increase in disease severity and decrease in the age of onset in each generation of affected families (López de Munain et al., 1994).

Repeat Expansion Mechanisms in DM

As previously mentioned, somatic mosaicism is an important feature of myotonic dystrophies; tandem repeats increase with aging in an unsynchronized fashion, leading to cells with different repeat lengths in their genomes. Furthermore, somatic instability is prevalent in highly affected tissues, such as skeletal muscle (Morales et al., 2012).

Slippage of DNA polymerase during DNA replication was at first considered the main mechanism through which repeat sequences are expanded in myotonic dystrophies. Therefore, highly proliferative cells would have a higher tendency to expand DNA repeat sequences, which contradicts the fact that the longest tandem repeats are found in severely affected tissues that happen to be post-myototic. In this regard, DNA repair mechanisms, which are also active in non-cycling cells, have been found to notably contribute to this phenomenon (van den Broek et al., 2002; Savouret et al., 2003; Kovtun et al., 2004; Seriola et al., 2011; Gomes-Pereira et al., 2014), which could explain the elongation of tandem repeats in non-proliferating cells in culture (Gomes-Pereira et al., 2014).

DNA tandem repeats in DM acquire secondary conformations, usually forming hairpin structures. DNA repair proteins recognize these structures and may abnormally repair them, varying the repeat length (McMurray, 2008). Knock-out animal models for components of the DNA repair machineries, as well as silencing these genes in patient-derived cultures, reduce or even abrogate the elongation of repeat expansion sequences. For example, it has been established that Msh2, a component of the mismatch repair (MMR) machinery, is required for triplet expansion in DM1-derived pluripotent stem cells (iPS) cells, as its silencing blocks the expansion of the triplet repeat sequence (Du et al., 2013). Knock-down and overexpression experiments of proteins involved in Base Excision Repair (BER) systems in yeast also indicate that these proteins might play a role in the expansion of repeat sequences (Refsland and Livingston, 2005; Subramanian et al., 2005). However, this fact has not been fully demonstrated in mice due to the lethality of knock-out mouse models for these proteins (Xanthoudakis et al., 1996; Tebbs et al., 1999).

The fact that skeletal muscle is composed of postmitotic cells suggests that these DNA repair systems may contribute in a major way to the expansion of repeat sequences in these tissues. Interestingly, a study performed by Vahidi Ferdousi et al. (2014) shows that double-strand breaks produced by irradiation are more rapidly repaired in muscle satellite cells than their progeny, thus indicating that satellite cells possess a more efficient DNA repair system, which usually acts through non-homologous

end joining. Therefore, it could be hypothesized that triplet expansions are predominantly elongated in satellite cells due to the high activity of the DNA-repair system in these cells. This is consistent with the muscle degeneration seen in DM patients, as dysfunction of satellite cells would disrupt muscle regeneration.

Recent studies have shown that, in addition to DNA repair molecules, the 26S proteasome also participates in the expansion of tandem repeat sequences (Debacker et al., 2012; Concannon and Lahue, 2013). Importantly, the proteolytic activity of 26S, rather than the stress-induced activation of the ubiquitin-proteasome system (UPS), is implicated in this elongation process. Indeed, DNA repair mechanisms could interact with the proteasome to induce repeat expansions through Rad23, a protein involved in both nucleotide excision repair (NER) system and the carriage of ubiquitinated proteins to the proteasome (Concannon and Lahue, 2014).

Pathogenic Mechanisms in DM

It is remarkable that severely affected tissues in DM possess very low mitotic rates. Interestingly, *in vitro* experiments have shown that nuclear foci tend to predominantly accumulate in non-cycling cells. Cycling DM2 fibroblasts accumulate nuclear foci during interphase but these are cleared out during mitosis, leading to mitosis-dependent cycles of foci formation and disassembly. On the contrary, mitotically arrested fibroblasts accumulate more foci, which keep enlarging progressively (Giagnacovo et al., 2012). In this regard, muscle biopsies of DM2 patients have confirmed that the size of myonuclear foci increases in an age-dependent manner (Giagnacovo et al., 2012). Therefore, this could help clarify why tissues and cell types with low mitotic rates are preferentially affected in these diseases.

Several mouse models have been created to study the pathogenic mechanisms of DMs. These models can be categorized into 4 subgroups regarding the genetic modifications that have been introduced in order to mimic different molecular aspects of the diseases: (i) introduction of unstable CTG repeat sequences; (ii) overexpression of toxic CTG/CCTG repeats; (iii) inactivation of the genes located in the DM1 locus; and (iv) mice models of MBNL inactivation or CUGBP1/CELF overexpression (Gomes-Pereira et al., 2011).

Based on these studies, three pathogenic mechanisms that link the nucleotide expansion with the clinical manifestation of myotonic dystrophies have been proposed: (a) DMPK and CNBP haploinsufficiency; (b) *Cis* alteration of neighboring genes; and (c) RNA-induced toxicity (Figure 3).

Haploinsufficiency

Haploinsufficiency refers to the deleterious effect of having only one wild type copy of a gene in the phenotype of the organism. DM patients carry only one wild type copy of the *MDPK* or *CNBP* gene, as the other copy harbors the tandem repeats that form nuclear foci, therefore reducing the amount of DMPK or CNBP protein synthesized.

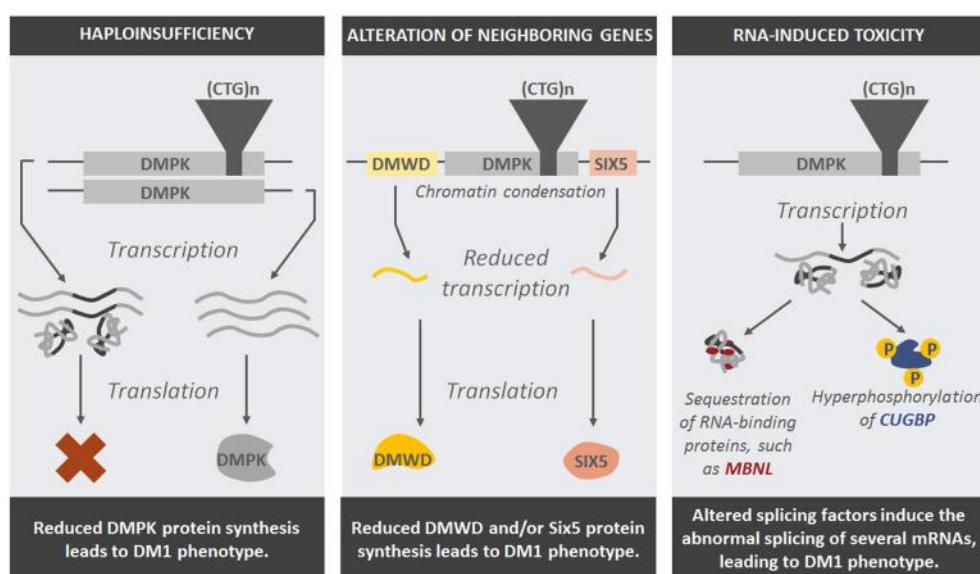


FIGURE 3 | Representation of potential pathogenic mechanisms that explain the effect of DNA expansions in DM1-affected cells and the phenotype seen in patients.

DMPKs is a serine-threonine kinase, whose function in skeletal muscle is not fully understood. It has been shown to localize in the nuclear membrane, where it interacts with lamin-A/C and its deficiency leads to nuclear envelope instability (Harmon et al., 2011). However, it has also been found in the cytoplasm and the cellular membrane during cell division and cardiomyocyte differentiation (Harmon et al., 2008). Depletion of DMPK reduces myogenin expression and prevents proper myoblast differentiation (Harmon et al., 2008). Other studies have also shown that DMPK can phosphorylate the myosin-binding subunit of myosin phosphatase (MYPT1), which inhibits myosin phosphatase (PP1c) activity (Murányi et al., 2001). DMPK also phosphorylates the SERCA2a inhibitor Phospholamban, thus regulating calcium uptake in cardiomyocytes (Kaliman et al., 2005), and Phospholemmann, a regulator of Na⁺, K⁺-ATPase (Mounsey et al., 2000).

As for DM2, CNBP is a nucleic-acid binding protein that mainly binds single stranded DNA and RNA, and modulates the transcription of genes involved in Wnt signaling pathway (Margarit et al., 2014).

The proposed pathogenic mechanism hypothesizes that reduced DMPK and CNBP/Znf9 levels in DM1 and DM2, respectively, cause the onset of the diseases. Consistent with this, decreased amounts of DMPK transcripts and protein have been detected in DM1 patients, and this decrease inversely correlated with the CTG repeat length (Fu et al., 1993).

In this regard, DMPK knock-out mice fail to reproduce the multisystem phenotype of DM1 patients, and only develop late-onset myopathy (Jansen et al., 1996; Reddy et al., 1996; Berul et al., 1999), suggesting that haploinsufficiency of DMPK is not the primary mechanism that initiates this disease.

On the contrary, *Znf9^{+/−}* mice (lacking one CNBP allele) develop a multisystem phenotype resembling DM, including muscle wasting, heart failure and cataracts (Chen et al., 2007), which suggests that CNBP insufficiency may have a role in the pathologic mechanisms of DM2 (Raheem et al., 2010). However, other studies have yielded contradictory results, indicating that aberrant CCTG expansions do not alter CNBP protein levels, and thus, CNBP deficiency would not contribute to the onset of the disease (Margolis et al., 2006).

Cis-Alteration of Neighboring Genes

Tandem repeats produce changes in chromatin structure. Indeed, nucleosomes tend to localize in these repeat sequences, inducing chromatin condensation (Wang et al., 1994; Volle and Delaney, 2012), which could have a notable impact on the transcriptional activity of DMPK and ZNF9 flanking genes (Klesert et al., 1997; Thornton et al., 1997; Westerlaken et al., 2003). Similar to the haploinsufficiency of the repeat-containing genes in myotonic dystrophies, the altered expression of genes located in the neighborhood of the disease-causing genes could also contribute to the molecular mechanisms leading to these diseases.

In line with this, mRNA level of SIX5 (also known as DMAHP), a transcription factor coding gene located downstream of DMPK, is reduced in DM1 patients (Klesert et al., 1997; Thornton et al., 1997; Westerlaken et al., 2003). Expression of DMWD, an upstream DMPK flanking gene, also seems to be somewhat reduced in the cytoplasm of DM cells, although nuclear levels remain unchanged (Alwazzan et al., 1999; Frisch et al., 2001).

In order to ascertain the contribution of reduced levels of SIX5 to the development of DM1, a knock-out mouse model of SIX5 was created. Besides showing an increased susceptibility to developing cataracts (Klesert et al., 2000; Sarkar et al., 2000),

reduced fertility in males (Sarkar et al., 2004) and altered cardiac function (Wakimoto et al., 2002), these mice do not show multisystem involvement and thus fail to recapitulate the dystrophic phenotype of DM1 patients.

RNA-Induced Toxicity

The third pathogenic mechanism proposes that repeat expansions, once translated into RNA, exert a gain-of-function toxic effect in the cells. The expanded CUG-containing transcripts form secondary structures of a hairpin shape (Michałowski et al., 1999) and sequester specific RNA-binding proteins that participate in pre-mRNA transcription and maturation, such as MBNL (Muscleblind-like) (Miller et al., 2000), thus leading to splicing defects in both DM1 and DM2 patients (Du et al., 2010; Malatesta and Meola, 2010). Double-strand RNA structures also abnormally activate the RNA-dependent protein kinase R (PKR), which in turn hyperphosphorylates CUG-BP/CELF1 protein and alters its function (Tian et al., 2000). This protein is involved in the splicing of several genes directly implicated in the multisystem phenotype of DM patients, such as cardiac Troponin (cTNT), IR and chloride channel 1 (CLCN1; Osborne et al., 2009). Overactivation of PKR also inactivates its substrate eIF2 α , inhibiting the translation of specific mRNAs, such as the DNA repair factor MRG15 (Huichalaf et al., 2010).

In order to assess the contribution of foci formation to the multisystem involvement seen in DM patients, several mouse models have been created carrying variable repeat length sequences and tissue specificity. In all these models the toxicity of foci has been tested, and such models have helped to elucidate how these aggregates play a key role in the onset of the disease. For example, HSA mice, which harbor 5 (short repeat length, HSA^{SR}) or 250 (long repeat length, HSA^{LR}) CTG repeats in the Human Skeletal Actin gene (HSA), thus inducing a muscle-specific expression of tandem repeat-containing RNAs, have clearly established the role of RNA-protein aggregates in the development of the disease, as mice harboring long repeats developed a DM-like phenotype of muscle degeneration (Mankodi et al., 2000). RNA toxicity has been confirmed with transgenic mice harboring long triplet repeats in the DMPK gene. Such mice develop multisystem abnormalities mimicking the human DM phenotype, with predominant involvement of muscles and the CNS, although the resulting phenotype is milder than in other mouse models of the disease (Seznec et al., 2001).

Therefore, all three mechanisms may participate to some extent in the onset of a range of symptoms in DM patients, although RNA toxicity seems to contribute most notably to the multisystem degeneration seen in DM patients.

Age-Related Genomic Events in DM

Cellular aging is accompanied by accumulating DNA damage (Moskalev et al., 2013), which includes point mutations, translocations, and double strand breaks, among others. These mutations can seriously affect the functionality of several cell types, such as the regenerative capacity of stem cells. Cells possess

several mechanisms to cope with these abnormalities, either by activating DNA repair mechanisms or inducing cell death. Unfortunately, DNA damage increases with aging partially due to decreased efficiency of DNA repair systems (Gorbunova et al., 2007).

As previously mentioned, these DNA repair mechanisms participate in the expansion of tandem repeats, which cause not only DM but also other degenerative diseases such as Huntington's Disease and Friedreich's ataxia. Besides DNA repair mechanisms, telomere maintenance could also be implicated in muscle aging and DM.

Chromosomal ends, also known as telomeres, are composed of highly repetitive sequences that play an important role in the maintenance of chromosomal structure. Importantly, these DNA ends cannot be replicated by common DNA polymerases and thus, they continue to shorten in every cell division. Telomere shortening has been observed in most cell types during the aging of both human and mice tissues (Blasco, 2007) and has become a cellular marker of aging. However, some cell types, such as most adult stem cells, express telomerase, a specific enzyme able to replicate telomeric sequences, counteracting their shortening (Vaziri et al., 1994; Chiu et al., 1996; Morrison et al., 1996; Espejel et al., 2004; Ferrón et al., 2004; Flores et al., 2005).

Absence of telomere shortening in satellite cells of aged mice, together with the insignificant reduction of telomerase activity in aged muscle stem cells, indicates that satellite cells possess mechanisms to maintain telomeres, and thus, the age-related reduction of their regenerative potential is telomere independent. On the contrary, myogenic differentiation of these satellite cells abolishes their telomerase activity (O'Connor et al., 2009).

In vitro studies with human congenital DM1 samples show that despite DM1-affected satellite cells having a higher telomere shortening rate, these cells enter senescence prior to reaching critically short telomere lengths (Bigot et al., 2009; Thornell et al., 2009). In these cases, p16-dependent signaling seems to induce the senescence of satellite cells. Thus, telomere shortening, even though it is altered in DM1 patients, does not seem to play a role in the pathology of this disease. On the contrary, telomere shortening could influence the regenerative capacity of DM2-affected satellite cells, as will be discussed below Renna et al. (2014).

Epigenetic Modifications in DMs and Aging

Epigenetic modifications encompass post-translational modifications of DNA and histones that lead to chromatin remodeling processes. Different trends of these modifications have been associated with aging, such as increased histone H4K16 acetylation, H4K20 trimethylation and H3K4 trimethylation, and decreased H3K9 methylation or H3K27 trimethylation (Fraga and Esteller, 2007; Han and Brunet, 2012; López-Otín et al., 2013). Importantly, the family of histone deacetylases known as sirtuins have been shown to possess outstanding antiaging potential (Kaeberlein, 2008; Houtkooper et al., 2012; Imai and Guarente, 2014).

Epigenetic modifications also seem to correlate with the age-dependent loss of the regenerative potential of stem cells (Liu and Rando, 2011). This also applies to muscle satellite cells, in which H3K27 trimethylation seems to increase overall in their genome, along with reduced histone biosynthesis (Liu et al., 2013).

Although epigenetic modifications in DM-affected satellite cells have not yet been studied specifically, triplet expansions seem to induce different DNA methylation patterns. In this regard, the DMPK locus shows a variably methylated sequence upstream of the triplet expansion, whereas this sequence is unmethylated in healthy controls (Ghorbani et al., 2013). Interestingly, the expanded sequence and the downstream sequence are not methylated (López Castel et al., 2011). Methylation is more frequently found in tissues of congenital and young-onset patients, although it is not limited to cells with the largest expansions or to specific tissues (López Castel et al., 2011). In an attempt to elucidate the consequences of these methylations, disruption of CTCF binding to its DNA binding sites, which flank the DM1 locus, has been postulated to explain the reduced transcription of DMPK and Six5 genes (Filippova et al., 2001; He and Todd, 2011). However, CTCF binding to DNA seems to be unaffected in a mouse model of DM1, despite the methylation and heterochromatinization found in the DM1 locus (Brouwer et al., 2013). These authors alternatively propose proliferating cell nuclear antigen (PCNA), which also binds DNA repeat sequences, as a player in the triplet-expansion mechanism.

The epigenetic modifications resulting from triplet expansions require more precise study in order to determine their contribution to the development of the disorder. The study of these modifications in muscle satellite cells would contribute to unravel the role of epigenetic modifications in the regenerative capacity of DM-affected satellite cells.

Is Muscle Wasting in Myotonic Dystrophy a Matter of Premature Aging?

Satellite Cells and Muscle Regeneration in DM and Aging

Satellite cells play a key role in the maintenance of muscle structure and function both in homeostasis and after acute damage. The age-related dysfunction of satellite cells has been postulated to be a key cause of sarcopenia, apart from also being implicated in other muscle wasting conditions such as muscular dystrophies (Day et al., 2010).

Satellite cells maintain skeletal muscle structure and functionality by differentiating into myogenic cells that regenerate muscle tissue (Lepper et al., 2011; Murphy et al., 2011; Sambasivan et al., 2011). In adulthood, these cells are quiescent and locate in the periphery of muscle fibers, between the basal membrane and the sarcolemma (Mauro, 1961). When muscle regeneration is required, these cells activate the cell cycle and proliferate. Most of these activated satellite cells enter the myogenic program and differentiate into myoblasts that fuse to each other or to preexisting myotubes, regenerating the damaged tissue. However, a small subpopulation of these cells repopulates the satellite cell niche (Chargé and Rudnicki, 2004; Yin et al., 2013).

Age-dependent regenerative dysfunction of skeletal muscle mainly depends on the decreased amount and functionality of satellite cells (Shefer et al., 2006; Brack and Rando, 2007; Collins et al., 2007). However, it has been widely debated if this reduced regenerative potential is due to extrinsic factors, such as age-dependent alterations of the satellite cell niche and circulating factors (Brack and Rando, 2007; Gopinath and Rando, 2008; Urciuolo et al., 2013), or due to intrinsic defects of these stem cells (Bernet et al., 2014; Sousa-Victor et al., 2014).

With regard to extrinsic factors, satellite cells are responsive to a wide variety of circulating *biomolecules*, whose concentration is constantly being modified in an age-dependent manner. Experiments involving surgical sharing of the circulating system (also known as heterochronic parabiosis) between aged and young mice have revealed that aged serum harbors factors that reduce the regenerative capacity of young satellite cells, whereas young serum is able to partially revert the myogenic-to-fibrogenic transition of aged muscle stem cells, thus reducing the muscle fibrosis seen in old muscles (Conboy et al., 2005; Brack and Rando, 2007). *In vitro* experiments performed with human satellite cells have confirmed this fact. These findings show that age-dependent systemic factors act on the regenerative potential of satellite cells (Carlson and Conboy, 2007; Carlson et al., 2009).

The satellite cell *niche*, composed of cells and extracellular matrix located in the close vicinity of satellite cells, also affects satellite cell functionality, probably through secreted factors as well as cell-to-cell interactions (Murphy et al., 2011; Naito et al., 2012). Moreover, aging alters the pattern of secreted molecules and cellular interactions, thus affecting satellite cell biology. For example, age-dependent increases in basic fibroblast growth factor (bFGF) activate satellite cells in homeostasis, inducing their differentiation, and consequently, the satellite cell pool is depleted (Chakkalakal et al., 2012). On the other hand, matrix molecules, such as extracellular fibronectin, participate in the activation of Wnt signaling in satellite cells, promoting their symmetric division (Bentzinger et al., 2013).

Apart from environmental factors, satellite cell-intrinsic factors also play a central role in the maintenance of the regenerative capacity of these cells. Recently, Sousa-Victor et al. (2014) have demonstrated that the pronounced decline in the regenerative potential of satellite cells in very old (geriatric) mice is caused by the induction of p16^{Ink4a}, which drives cells from quiescence to irreversible senescence. p38 signaling also occupies a central role in the functionality of satellite cells. In this regard, activation of p38 signaling in dividing aged satellite cells induces the differentiation of both daughter cells, whereas in young animals, asymmetric localization of p38 enables satellite cells to divide asymmetrically, thus favoring the maintenance of the quiescent satellite cell pool (Bernet et al., 2014; Cosgrove et al., 2014).

Therefore, both satellite cell-extrinsic and -intrinsic factors affect the regenerative potential of muscle stem cells during aging and these factors may also play a role in DM-affected satellite cells. Indeed, it has been shown that cell-intrinsic events, such as nuclear foci accumulation, affect DM cell functionality (Malatesta, 2012), whereas the multisystem dysfunction seen in

these diseases may probably alter circulating factors, impairing the regenerative potential of these cells.

Several studies have aimed to study the involvement of satellite cells in DM. In this regard, histopathologic analysis of severely affected distal muscles vs. slightly affected proximal muscles of DM1 patients has shown a two-fold increase in the number of satellite cells in severely affected muscles. Interestingly, telomere length is not altered and the number of regenerative fibers is low in both distal and proximal muscles (Thornell et al., 2009). In this regard, *in vitro* proliferative capacity of DM1 satellite cells is considerably reduced, and their entry into senescence is telomere-independent (Thornell et al., 2009). Indeed, this has been corroborated with *in vitro* cultured satellite cells from congenital DM fetuses and newborns. These satellite cells show a considerably lower proliferative rate than age-matched controls, besides having activated senescence-associated beta galactosidase, high levels of cyclin D1 and hypophosphorylated Rb. Interestingly, these cells also enter senescence prior to reaching critically short telomere lengths and express p16^{Ink4a}, a Cdk4 inhibitor that induces cell cycle arrest (Bigot et al., 2009). It remains to be clarified which cellular events induce p16^{Ink4a} expression, although disturbed DNA functioning due to the formation of unusual structures, as well as increased free radicals and oxidative stress might be involved in the activation of this gene.

Besides the involvement of the proliferative capacity of DM satellite cells, the myogenic program is also abnormal in these cells. Indeed, there is evidence of the defective differentiation and maturation of DM1 myogenic progenitors *in vitro*, resulting in smaller and thinner myotubes, with a 30% lower fusion index and the lack of expression of mature myosin forms (Furling et al., 2001). This lack of fiber maturation has also been confirmed in DM1 muscle biopsies, where late myogenic differentiation markers are not fully expressed (Vattemi et al., 2005). Moreover, DM1 myoblasts seem to have impaired cell cycle withdrawal, probably due to the inability to induce the expression of p21 (Timchenko et al., 2001).

Satellite cells in DM2 show an intermediate phenotype between DM1 and healthy satellite cells. Renna et al. (2014) suggest that DM2 satellite cells also show premature entry into senescence, although later than DM1 satellite cells. Importantly, senescence seems to correlate with telomere shortening rather than the induction of p16^{Ink4a} (Renna et al., 2014). In contrast to DM1 progenitors, DM2 progenitors did not show any differentiation defects and CGUBP1 levels were also unchanged. However, DM2 cells had abnormal IR splicing (Pelletier et al., 2009). In this regard, the interaction of CUGBP1 with eIF2 α and Cyclin D3-CDK4/6 seems to be crucial to achieve a correct myogenic differentiation. Reduction of these interactions in DM1 cells could explain the impaired differentiation of these cells (Salisbury et al., 2008).

This reduced differentiation and premature senescence of satellite cells resembles physiological satellite cell aging. Moreover, age-related cell-senescence features, such as cytoplasmic vacuolization, accumulation of heterochromatin

and impaired pre-mRNA maturation (Malatesta and Meola, 2010) have also been found in DM cells (Malatesta et al., 2011a,b).

As previously mentioned, secreted factors also have an impact on muscle regeneration during aging, and could also play a role in DM. In this regard, congenital DM1 muscle progenitor cells with long triplet expansions seem to secrete prostaglandin, which in turn hampers myogenic differentiation, probably by lowering intracellular calcium levels (Beaulieu et al., 2012).

Loss of Proteostasis in Aging and DM

Proteostasis encompasses cellular mechanisms that preserve the stability and functionality of its proteome in order to prevent the accumulation of damaged proteins and ensure continuous renewal of intracellular proteins. Many studies have demonstrated that protein homeostasis collapses during aging, leading to the accumulation of unfolded, aggregated and misfolded proteins, a phenomenon that causes several age-related diseases (Powers et al., 2009). Cells have various mechanisms to tackle these protein failures. The three principal proteostatic systems are the UPS, the autophagy-lysosomal system and chaperones, the efficiency of all of which decreases with aging (Calderwood et al., 2009; Tomaru et al., 2012). Consequently, old cells carry more non-enzymatic posttranslational protein modifications and accumulate more cross-linked and aggregated proteins than young cells (Soskić et al., 2008).

The Ubiquitin-Proteasome System

The UPS actively participates in the regulation of protein synthesis and degradation. The age-dependent decay of UPS efficiency may be the result of the reduced expression of proteasome subunits, their inadequate assembly, and/or reduced ATP availability due to mitochondrial dysfunction (Chondrogianni et al., 2014).

The activity of UPS is reduced in several aged mammals, such as humans, mice, rats and sheep. However, contrary to what could be expected, some aged tissues such as muscle, show increased expression of UPS subunits (Ferrington et al., 2005), which could be a compensatory effect for reduced constitutive proteasomal activity (Husom et al., 2004). Maintenance of proteostasis in stem cells may also play an important role in organismal aging (Vilchez et al., 2014). Indeed, proteasome activation is a conserved mechanism that regulates aging and longevity (Chondrogianni et al., 2014).

The breakdown of proteostasis has been linked to several disorders, including myotonic dystrophy. UPS is increased in skeletal and cardiac muscle of transgenic DM mice with 550 CTG repeats, triggered by the up-regulation of Fbx032/Atrogin-1 and/or Trim63/Murfl. These mice develop progressive muscle weakness between 3 and 10 months of age (Vignaud et al., 2010). Overactivation of UPS has also been confirmed in the DMSXL mouse model, which exhibits more than 1.000 CTG repeats. UPS activity was considerably increased at 4 months of age in these mice, suggesting that this proteolytic pathway could play a role in the physiopathological remodeling of muscle (Huguet et al., 2012).

Proteomic analyses have also confirmed that protein degradation is altered in DM2 myotubes (Rusconi et al., 2010). Indeed, an overall reduction in ubiquitinated proteins as well as reduced proteasome subunits have been found in DM2 myotubes (Rusconi et al., 2010). DM2 myoblasts degrade faster a variety of short-lived proteins, such as c-myc and p21, due to increased UPS activity that results from RNA CCUG repeats that bind the 20S core complex (Salisbury et al., 2009). Skeletal muscles of DM2 patients also show a deregulated *neural precursor cell expressed developmentally down-regulated protein 4* (NEDD4) ubiquitin ligase-PTEN pathway, which could contribute to the increased risk of statin-adverse reactions in patients with DM2, due to PTEN accumulation in highly atrophic muscle fibers (Screen et al., 2014).

It is worthy of note that besides UPS overactivation in DM, the 26S proteasome itself is implicated in trinucleotide expansion, thus favoring the expansion of these pathogenic repeat sequences (Concannon and Lahue, 2014).

Autophagy

Autophagy mediates the degradation of cellular components in order to recycle them or to obtain energy. Indeed, autophagy is activated during starvation and induces the degradation of cellular components, providing the cell with energy and thus promoting cell survival during a period of low nutrient availability. In contrast to autophagy, mTOR signaling is activated by high nutrient availability, such as insulin and amino acids, and activates cell division and protein synthesis. Importantly, age-dependent decline of autophagy disrupts cellular proteostasis.

In skeletal muscle, autophagy seems to participate in the activation of quiescent satellite cells, probably providing the additional energy required for this process (Tang et al., 2014). Thus, age-related dysfunction of autophagy could undermine satellite cell activation with aging.

In vitro myoblast cultures of DM1 patients show that nearly half of the myoblasts undergo abnormal differentiation. Strikingly, cells that fail to differentiate show autophagic features after 6 days in culture, with increased cellular volume and a high density of autophagic vacuoles as compared to control and DM1 differentiated cells (Beffy et al., 2010). Senescence was ruled out as an activator of autophagy in this study, as the percentage of cells expressing senescence-associated beta galactosidase was similar in DM1 cells and controls.

Autophagy may also be abnormal in DM1-affected neurons. In this regard, autophagy has been found to be activated and mTOR signaling partially inhibited in DM1-hESC-derived neurons as compared to wild type-hESC-derived neurons. Phosphorylation of the mTOR downstream components GSK3 α/β and rpS6 were decreased in these cells. Importantly, reduction of mTOR signaling was p53-independent, therefore suggesting that inhibition of mTOR is not induced by cellular stress (Denis et al., 2013). In line with this, GSK3 β has been found to be overactivated in muscles of the DM1 mouse model HSA^{LR} prior to the onset of muscle wasting, and GSK3 β blockers improved skeletal muscle strength and reduced myotonia in this mouse model, suggesting that these inhibitors could have a

beneficial effect on the treatment of DM1 by alleviating muscle wasting (Jones et al., 2012).

It has been speculated that autophagy could be a mechanism either to avoid apoptosis or to protect cells against metabolic stress. However, transfection of C2C12 myoblasts with the human DMPK-A isoform not only shows increased autophagy, but also enhances apoptosis (Oude Ophuis et al., 2009). Therefore, autophagy in DM must be thoroughly studied in order to determine the causes and the effects of its overactivation in these patients.

Chaperones

Chaperones exert a key function in proteostasis by folding peptides, refolding incorrectly folded proteins and unfolding damaged proteins to facilitate their degradation. Heat Shock Proteins (HSPs) constitute a subgroup of chaperones that are specifically induced by different cell stressors, such as protein damage. As expected, chaperones play a central role in protecting cells from protein damage and cell death during aging (Calderwood et al., 2009). Moreover, experiments performed in *S. cerevisiae* have shown that overexpression of specific HSPs, such as HSP104, increases protein disaggregation, reduces protein accumulation and restores UPS in aged cells (Andersson et al., 2013).

The muscle-specific HSPs HSPB3 and HSPB2, the latter also known as Myotonic dystrophy Protein Kinase Binding Protein (MKBP), seem to occupy a central role in muscle regeneration. HSPB2 specifically binds and activates DMPK, which contributes to muscle maintenance (Suzuki et al., 1998; Prabhu et al., 2012). HSPB2 and HSPB3 are induced by MyoD during myogenic differentiation, which strongly suggests that they play central roles in muscle regeneration, probably through the interaction of MKBP with DMPK (Sugiyama et al., 2000). Other HSPs, such as HSP70, may also play muscle-specific roles, as it is induced in type 1 fibers (Locke et al., 1991).

Expression of HSPB2/MKBP is specifically up-regulated in the skeletal muscle of DM1 patients, probably in order to partially compensate for the reduced amount of DMPK (Sugiyama et al., 2000). Therefore, an increase in chaperone activity would potentially benefit the maintenance of skeletal muscle functionality in both DM-affected and aged muscles.

Mitochondrial Dysfunction

The Mitochondrial Free Radical Theory of Aging (MFRTA), which proposes that mitochondrial free radicals cause oxidative damage that gives rise to cellular aging, has been postulated as one of the main hypotheses to explain how cells age (Sanz and Stefanatos, 2008). Mitochondria play an important role in mediating and amplifying the oxidative stress that drives the aging process (Bratic and Larsson, 2013).

The largest isoform of myotonic dystrophy protein kinases, DMPK-A, supplies antioxidants and antiapoptotic signals needed for correct muscle fiber function and differentiation (van Herpen et al., 2005; Pantic et al., 2013). However, anchorage and accumulation of DMPK-A in the mitochondrial outer membrane can lead to mitochondrial fragmentation and the formation of

perinuclear clusters of morphologically altered mitochondria, finally inducing the activation of autophagy (Oude Ophuis et al., 2009).

DM-patients' muscles show a reduced expression of DMPK, mitochondrial accumulation in degenerated myofibrils and disorganization of the sarcoplasmic reticulum (Ueda et al., 1999). On the other hand, these muscles show reduced Coenzyme Q10 (CoQ10) levels, a component of the electron transport chain that participates in aerobic cellular respiration, generating energy as ATP (Siciliano et al., 2001). Blood samples have confirmed an inverse correlation between CoQ10 levels and CTG expansion length in DM patients (Tedeschi et al., 2000).

As for DM2, proteomic analyses of myotubes have detected abnormalities in two proteins involved in mitochondrial fatty acid degradation and another two proteins involved in the import, chaperonin and quality control functions of mitochondria (Rusconi et al., 2010). The elongation factors Tu and Ts, two posttranslational proteins that participate in the mitochondrial translational machinery, were also reduced in DM2 cultures. Mutations and/or reductions in these proteins are associated with muscle hypotonia and decline of motor skills (Valente et al., 2007). Therefore, mitochondrial dysfunction seems to be common to both muscular aging and DM.

Deficiencies in Nutrient Sensing

DM patients show several metabolic defects that are also common in aged individuals, such as glucose resistance, hyperinsulinemia and in some cases, the development of diabetes mellitus. Studies performed with large DM1 samples have revealed that there are metabolic dysfunctions associated with this disease, such as primary hyperparathyroidism, calcium metabolism, thyroid insufficiency, hypogonadism, hyperprolactinemia or diabetes. Some of these dysfunctions seem to correlate with the length of the repeat expansion (Ørnsgreen et al., 2012).

Hyperphosphorylation of CUGBP, which is a feature of DM, leads to abnormal splicing of the IR mRNA, lacking exon 11 (Osborne et al., 2009). The IR is mainly expressed in skeletal muscle and its binding to the ligands insulin and IGF-I activates metabolic pathways implicated in muscle hypertrophy, whereas binding to IGF-II induces mitosis. This binding affinity is isoform-dependent; the form that lacks exon 11 (immature form), is mainly expressed in embryonic tissues and shows high affinity towards IGF-II, compared to insulin and IGF-I. Due to the abnormal splicing, DM skeletal muscles are characterized by a predominant expression of the immature isoform, which leads to insulin insensitivity (Savkur et al., 2001, 2004). This splicing defect seems to be independent of muscle fiber type, as both fiber types show a reduced expression of the adult IR isoform (Santoro et al., 2013), despite DM muscle wasting being fiber-type dependent (Vihola et al., 2003; Pisani et al., 2008).

These splicing abnormalities have been observed in muscle tissue and myotube cultures of both DM1 and DM2 patients prior to the development of muscle histopathology, which indicates that DM1 and DM2 share common pathogenic mechanisms and that these splicing abnormalities appear before myofiber degeneration (Santoro et al., 2013).

Insulin secretion has also been found to be abnormal in DM patients, probably due to loss of calcium homeostasis that regulates insulin secretion by pancreatic beta-cells (Savkur et al., 2001).

Thus, deficiencies in nutrient sensing are shared by both physiological aging and DM patients, which leads to the use of common therapeutic approaches to treat both conditions.

Concluding Remarks

Myotonic dystrophies represent a new paradigm of how a genetically determined disease initiates a cascade of events that lead to a wide variety of symptoms (myotonia, cataracts, heart dysfunction, baldness, etc.) that resemble the multisystem involvement induced by aging.

Different pathogenic mechanisms exist to explain how these tandem expansions in the genome of affected patients lead to the DM phenotype, although it has not yet been clearly defined to what degree each mechanism contributes to the development of the disease.

An important feature of myotonic dystrophies and aging resides in their progressive nature. The described molecular events, such as genomic instability, alteration of autophagy or mitochondrial dysfunction, among others, induce cell damage that continues to accumulate throughout life (Figure 4).

Satellite cells have been hypothesized to be the main contributors to muscle regeneration. Numerous studies have established that both cell-intrinsic and environmental factors induce the age-related decline of their regenerative capacity in muscle aging and sarcopenia. However, it is still unclear how these cells behave in different muscle dystrophies and if their

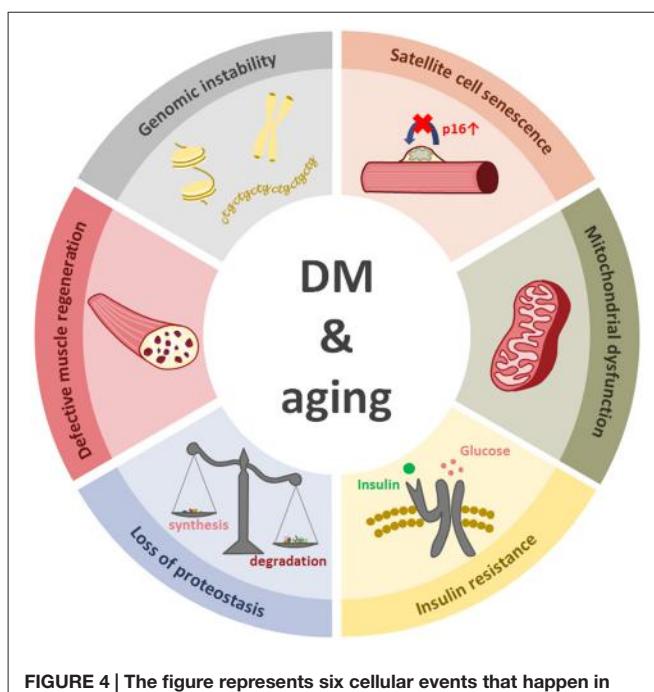


FIGURE 4 | The figure represents six cellular events that happen in both aging and myotonic dystrophy.

loss of regenerative potential is crucial in muscle wasting seen in affected patients.

The molecular pathologic mechanisms of DM, as well as the aging-related events reviewed above, strongly suggest that satellite cell dysfunction could be a major contributor to the development of muscle wasting in these patients and thus, that these cells could become potential targets for the treatment of both age-related and DM-induced muscle dysfunction. It is worth highlighting that skeletal muscle fibers also present proteostatic and mitochondrial abnormalities that resemble general aging processes. Thus, DM patients and animal models can be considered *bona fide* models of aging, and this should be kept in mind when designing treatments to treat both myotonic dystrophy patients and aging-derived disorders.

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Author Contributions

All authors have contributed to the design, data collection and writing of the manuscript. Authors have critically revised the work and they have approved the submitted version of the review.

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MicroRNAs modulated by local mIGF-1 expression in mdx dystrophic mice

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Duchenne muscular dystrophy (DMD) is a X-linked genetic disease in which the absence of dystrophin leads to progressive lethal skeletal muscle degeneration. It has been demonstrated that among genes which are important for proper muscle development and function, micro-RNAs (miRNAs) play a crucial role. Moreover, altered levels of miRNAs were found in several muscular disorders, including DMD. A specific group of miRNAs, whose expression depends on dystrophin levels and whose deregulation explains several DMD pathogenetic traits, has been identified. Here, we addressed whether the anabolic activity of mIGF-1 on dystrophic muscle is associated with modulation of microRNAs expression. We demonstrated that some microRNAs are strictly linked to the dystrophin expression and are not modulated by mIGF-1 expression. In contrast, local expression of mIGF-1 promotes the modulation of other microRNAs, such as miR-206 and miR-24, along with the modulation of muscle specific genes, which are associated with maturation of regenerating fibers and with the stabilization of the differentiated muscle phenotype. These data suggest that mIGF-1, modifying the expression of some of the active players of muscle homeostasis, is able, even in absence of dystrophin expression, to activate circuitries that confer robustness to dystrophic muscle.

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Introduction

Duchenne muscular dystrophy (DMD) is an X-linked genetic disease caused by mutations in the dystrophin gene, leading to progressive lethal muscle degeneration, chronic inflammatory response, and fibrosis (Deconinck and Dan, 2007).

Recent works have shown that micro-RNAs (miRNAs), which are important regulatory elements for proper muscle development and function (Eisenberg et al., 2009; Gütler and Russell, 2010; Sharma et al., 2014), might play crucial roles in the pathogenesis of several muscular disorders, including DMD (Eisenberg et al., 2007; Chen et al., 2009). MiRNAs are small, ~22 nucleotides long, non coding RNA that function as regulatory molecules, silencing their cognate target genes (Bartel, 2004).

In a previous work, we contributed to identify a specific group of miRNAs whose expression depends on dystrophin levels and whose deregulation explains several DMD pathogenetic traits (Cacchiarelli et al., 2010). This class of miRNAs, poorly expressed in mdx, was upregulated in exon-skipping-treated animals and included muscle specific (miR-1 and miR-133) and more

ubiquitous (miR-29 and miR-30) miRNAs. Moreover, the negative modulation of the inflammatory miR-223 and the up-regulation of miR-29, which controls collagen deposition, was consistent with the observed amelioration of the dystrophin phenotype due to the rescue of dystrophin expression by exon-skipping approach (Cacchiarelli et al., 2010). In contrast, the restricted expression of the myomiR 206 in activated satellite cells before the onset of dystrophin synthesis, suggested that miR-206 is independent from the Dystrophin/nNOS-mediated pathway. Moreover, it has been demonstrated a common micro-RNA signature in skeletal muscle damage and regeneration induced by DMD and acute ischemia (Greco et al., 2009), suggesting an important role of miRNAs in physiopathological pathways regulating muscle response to damage and regeneration.

Among growth factors, the insulin-like growth factors 1 (IGF-1) has been implicated in many anabolic pathways in skeletal muscle. Different studies on the roles of IGF-1 isoforms in skeletal muscle growth and differentiation have provided new insights into the function of these signaling molecules in muscle homeostasis, and in the control of skeletal muscle growth and regeneration (Scicchitano et al., 2009).

We previously demonstrated that muscle restricted mIGF-1 transgene (MLC/mIGF-1) sustained muscle hypertrophy and regeneration in young and senescent skeletal muscle (Musarò et al., 2001; Pelosi et al., 2007), enhanced the recruitment of circulating stem cells in injured muscle (Musarò et al., 2004) and counteracted muscle wasting in mdx dystrophic mice (Barton et al., 2002). In particular, we found a reduction in myonecrosis and fibrosis in the muscles of mdx/mIGF-1 mice compared with age-matched mdx animals (Barton et al., 2002; Shavlakadze et al., 2004). Likewise, co-injection of the rAAV-microdystrophin and rAAV-mIGF-1 vectors resulted in increased muscle mass and strength, reduced myofibers degeneration, and increased protection against contraction-induced injury (Abmaya et al., 2005). However, no specific genes and regulatory circuitries that could account for the observed morpho-functional benefits in mdx/mIGF-1 muscle have been characterized.

In this work we took advantage of mdx/mIGF-1 mice to define whether, independently of dystrophin expression, the modulation of the dystrophic microenvironment by mIGF-1 expression has some effect on miRNAs expression.

Materials and Methods

Mice

Animal model used: 4 week-old C57Bl/10 (control strain) mice, 4 week-old mdx and mdx/mIGF-1 (Barton et al., 2002) mice. Mdx female mice (Jackson Laboratories) were bred with IGF-1 transgenic male mice (mIGF-1) (Musarò et al., 2001), resulting in a group of mice homozygous for the IGF-1 gene and the X-linked mdx mutation (mdx/mIGF-1) (Barton et al., 2002). Mice were maintained according to the institutional guidelines of the animal facility of the unit of Histology and Medical Embryology. All animal experiments were approved by the ethics committee of Sapienza University of Rome-Unit of Histology

and Medical Embryology and were performed in accordance with the current version of the Italian Law on the Protection of Animals.

RNA Extraction and Real Time PCR Analysis

Total RNA was prepared from liquid nitrogen powdered diaphragms homogenized with tissue lyser (QIAGEN) in TriRiagentTM (Sigma). To synthesize single-stranded cDNA, 10 ng of total RNA were reverse transcribed using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystem), while double-stranded cDNA was synthesized with the QuantiTect Reverse Transcription kit (Qiagen). miRNA and mRNA analysis were performed on an ABI PRISM 7500 SDS (Applied Biosystems), using specific TaqMan assays (Applied Biosystems). Relative quantification was performed using as endogenous controls U6 snRNA for miRNAs and HPRT1 for mRNAs. The relative expression was calculated using the 2-DDCt method (Livak and Schmittgen, 2001).

Protein Extraction, Western Blot Analysis

Diaphragm muscles from at least 3 animals/strain (wild type, mdx, and mdx/mIGF-1 mice) were homogenized in modified lysis buffer (Tris-HCl, pH 7.5/20 mM, EDTA/2 mM, EGTA/2 mM, sucrose/250 mM, DTT/5 mM, Triton-X/0.1%, PMSF/1 mM, NaF/10 mM, SOV₄/0.2 mM, cocktail protease inhibitors/1X (Sigma)). Muscle lysates were processed as previously described (Pelosi et al., 2007). Filters were blotted with antibodies against: HDAC2 (Santa Cruz Biotechnology, INC), HADAC4 (Santa Cruz Biotechnology, INC) and αTubulin (Sigma). Signals were captured by ChemiDoc-It® Imaging System (UVP, LLC) and densitometric analysis were performed with VisionWorks® LS Image Acquisition and Analysis Software (UVP, LLC).

Statistical Analysis

Statistical analysis was performed with GraphPad Prism Software. All data, if not differently specified, were expressed as mean ± SEM. Difference among groups were assessed with one-way ANOVA with a Bonferroni post test or Dunn's post Test, and between pairs with Mann-Whitney test or Student's *t* test assuming two-tailed distributions. Each data shown in qRT-PCR was performed on at least four different samples/animals in biological duplicates. Sample size was predetermined based on the variability observed in preliminary and similar experiments. All experiments requiring animal models were subjected to randomization based on litter. *p* < 0.05 is considered statistically significant.

Results

mIGF-1 Modulates the Expression of miRNAs and Factors Associated with Regeneration

In this study, we aimed to identify miRNAs involved in the pathologic phenotype of young (4 weeks of age) mdx mice and

to verify whether mIGF-1 expression was able to modulate the miRNAs' signature of dystrophic muscle.

Dystrophic-signature miRNAs has been divided into three main classes: degenerative miRNAs (miR-1, miR-29c, and miR-135a), regeneration miRNAs (miR-31, miR-34c, miR-206, miR-335, miR-449, and miR-494), and inflammatory miRNAs (miR-222 and miR-223) (Greco et al., 2009).

Moreover, these groups of miRNAs play important and crucial roles in tissue proliferation, differentiation, and homeostasis. In particular miR-1 and miR-206, classified as myomiRs on the basis of their selective expression in skeletal and cardiac muscles, regulate muscle satellite cells proliferation and differentiation, by repressing Pax-7 (Chen et al., 2010). miR-29 regulates collagens and elastin and therefore controls fibrogenesis (van Rooij et al., 2008). miR-31 might have different roles; it is strongly induced in ischemia damaged myofibers, it plays a fundamental role in postnatal vascular repair (Greco et al., 2009; Wang et al., 2014), regulates both dystrophin expression (Cacchiarelli et al., 2011) and the progression of satellite cells toward differentiation (Crist et al., 2012). miR-34c is strongly induced in ischemia damaged myofibers (Greco et al., 2009) and it promotes cell cycle withdrawal and apoptosis (Corney et al., 2007). miR-221 and miR-223

have antiangiogenic properties and play important role in the regulation of vascular inflammation (Poliseno et al., 2006). Moreover, miR-221 and miR-222 might regulate skeletal muscle differentiation (Fasanaro et al., 2010). miR-335 and miR-449 are potent mediators of cell differentiation (Lizé et al., 2011; Tomé et al., 2011), whereas miR-494 has been proven critical for the myocytes' adaptation and survival during hypoxia/ischemia (Han et al., 2011).

Based on the dystrophic-signature miRNAs (Greco et al., 2009) and on the specific properties of selected miRNAs, at first, we defined and compared the miRNAs expression profile of dystrophic diaphragm muscle derived from 4-week-old wild type and mdx mice.

We observed that all degenerative- miRNAs were down-regulated in diaphragm of 4-week-old mdx compared to wild type mice (Figure 1A), whereas most of the regeneration-miRNAs reveal a differentially expression pattern in the diaphragm of 4-week-old mdx mice, compared to wild type littermates (Figures 1B–F). In particular, miR-31 and miR-34c were not modulated (Figures 1B,C); miR-206 was up-regulated (Figure 1D) and miR-449 and miR-335 were down-regulated (Figures 1E,F) in the diaphragm of 4-week-old mdx mice. The inflammatory miRNAs (miR-222 miR-223) had a similar level of expression between mdx and

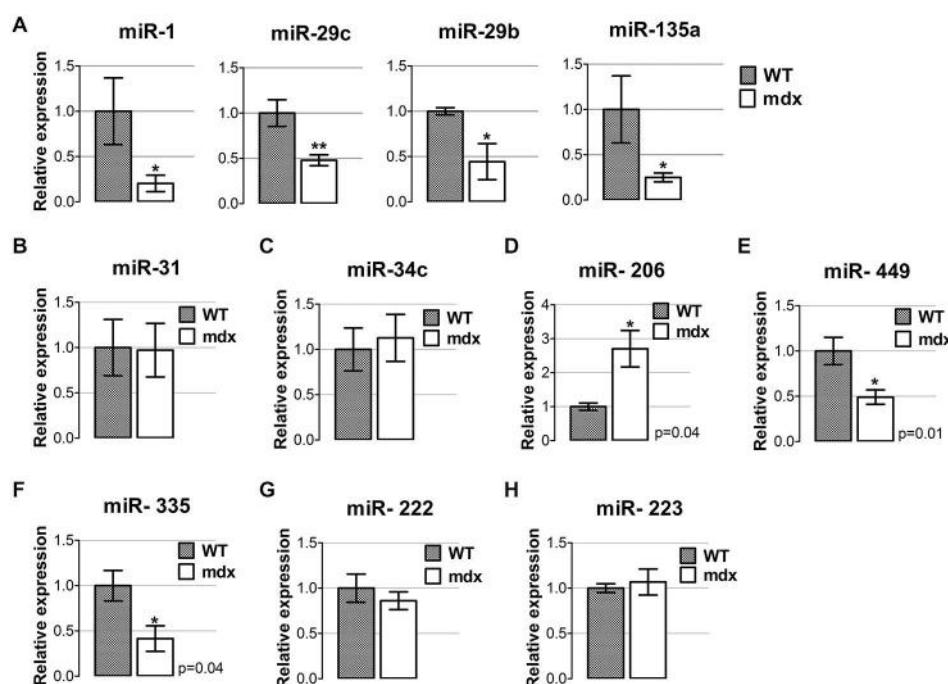


FIGURE 1 | miRNAs expression profile in diaphragm muscle of young mdx mice. (A) qRT-PCR analysis for the expression of miRNAs classified as degenerative miRNAs (Greco et al., 2009) (miR-1, miR-29c, miR-29b and miR-135a) performed on diaphragm muscles from wild-type (WT) and mdx mice at 4 weeks of age. All miRNAs were significantly down-regulated in dystrophic diaphragm of young mdx mice compared to WT. ** $p < 0.005$, * $p < 0.05$. **(B–F)** qRT-PCR analysis for the expression of miRNAs classified as regeneration miRNAs (Greco et al., 2009), such as miR-31 **(B)**, miR-34c

(C), miR-206 **(D)**, miR-449 **(E)** and miR-335 **(F)**, in diaphragm of 4-week old WT and mdx mice. **(G,H)** qRT-PCR analysis for the expression of miRNAs classified as inflammatory miRNAs (Greco et al., 2009), namely miR-222 **(G)** and miR-223 **(H)** in diaphragm muscles from WT and mdx mice at 4 weeks of age. For all graphs, relative expressions were normalized to U6 snRNA and shown with respect to WT set to a value of 1. Values represent mean \pm SEM; $n = 4\text{--}7$ per group. p values using Student's t test assuming two-tailed.

wild type mice. (Figures 1G,H). These data indicate that in diaphragm of young mdx mice the dystro-miRNAs have a specific pattern of expression that might correlate with the onset of dystropathology.

Muscle specific expression of mIGF-1 plays important anabolic role in skeletal muscle, promoting muscle growth and regeneration (Musarò et al., 2001, 2004; Pelosi et al., 2007) and counteracting muscle wasting in mdx dystrophic mice (Barton et al., 2002).

We verified whether mIGF-1 was able to modulate the expression of deregulated dystro-miRNAs in the diaphragm muscle of 4-week-old mdx mice.

QRT-PCR analysis revealed that miRNAs classified within the degenerative group, namely miR-1, miR-29b, miR-29c, and miR-135a (Greco et al., 2009) were expressed in similar manner in diaphragm muscle of both mdx and mdx/mIGF-1 mice (Figure 2A). This supports the evidence that there is a group of miRNAs that are strictly dependent on dystrophin expression (Cacchiarelli et al., 2010).

In contrast, mIGF-1 overexpression modulated regenerative miR-449 and miR-206 (Figures 2C,D) but not miR-335 expression (Figure 2B). In particular, we observed a down-regulation of miR-449 (Figure 2C), and an up-regulation of miR-206 (Figure 2D) in the diaphragm of 4-week-old mdx/mIGF-1 mice, compared to mdx littermates.

The up-regulation of the myomiR 206 suggests that mIGF-1 stabilizes the differentiated muscle phenotype, reducing the cycle of regeneration and degeneration and therefore the need to continuously activate satellite cells. We have recently demonstrated that in mdx mice, miR-206 facilitates satellite cell differentiation by restricting their proliferative potential through the repression of Pax-7 expression (Cacchiarelli et al., 2010). To support this hypothesis, we evaluated the expression of Pax-7, a marker of quiescent and activated satellite cells (Seale and Rudnicki, 2000), in both mdx and mdx/mIGF-1 mice. Pax-7 expression showed an inverse correlation with miR-206 levels, since it was lowly expressed in the diaphragm of mdx/mIGF-1 mice compared to mdx littermates (Figure 2E).

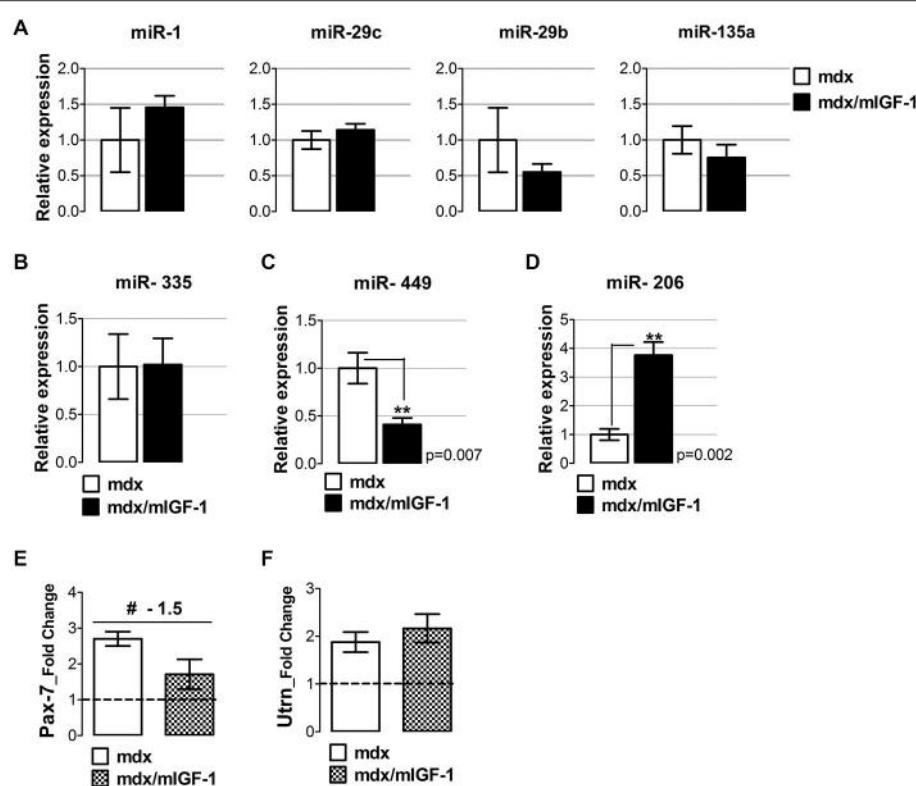


FIGURE 2 | mIGF-1 positively modulates regenerative miRNA expression in dystrophic muscle. (A) Total RNA from diaphragm of 4-week-old mdx/mIGF-1 mice and mdx littermates (control), were analyzed by qRT-PCR for degenerative-miRNAs expression (miR-1, miR-29c, miR-29b and miR-135a). Relative expressions were normalized to U6 snRNA and shown with respect to mdx set to a value of 1. Values represent mean \pm SEM; $n = 5\text{--}7$ per group. (B–D) Histograms show relative expression of regeneration- miRNAs: miR-335 (B), miR-449 (C), and miR-206 (D), measured by qRT-PCR in diaphragm of 4-week-old mdx and mdx/mIGF-1 mice.

Relative expressions were normalized to U6 snRNA and shown with respect to mdx littermates, set to a value of 1. Values represent mean \pm SEM; $n = 4\text{--}7$ per group. p values using Student's *t* test assuming two-tailed. (E,F) Graphs show the fold change of Pax-7 (E) and utrophin (F) expression levels from real time PCR analysis performed on diaphragm muscles of WT, mdx and mdx/mIGF-1 mice at 4 weeks of age. Relative expressions were normalized to HPRT1 and shown with respect to WT set to a value of 1 (dashed line). Values represent mean \pm SEM; $n = 4\text{--}6$ per group. ${}^{\#}p < 0.05$ using one way ANOVA.

Another potential target of miR-206 is utrophin (Rosenberg et al., 2006), a gene supposed to counteract the absence of dystrophin. Real time PCR analysis revealed that utrophin was up-regulated in both mdx and mdx/miGF-1 diaphragm (**Figure 2F**); however, we did not observe significant differences in utrophin gene expression between the two experimental models (**Figure 2F**), suggesting that the up-regulation of miR-206 did not impinge the expression of utrophin.

These data suggest that miGF-1 might enhance the differentiation program of dystrophic fibers, inducing an up-regulation of miR-206.

mIGF-1 Stimulates the Maturation of the Myogenic Program

To further support this hypothesis, we evaluated other relevant markers of the myogenic program and muscle maturation. A key myogenic factor that triggers myoblast differentiation is MyoD (Tassan-Got et al., 2005; Musarò, 2014), which resulted significantly up-regulated in the diaphragm of 4-week-old mdx/miGF-1 mice compared to that of mdx littermates (**Figure 3A**). Interestingly, MyoD showed a direct correlation with miR-206 levels (**Figure 2D**), confirming evidences that miR-206 is up-regulated by MyoD (Rosenberg et al., 2006) and targets Pax-7 mRNA (Cacchiarelli et al., 2010). Through this miR-206-mediated negative feedback mechanism, MyoD facilitates progression toward terminal differentiation (Chen et al., 2010; Hirai et al., 2010).

Myogenin is the myogenic factor that function downstream of MyoD and plays a critical role in the terminal differentiation of myoblasts (Nabeshima et al., 1993). Myogenin expression resulted significantly up-regulated in the diaphragm of mdx/miGF-1 mice compared to mdx littermates (**Figure 3B**).

The final stage of skeletal muscle differentiation and maturation program is dependent on the concerted action of myogenic factors, such as MEF-2C and MRF4.

Real time PCR analysis revealed a significant increase of both MEF-2C and MRF4 transcripts in the diaphragm from 4-week-old mdx/miGF-1 mice, compared to mdx littermates (**Figures 3C,D**), suggesting that miGF-1 favors the completion of the myogenic program.

To support this hypothesis, we analyzed relevant markers of a mature muscle phenotype such as β -enolase (ENO3) and MCK, which are down-stream myogenic factors of MEF-2C and myogenin. MCK and ENO3 expression levels were significantly enhanced in the diaphragm from 4-week-old mdx/miGF-1 mice, compared to mdx littermates (**Figures 3E,F**).

These data suggest that the maturation of the myogenic program, which is affected by the absence of dystrophin expression, is promoted by miGF-1 expression.

To further support the pro-myogenic activity of miGF-1, we analyzed the expression of another key player that functions during both differentiation and homeostatic maintenance of skeletal muscle tissues, namely the non-muscle-specific miR-24

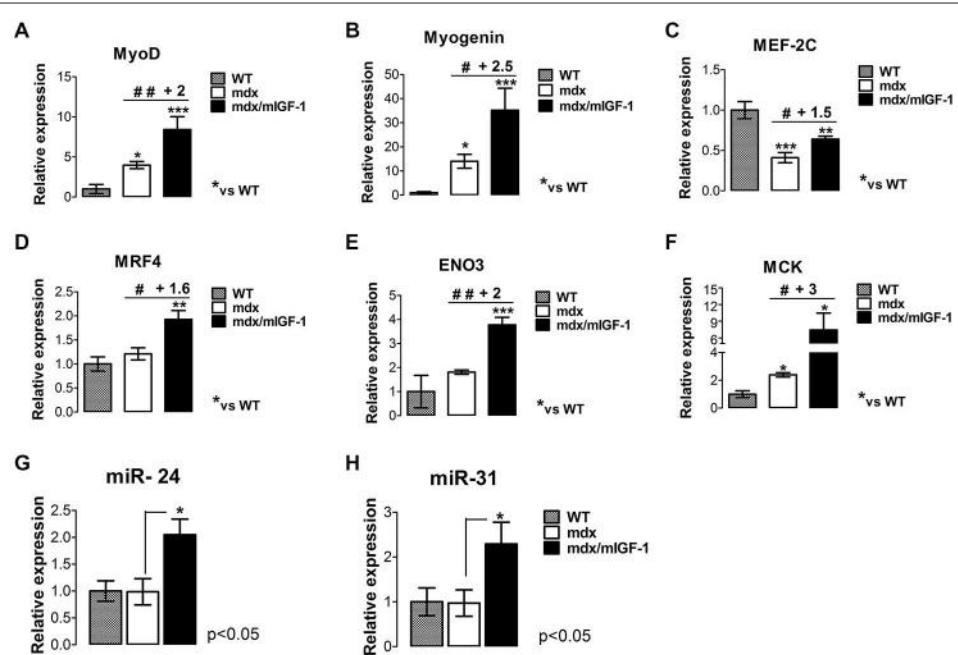


FIGURE 3 | mIGF-1 promotes maturation of the myogenic program in diaphragm muscle of mdx mice. **(A–F)** Expression of MyoD **(A)**, Myogenin **(B)**, MEF-2C **(C)**, MRF4 **(D)**, β -enolase (ENO3) **(E)**, and MCK **(F)**, evaluated by real time PCR analysis in diaphragms of indicated genotypes at 4 weeks of age. Relative expressions were normalized to HPRT1 and shown with respect to WT, set to a value of 1. Values represent mean \pm SEM; n = 4–7 per group.

*p < 0.05, **p < 0.005, ***p < 0.0005, #p < 0.05, ##p < 0.005 using one way ANOVA. **(G,H)** Histograms show miR-24 **(G)** and miR-31 **(H)** expression, analyzed by qRT-PCR in diaphragm muscles from WT, mdx, and mdx/miGF-1 mice at 4 weeks of age. Expression levels were normalized to U6 snRNA and shown with respect to WT set to value of 1. For all graphs, values represent mean \pm SEM; n = 5–7 per group. p values using one way ANOVA.

(Sun et al., 2008). In fact, in addition to being strongly induced during myogenesis, miR-24 expression is maintained at high levels in terminally differentiated muscle tissues (Sun et al., 2008). **Figure 3G** shows that miR-24 was significantly up-regulated in the diaphragm of 4-week-old mdx/mIGF-1 mice compared to mdx littermates.

Of interest was also the observation that local expression of mIGF-1 enhanced miR-31 expression in the diaphragm of mdx/mIGF-1 mice, compared to mdx littermates (**Figure 3H**). It has been demonstrated that miR-31 targets Myf5, maintaining the quiescence of satellite cell. In activated satellite cells the levels of miR-31 are reduced, leading to Myf5 protein accumulation and satellite cells activation/proliferation (Sun et al., 2008; Crist et al., 2012).

mIGF-1 Prevents the Activation of a Chronic Inflammatory Response

Muscle necrosis and inflammation became significantly apparent at 3–4 weeks of age in mdx mice (Tidball and Villalta, 2010). It has been reported that miR-222 and miR-223, classified as inflammatory miRNAs (Greco et al., 2009) were highly expressed in damaged areas of the ischemic muscle and adult mdx mice, whereas they were not induced in muscles of newborn mdx mice (Greco et al., 2009). We did not observe significant change in miR-222 and miR-223 expression in mdx nor mdx/mIGF-1 mice (**Figures 4A,B**), suggesting that these two specific miRNAs were not modulated in the diaphragm of 4-week-old dystrophic mice and by the expression of mIGF-1.

It is known that M1 macrophages predominate during the early, acute stage of inflammation in mdx muscle (Villalta et al., 2009) and the proinflammatory cytokine tumor necrosis factor alpha (TNF- α), strongly contributes to necrosis in the dystrophin-deficient fibers of the mdx mice (De Paepe and De Bleecker, 2013). Real time PCR analysis revealed a significant down-regulation of TNF- α in the diaphragm of mdx/mIGF-1 mice, compared to mdx littermates (**Figure 4C**). This suggests that mIGF-1, modulating specific factors of M1 phenotype, might attenuate the severity of muscle pathology in muscular dystrophy. To further support this hypothesis we analyzed miR-16 expression, which induces TNF- α mRNA degradation (Jing et al., 2005). miR-16 was indeed strongly downregulated in the diaphragm of 4-week-old mdx compared to wild type, while its expression was rescued in mdx/mIGF-1 diaphragm (**Figure 4D**).

Another chemokine that mediates the cytotoxic activities of M1 macrophages in DMD is MCP-1 (Villalta et al., 2009). Real time PCR analysis revealed a significant downregulation of MCP-1 in the diaphragm of mdx/mIGF-1 mice compared to mdx littermates (**Figure 4E**), indicating a reduction of macrophage-dependent inflammatory response in dystrophic niche. We did not observe any modulation of the inflammatory cytokines in the diaphragm of both healthy wild type and mIGF1 transgenic mice (data not shown).

These data suggest that mIGF-1 contributes to amelioration of dystrophic niche, interferes with the activation of a chronic inflammatory program, and guarantees a functional homeostatic maintenance of dystrophic muscle.

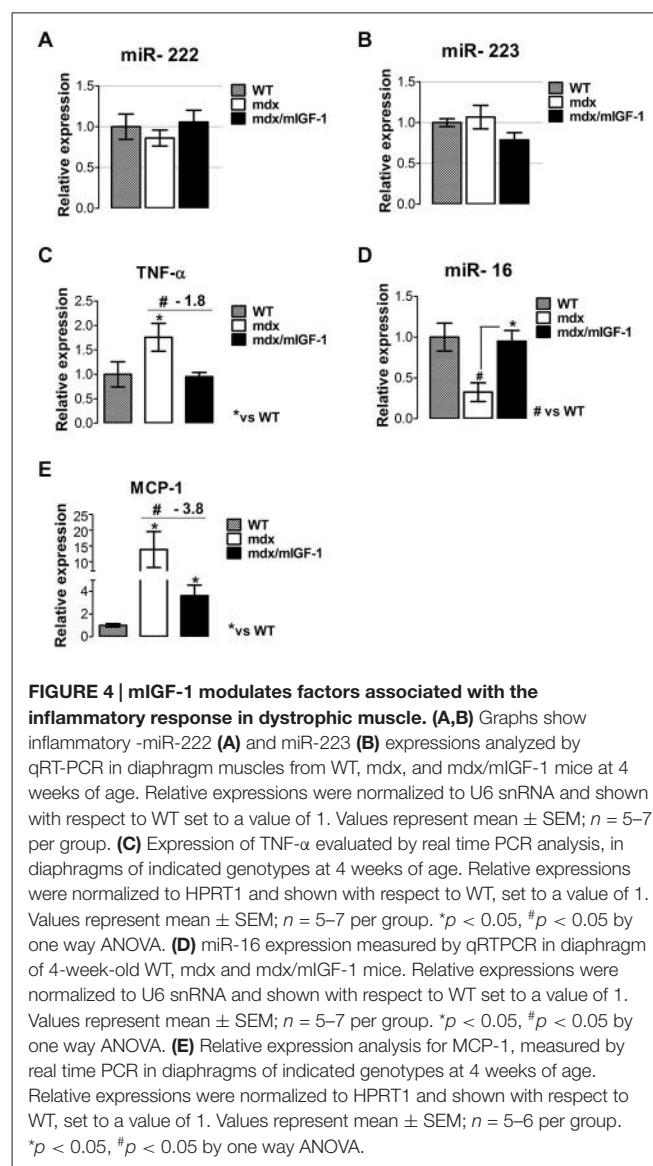


FIGURE 4 | mIGF-1 modulates factors associated with the inflammatory response in dystrophic muscle. **(A,B)** Graphs show inflammatory -miR-222 **(A)** and miR-223 **(B)** expressions analyzed by qRT-PCR in diaphragm muscles from WT, mdx, and mdx/mIGF-1 mice at 4 weeks of age. Relative expressions were normalized to U6 snRNA and shown with respect to WT set to a value of 1. Values represent mean \pm SEM; $n = 5\text{--}7$ per group. **(C)** Expression of TNF- α evaluated by real time PCR analysis, in diaphragms of indicated genotypes at 4 weeks of age. Relative expressions were normalized to HPRT1 and shown with respect to WT, set to a value of 1. Values represent mean \pm SEM; $n = 5\text{--}7$ per group. * $p < 0.05$, # $p < 0.05$ by one way ANOVA. **(D)** miR-16 expression measured by qRT-PCR in diaphragm of 4-week-old WT, mdx and mdx/mIGF-1 mice. Relative expressions were normalized to U6 snRNA and shown with respect to WT set to a value of 1. Values represent mean \pm SEM; $n = 5\text{--}7$ per group. * $p < 0.05$, # $p < 0.05$ by one way ANOVA. **(E)** Relative expression analysis for MCP-1, measured by real time PCR in diaphragms of indicated genotypes at 4 weeks of age. Relative expressions were normalized to HPRT1 and shown with respect to WT, set to a value of 1. Values represent mean \pm SEM; $n = 5\text{--}6$ per group. * $p < 0.05$, # $p < 0.05$ by one way ANOVA.

mIGF-1 Modulates Factors Associated with Adipogenic Differentiation

DMD is characterized by membrane fragility, myofibers necrosis and replacement of skeletal muscle by fibrous and fatty connective tissue, due to failed regeneration (Grounds et al., 2008). In order to verify whether mIGF-1 acts as an environmental cues controlling adipogenic differentiation, we analyzed relevant markers of molecular mediators of adipogenic phenotype, including PPAR γ and HADCs. PPAR γ is a master gene involved in adipogenic differentiation (Joe et al., 2010; Uezumi et al., 2010). In addition, it has been demonstrated that the reduction of adipogenic differentiation in young mdx mice, through Histone Deacetylase inhibition, counteracts DMD progression (Mozzetta et al., 2013; Saccone et al., 2014).

Real time PCR analysis revealed a strong down-regulation of PPAR γ in the diaphragm of 4-week-old mdx/mIGF-1

mice, compared to mdx littermates (**Figure 5A**). Moreover, we analyzed the expression of the upstream modulators of the fibro-adipogenic progenitors (FAPs) phenotype in young mdx mice, such as HADCs and myomiRs (Colussi et al., 2008; Cacchiarelli et al., 2010; Saccone et al., 2014). While HDAC4 did not show change, we observed a significantly reduction of HADC2 in mdx/miGF-1 compared to mdx muscle (**Figures 5B,C**). Moreover, myomiR-206 (**Figure 2D**) and myomiR-133a (**Figure 5D**), which ultimately lead to the activation of a pro-myogenic program at the expense of the fibro-adipogenic phenotype (Saccone et al., 2014), were strongly up-regulated in mdx/miGF-1 compared to mdx diaphragm. The molecular mechanisms that, in concert with environmental cues, control the identity and activity of muscle cells involve the BAF60C-based SWI/SNF complex (Saccone et al., 2014). We observed a significant up-regulation of BAF60C in the muscle of mdx/miGF-1 mice compared to mdx littermates (**Figure 5E**).

These data suggest that miGF-1 creates a qualitative environment that favors muscle differentiation and maturation.

Discussion

The major findings of this study indicate that muscle-specific expression of IGF-1 (miGF-1) can counter aspects of the muscular dystrophy associated with the loss of dystrophin,

modulating relevant molecules of the genetic and epigenetic circuitries in the mdx dystrophic mouse model.

The mdx mouse strain, lacking a functional dystrophin gene, has served as the animal model for human Duchenne and Becker muscular dystrophies (Hoffman et al., 1987). Moreover, it provides a convenient system to test possible therapeutic interventions as well as to select molecular markers that could be useful to monitor disease progression and therapeutic outcomes (Grounds et al., 2008).

The choice to analyse the effect of miGF-1 in the diaphragm of young (4 weeks of age) dystrophic mdx mice was based on the evidence that at this age there is an acute onset of pathology (increased myofiber necrosis and elevated blood CK), in which mdx mice display muscle weakness similarly to DMD patients (Grounds et al., 2008). Moreover, the diaphragm represents one of the most severely compromised muscle in mdx mice, more closely resembling the severe pathology of DMD (Stedman et al., 1991; Lynch et al., 1997; Grounds et al., 2008).

Among epigenetic factors, miRNAs represent a class of highly conserved small molecules of about 20–23 nucleotides long that regulate gene expression at post-transcriptional level. miRNAs participate in the regulation of several essential biological processes such as cell proliferation and apoptosis, cell differentiation, stress response, and immune regulation (Sayed and Abdellatif, 2011). Several miRNAs show a tissue or developmental

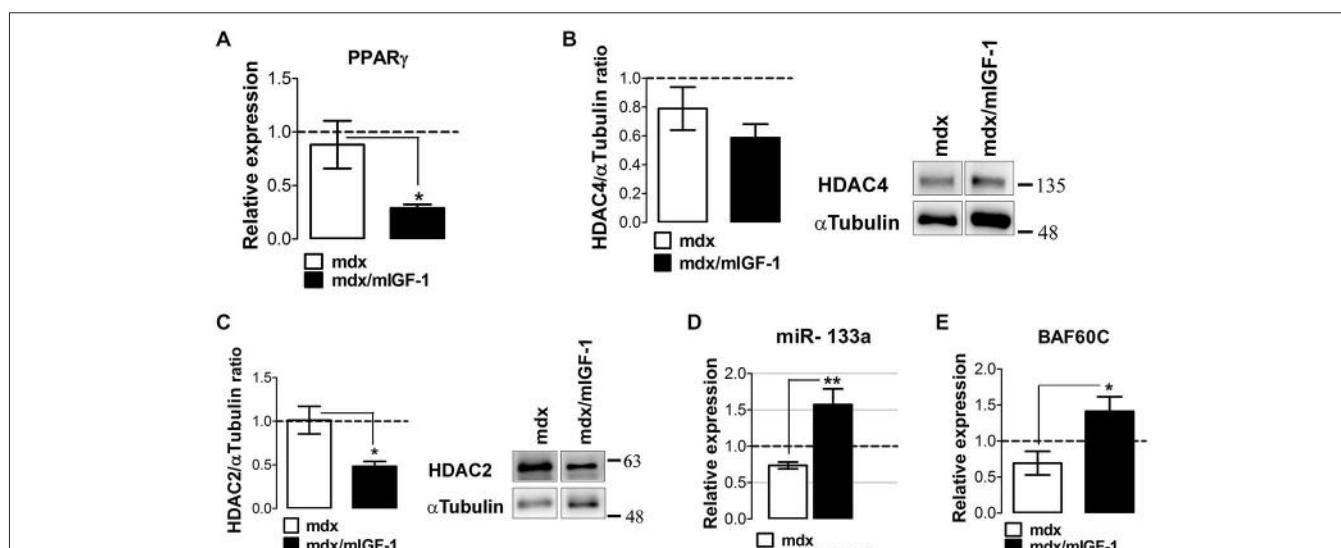


FIGURE 5 | miGF-1 modulates factors associated with adipogenic differentiation. **(A)** Graph shows the fold change of PPAR γ expression levels from qPCR analysis performed on diaphragm muscles of WT, mdx and mdx/miGF-1 mice at 4 week of age. Relative expressions were normalized to HPRT1 and shown with respect to WT, set to a value of 1 (dashed line). Values represent mean \pm SEM; $n = 4\text{--}6$ per group. * $p < 0.05$ using one way ANOVA. **(B,C)** Representative images of western blot analysis for the expression of HADAC4 (right panel, **B**) and HADAC2 (right panel, **C**) on diaphragm of 4-week-old wild type (WT), mdx, and mdx/miGF-1 mice. Left panels show densitometric analysis. Expression levels were normalized to HPRT1 and shown with respect to WT, set to a value of 1 (dashed line). Values represent mean \pm SEM; $n = 5\text{--}6$ per group. * $p < 0.05$ using one way ANOVA.

SEM; $n = 5\text{--}6$ per group. * $p < 0.05$ using one way ANOVA. In **(B)** and **(C)** the lanes were run on the same gel but were non contiguous. **(D)** miR-133a expression measured by qRT-PCR in diaphragm of 4-week-old wild type (WT), mdx and mdx/miGF-1 mice. Relative expressions were normalized to U6 snRNA and shown with respect to WT set to a value of 1 (dashed line). Values represent mean \pm SEM; $n = 4\text{--}7$ per group. ** $p < 0.005$, by one way ANOVA. **(E)** Graph shows the fold change of BAF60C expression levels from qPCR analysis performed on diaphragm muscles of WT, mdx and mdx/miGF-1 mice at 4 week of age. Relative expressions were normalized to HPRT1 and shown with respect to WT, set to a value of 1 (dashed line). Values represent mean \pm SEM; $n = 4\text{--}5$ per group. * $p < 0.05$ using one way ANOVA.

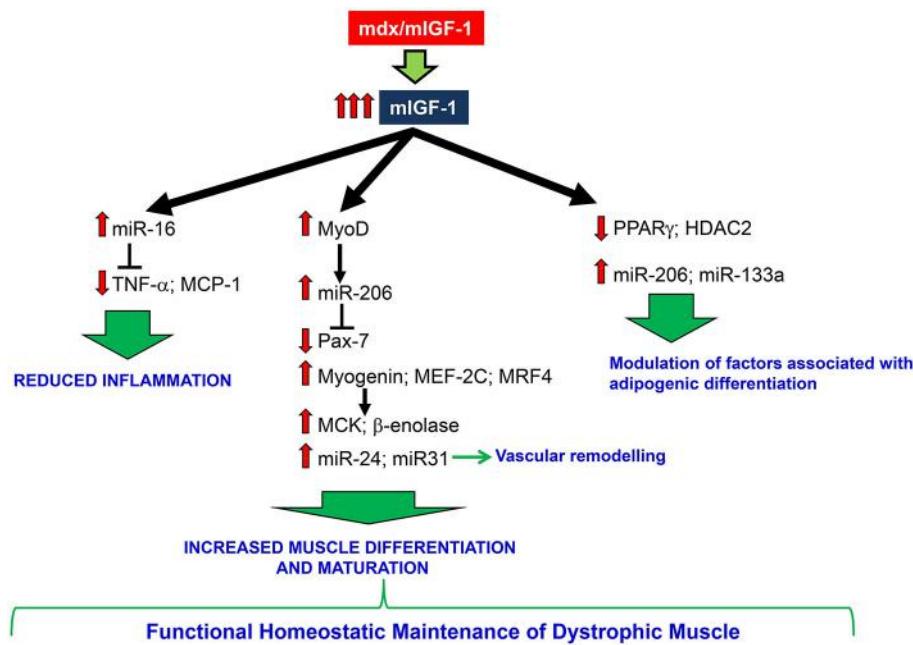


FIGURE 6 | A schematic model depicting the effects of mIGF-1 overexpression on dystrophic muscle. mIGF-1 overexpression ameliorates dystrophic niche, reducing inflammation, modulating factors associated with

adipogenic differentiation, and improving differentiation and maturation of regenerating myofibers. All of this contributes to functional homeostatic maintenance of dystrophic muscle.

specific expression pattern and are present in complex regulatory networks to govern stem cells function, tissue differentiation and maintenance of cell identity during development and adult life (Fazi and Nervi, 2008). Thus, altered expression of miRNAs may be associated with different pathologies.

Several studies have analyzed the expression of miRNAs in the dystrophic mdx mouse model, (Greco et al., 2009; Cacchiarelli et al., 2010; Roberts et al., 2012). The findings point to an unexpected layer of complexity in the mdx mouse miRNA transcriptome. The differences in miRNAs expression, observed in different studies using the same mdx mouse model, can be justified considering different parameters, namely the rescue of the dystrophin-mediated signaling, the age of mdx mice, and the severity of muscle districts (McCarthy et al., 2007; Grounds et al., 2008; Yuasa et al., 2008; Greco et al., 2009; Cacchiarelli et al., 2010).

In our study, we verified whether mIGF-1 expression was able to modulate dystrophic-signature miRNAs in the diaphragm muscle of 4-week-old mdx mouse model.

It has been demonstrated that when dystrophin synthesis was restored the levels of miR-1, miR-133a, miR-29c, miR-30c, and miR-206 increased, while miR-23a expression did not change (Cacchiarelli et al., 2010). At variance with the other myomiRs, miR-206 was highly expressed in mdx as well as in exon-skipping-treated animals (Cacchiarelli et al., 2010). On the other hand, local injection of the NO-donor nitroglycerin (NTG) in mdx mice increased miR-1 and miR-29 expression, whereas did not modulate miR-206 (Cacchiarelli et al., 2010).

In our study, we revealed that mIGF-1 expression was not able to modulate miR-29 and miR-1 expression in the mdx mouse model, further indicating that the expression of these miRNAs is strictly linked to the dystrophin rescue (Cacchiarelli et al., 2010).

Our data with mdx/mIGF-1 mice also support the evidences that miR-206, which it has been demonstrated to be expressed before dystrophin synthesis starts (Cacchiarelli et al., 2010), well correlates with its expression being independent from the Dystrophin/nNOS/HDAC2 pathway and might depict the potential of muscle regeneration and maturation activated by mIGF-1.

On the contrary, McCarthy et al. (2007) proposed that increased miR-206 expression may contribute to the chronic pathology of mdx diaphragm. In addition, miR-206 seems to be primarily involved in satellite cell impairment of dystrophic dogs, although its precise role needs to be elucidated (La Rovere et al., 2014). The apparent discrepancy among these studies and our findings can be justified considering that the up-regulation of miR-206 in the diaphragm of mdx/mIGF-1 might be related to its specific function in muscle differentiation instead in muscle pathology. This consideration is supported by the evidence that the other markers of muscle differentiation and maturation are positively modulated in the mdx/mIGF-1 mice compared to mdx littermates.

The transition from cell proliferation to differentiation and maturation involves the downregulation of proliferative-associated genes. Sustained expression of Pax-7 in satellite cells delays the onset of myogenesis, and elevated expression of Pax-7 in primary myoblasts inhibits the expression of

MyoD, preventing myogenin induction and muscle terminal differentiation (Olguin and Olwin, 2004; McFarlane et al., 2008).

We observed, in mdx/mIGF-1 mice compared to mdx animals, a downregulation of Pax-7 expression, associated with an increased in MyoD, myogenin, MCK and β -enolase expression, which represent relevant markers of differentiated and more mature muscle phenotype (Musarò, 2014). In addition, these data were strengthened by the up-regulation of miR-24 in mdx/mIGF-1 mice compared to mdx littermates. miR-24 is a non-muscle-specific miRNA involved in myogenesis; it is highly expressed in terminally differentiated muscle and it functions during both differentiation and homeostatic maintenance (Sun et al., 2008).

Our data are consistent with a model in which mIGF-1 stimulates muscle differentiation and maturation (Musarò and Rosenthal, 1999), by promoting the up-regulation of MyoD that, in turn, activates the expression of miR-206 (Rosenberg et al., 2006). miR-206 potently enhances the myogenic program by limiting and refining the expression of Pax-7 in myogenic progenitor cells (Chen et al., 2006, 2010; Cacchiarelli et al., 2010).

All of these data suggest that the maturation of the myogenic program and the homeostatic maintenance of dystrophic muscle tissues, which are severely affected by the absence of dystrophin expression, are facilitated by mIGF-1 expression. This might result in reduction in the cycle of regeneration and degeneration, which characterize the mdx dystrophic muscle, and therefore the need to continuously activate satellite cells. DMD is a disease of accelerated damage to muscle that causes the satellite cells to eventually be used up. Thus, mIGF-1, stabilizing the muscle phenotype, reduces the need to continuous use satellite cells, delaying the progression of disease.

Dystrophin is expressed not only in muscle cells but also in vascular endothelial cells (ECs). In DMD, the signaling defects produce inadequate tissue perfusion caused by functional ischemia due to a diminished ability to respond to shear stress induced endothelium-dependent dilation. It has been recently demonstrated that vascular densities is decreased and angiogenesis impaired in the muscles of mdx mice (Matsakas et al., 2013; Palladino et al., 2013; Shimizu-Motohashi and Asakura, 2014). Thus, increasing the density of the underlying vascular network in dystrophic muscle might be relevant therapeutic approach to reduce functional ischemia and to strength the muscle niche (Ennen et al., 2013).

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In our study, we observed that mIGF-1 modulates miR-31, which is involved in vascular remodeling, regulating the activity of endothelial progenitor cells, and it plays a fundamental role in postnatal vascular repair (Greco et al., 2009; Wang et al., 2014). We can speculate that mIGF-1, modulating relevant players of vascular remodeling, might enhance vascularization in mdx mice.

Control of the inflammatory response is a critical component of efficient muscle regeneration. A balance must therefore be struck between excessive and insufficient inflammatory action. Sustained inflammatory response represents one of the pathogenic events associated with muscular dystrophy (Tidball and Wehling-Henricks, 2005; Tidball and Villalta, 2010). Release of cytokines, especially TNF- α during the inflammatory response has a strong influence on the normal progression of the proliferative stage of inflammatory cells, and on the transition from acute to chronic inflammatory response (Waheed et al., 2005; De Paepe and De Bleeker, 2013).

In our study, we revealed that mIGF-1 up-regulated miR-16, which in turn stimulates the degradation of TNF- α and the inhibition of MCP-1 expression.

Of note, TNF- α and MCP-1 are significantly associated with clinical outcome of DMD patients (De Pasquale et al., 2012).

Overall our study provides additional insights into the complex effects of mIGF-1 on muscle homeostasis and diseases and reveals the potential miRNA signature associated with mIGF-1 expression in mdx dystrophic mice. However, although these results point towards some mechanisms of action of mIGF-1, the elevated number of potential targets of these miRNAs make these mechanisms only mere suggestions.

Our work is consistent with a model (Figure 6) in which overexpression of mIGF-1 confers robustness to dystrophic muscle, impedes the activation of a chronic inflammatory response, activates the circuitry of muscle differentiation and maturation. This results in a functional homeostatic maintenance of dystrophic muscle.

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Feasibility of resistance training in adult McArdle patients: clinical outcomes and muscle strength and mass benefits

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We analyzed the effects of a 4-month resistance (weight lifting) training program followed by a 2-month detraining period in 7 adult McArdle patients (5 female) on: muscle mass (assessed by DXA), strength, serum *creatine kinase* (CK) activity and clinical severity. Adherence to training was $\geq 84\%$ in all patients and no major contraindication or side effect was noted during the training or strength assessment sessions. The training program had a significant impact on total and lower extremities' lean mass ($P < 0.05$ for the time effect), with mean values increasing with training by +855 g (95% confidence interval (CI): 30, 1679) and +547 g (95% CI: 116, 978), respectively, and significantly decreasing with detraining. Body fat showed no significant changes over the study period. Bench press and half-squat performance, expressed as the highest value of average muscle power (W) or force (N) in the concentric-repetition phase of both tests showed a consistent increase over the 4-month training period, and decreased with detraining. Yet muscle strength and power detraining values were significantly higher than pre-training values, indicating that a training effect was still present after detraining. Importantly, all the participants, with no exception, showed a clear gain in muscle strength after the 4-month training period, e.g., bench press: +52 W (95% CI: 13, 91); half-squat: +173 W (95% CI: 96, 251). No significant time effect ($P > 0.05$) was noted for baseline or post strength assessment values of serum CK activity, which remained essentially within the range reported in our laboratory for McArdle patients. All the patients changed to a lower severity class with training, such that none of them were in the highest disease severity class (3) after the intervention and, as such, they did not have fixed muscle weakness after training. Clinical improvements were retained, in all but one patient, after detraining, such that after detraining all patients were classed as class 1 for disease severity.

Keywords: rhabdomyolysis, muscle atrophy, muscle weakness, glycogenosis, weight lifting, exercise is medicine

INTRODUCTION

There is an urgent need to improve the treatment and prevention of aging sarcopenia as well as the muscle atrophy commonly associated with chronic disorders. Although several pharmacological therapies have been proposed to minimize sarcopenia and atrophy, they are not free of adverse side effects (Sanchis-Gomar et al., 2014). Physical exercise, particularly resistance (strength) exercise (i.e., movement, such as weight lifting or exercises with resistance bands, performed against a specific external force that is regularly increased during training), is an alternative approach to reverse muscle atrophy, although it is frequently overlooked in the clinical setting. Yet this type of exercise should form part of the routine exercise prescription to maintain and improve health and functional status in most, if not all population groups (Garber et al., 2011).

McArdle disease (glycogenosis (or glycogen storage disease) type V or *myophosphorylase* deficiency; OMIM® database number 232600), is an inborn disorder of skeletal-muscle carbohydrate metabolism characterized by failure of muscle glycogen breakdown. McArdle disease is a challenging disease model in which to study the feasibility and effects of resistance exercise in debilitated people: this disorder is arguably the paradigm of human exercise intolerance and rhabdomyolysis (Lucia et al., 2008). The latter is reflected by the efflux of intra-muscle proteins to the bloodstream, e.g., *creatine kinase* (CK) and myoglobin. Thus, high serum CK activity (typically $> 1,000 \text{ U} \cdot \text{L}^{-1}$) caused by exercise is a common finding in these patients, which can be accompanied by myoglobinuria, typically referred to as "dark urine" (Santalla et al., 2014). Additionally, fixed muscle weakness is an incident problem as patients age (Santalla et al., 2014). Despite some

studies showing the benefits of light-moderate intensity aerobic exercise in patients with McArdle disease (Haller et al., 2006; Maté-Muñoz et al., 2007; Perez et al., 2008), at present, clinicians in charge of these patients discourage performance of strenuous exercise, particularly resistance exercise (e.g., weight lifting), owing to a potential increased risk of severe rhabdomyolysis, which might eventually lead to acute renal failure in the most severe cases (Lucia et al., 2008). Yet preliminary data from our group indicated increases in dynamic muscle strength with no myoglobinuria in a 14-year-old male patient with McArdle disease in response to a 6-week, supervised light-moderate intensity weight lifting training program (García-Benítez et al., 2013). However, muscle mass, an important health indicator, was not determined.

We assessed the effects of a 4-month resistance training program followed by a 2-month detraining period in a group of adult McArdle patients on the following outcomes: muscle mass and strength, serum CK activity, and clinical severity.

METHODS

PATIENTS

Before entering the study, written informed consent was obtained from each participant, and the study was approved by the local human investigations committee and review board. Inclusion criteria were: adult with no disease contraindicating exercise other than McArdle disease, belonging to class severity 1–3 (Martinuzzi et al., 2003) (see below), living in the Madrid area (or willing to move there for the duration of the study period) to participate in all testing and training sessions. Eight McArdle patients (5 female) originally volunteered to participate in this study but one of them (male) withdrew because he had to move to a different country due to professional obligations after the first strength assessments. Genetic diagnosis was confirmed in all patients, i.e., they harbored documented pathogenic genotypes in the gene (*PYGM*) encoding *myophosphorylase* (Lucia et al., 2012), as shown in Table 1 (main demographic and clinical characteristics). All subjects reported symptoms of exercise intolerance since childhood and 4 belonged to the highest severity class, i.e., class 3 (that is, they had fixed muscle weakness) (Martinuzzi et al., 2003).

DESIGN

All study outcomes were assessed in each patient at 3 time points: before (baseline) and after the 4-month training period (“post-training”), and after detraining. Muscle strength and serum CK activity (see below) were also measured at the end of the 1st, 2nd and 3rd month of the training period. Owing to the relative rare nature of the disease (prevalence of ~1/167,000 in Spain) (Lucia et al., 2012), it was not possible to gather a sufficient number of patients to conduct a randomized controlled trial. Thus, a quasi-experimental reversal design was used, in which each subject acts as their own control (Thomas et al., 2005).

TRAINING PROGRAM

All training sessions and strength evaluations were performed in the same setting, i.e., in the gymnasium of the *Universidad Europea* (Madrid, Spain) and were supervised by experienced professionals (fitness instructors with a Master degree in Sports Science, 1 instructor/patient). Two familiarization sessions were performed by each subject, prior to starting the training program, which included 2 weekly sessions for 4 months (total of planned sessions = 32). A recovery period of at least 48 h was allowed between sessions, and the vast majority of the sessions were performed during week days. Make-up sessions were allowed (including during weekend days) when 1 session was missed and if fulfilling the criteria of ≥48 h of recovery between sessions.

Before each training or strength assessment session (see below), patients performed 2 consecutive warm-up sessions of 12-minute duration each, the first on an arm-crank ergometer and the second on a cycle-ergometer, in order to trigger the occurrence of the “second wind” (that is, the attenuation of early fatigue, increased risk of contractures and rhabdomyolysis that commonly occurs after 7–8 min of dynamic exercise in these patients (Vissing and Haller, 2003)) in both upper and lower body muscles, respectively. The end of the warm-up was followed by ingestion of a commercialized sports drink (330 mL, containing ~30 g of sucrose). All training and strength assessment sessions were followed by passive stretching exercises and hydration with plain water. We did not perform dietary analysis but patients were instructed to consume a high proportion (65%) of complex

Table 1 | Main characteristics of the study participants at the start of the study.

Subject	Sex	Age (years)	BMI (kg·m ⁻²)	PYGM genotype	Diagnostic corroborated by muscle biopsy*	Resting CK (U·L ⁻¹)	Fixed muscle weakness	Clinical severity class
A	Female	36	18.3	<i>R50X/p.V456M</i>	Yes	1076	Yes	3
B	Female	34	30.6	<i>R50X/W798R</i>	Yes	3938	Yes	3
C	Male	23	21.7	<i>G205S/c.1768+1G > A</i>	No	1556	No	1
D	Male	29	23.7	<i>G205S/c.1768+1G > A</i>	Yes	2050	No	2
E	Female	36	21.9	<i>p.R50X/p.R50X</i>	Yes	1373	No	2
F	Female	58	27.4	<i>R50X/p.K754fsX49</i>	Yes	1211	Yes	2
G	Female	53	29.1	<i>G205WS/R590H</i>	Yes	543	Yes	3

Abbreviations: BMI, body mass index. * Negative histochemical reaction for myophosphorylase and no myophosphorylase activity.

Note: Subjects C and D were brothers. Muscle fixed weakness affected mostly proximal/trunk (paraspinal, neck flexor, periscapular, proximal upper limb, axial or shoulder girdle) muscles in a symmetric manner in patients A, B and F, whereas it affected mostly lower extremities' muscles (also in a symmetric manner) in patient G. See text for description of clinical severity classes.

Table 2 | Mean ± SEM values of body composition assessed by dual energy x-ray absorptiometry (DXA).

Outcome	Pre-training	Post-training	Detraining	Time effect	Pre- vs. post-training	Pre- vs. detraining	Post- vs. detraining
Lean mass							
Total (g)	43,089 ± 1,997	43,944 ± 1,935	42,822 ± 2,099	P = 0.018	P = 0.043	P = 0.917	P = 0.018
Trunk (g)	21,179 ± 869	21,378 ± 833	20,928 ± 882	P = 0.772	-	-	-
Legs (g)	14,662 ± 825	15,209 ± 815	14,688 ± 889	P = 0.018	P = 0.043	P = 0.753	P = 0.018
Arms (g)	4,110 ± 353	4,206 ± 381	4,008 ± 398	P = 0.121	-	-	-
Body fat (g)	21,571 ± 2,880	21,473 ± 2,896	21,009 ± 2,625	P = 0.368	-	-	-

Significant P-values are in bold. Pairwise post hoc comparisons (Wilcoxon test) were only performed when a significant time effect was found.

carbohydrates (fruits, cereals, bread, pasta, rice) in the 2 meals (breakfast and lunch) that preceded each testing or training session (García-Benítez et al., 2013).

Except for the first 8 sessions of the program (where sets of 10 repetitions with very low loads were performed), the exercises composing sessions were performed for sets of a low number of repetitions (5–6) using a load (kg) eliciting a rating of perceived exertion (RPE) of 6–7 (on a 0 (= minimum effort) to 10 (= maximum effort) scale). Exercises were performed using a circuit involving large muscle groups and specific weight training equipment (Technogym; Gambettola, Italy), in the following order: bench press, leg press, pull down and abdominals. At the end of the 1st month, the leg and bench press exercises were gradually replaced by the half squat and bench press performed on a “multipower” machine (Technogym; Gambettola, Italy). The low number of repetitions allows the use of muscle phosphocreatine (PC) as the main energy substrate to fuel contraction, with no major reliance on muscle glycogen deposits and the circuit structure, with 2–3-min rest periods between each set of repetitions and exercises, was designed to allow PC to be resynthesized in a given muscle before this muscle was utilized again.

Passive stretching exercises were performed after each set of an exercise to attenuate muscle stiffness (3×30 s for each muscle group). The load was adjusted after the 1st month and thereafter was readjusted according to the results of the last strength assessment, with the purpose of reaching peak power with a similar number of sets (i.e., 4–6 sets for bench press and 6–8 sets for half-squat). The rate of increase in training load was constantly adjusted according to the patients' RPE. Thus, when the patient reported a RPE value of 6 for a given exercise in 2 consecutive sessions, the load for this particular exercise was increased (with the premise that RPE for the new load remained ≤ 7). On average, the load used for an exercise increased from the start to the end of the training program as follows: 5.4 kg → 12.8 kg (+139.7%) for chest press; 34.9 kg → 12.7 kg (+174%) for half-squat; 10.3 kg → 19.2 kg (+85.9%) for pull down; and 3.9 kg → 18.3 kg (+368.1%) for abdominal muscles.

OUTCOME ASSESSMENT

After familiarization with the equipment, participants performed “explosive” leg half-squats on a “Multipower machine”, which was connected to a linear encoder (T-Force Dynamic Measurement System, Ergotech, Murcia, Spain). The latter has previously proven valid to determine force (N) and power (W)

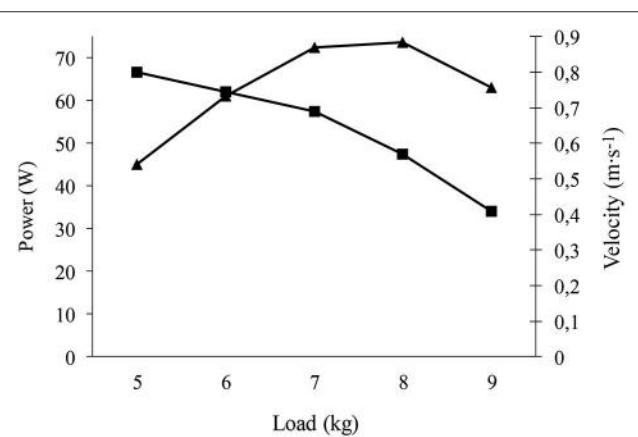


FIGURE 1 | Example of strength test (bench press) performed by one of the patients. The Figure shows that the velocity ($m\cdot s^{-1}$) decreases with increasing load (kg), while the average concentric-propulsive power initially increases to a load in which the decrease of velocity is so pronounced that power decreases. The highest average concentric-propulsive power of 73 W occurred at a load of 8 kg.

(González-Badillo and Sánchez-Medina, 2010). Patients performed 1 set of 3 repetitions at maximum speed with a 2-min recovery period. The load or “resistance” (kg) was increased by 2.5 kg in each successive set. Average muscle force and power output in the concentric-propulsive phase of the repetition were evaluated in each set (Sanchez-Medina et al., 2010). In this type of gradual resistance increase protocol, the developed muscle force increases with resistance, while the velocity of muscle contraction decreases. Power is the product of force \times velocity, and initially increases with resistance and then decreases when the resistance causes a substantial decrease in velocity. Thus, the test is stopped when the decrease in velocity is so pronounced that it causes a decrease in average muscle concentric power (see Figure 1 for an example). For statistical analyses we recorded the highest value of average power (W) in the concentric-propulsive phase, which typically coincides with the start of a decline in this variable together with the occurrence of the highest value of average force (N). We also recorded the load (kg) at which maximum average power was generated. The RPE after each set never exceeded the value of 7.

Total and regional body composition was assessed by dual energy x-ray absorptiometry (DXA; Hologic Serie Discovery QDR, Software Physician's Viewer, APEX System Software Version

3.1.2. Bedford, MA, USA). Body mass composition was calculated from whole-body scans. Whole-body scans were submitted to a regional analysis to determine the composition of the arm, leg and trunk regions. The arm region included the hand, forearm and arm, and was separated from the trunk by an inclined line crossing the scapulo-humeral joint, such that the humeral head was located in the arm region. The leg region included the foot, the lower leg and the upper leg. It was separated from the trunk by an inclined line passing just below the pelvis, which crossed the neck of the femur. The trunk region included the entire body except the arms, legs and head regions. The head region comprised all skeletal parts of the skull and cervical vertebra above a horizontal line passing just below the jawbone. With this analysis, regional body fat and lean mass can be assessed with a coefficient of variation below 5% (Calbet et al., 1998).

Peripheral venous blood was collected from all subjects to determine serum total CK activity, a widely used marker of skeletal muscle damage (Sorichter et al., 1999). CK activity was determined using a standard photometric analyzer (Hitachi 911, Boehringer Mannheim, Mannheim, Germany), at baseline (under “resting” conditions, that is, after 24 or more hours with physical activities restricted to the minimum) at the following time points: pre-training, before each strength assessment (end of 1st, 2nd, 3rd and 4th month), and after detraining. Serum total CK activity was also measured 1 h after each strength assessment (end of 1st, 2nd, 3rd and 4th month). The mean baseline values in our laboratory for adult male and female McArdle patients are $3,069 \pm 2,356$ and 1686.2 ± 1964.8 U·L⁻¹, respectively, whereas the mean baseline values for healthy aged-matched controls are 151 ± 48 and 97.7 ± 35.7 U·L⁻¹, respectively.

Identification of patient clinical features allowed us to allocate them to one of the following clinical severity classes according to the most commonly used phenotype severity scale (Martinuzzi et al., 2003): “0 = asymptomatic or virtually asymptomatic (mild exercise intolerance, but no functional limitation in any daily life activity); 1 = exercise intolerance, contractures, myalgia, and limitation of acute strenuous exercise, and occasionally in daily life activities; no record of myoglobinuria, no muscle wasting or weakness; 2 = same as 1, plus recurrent exertional myoglobinuria, moderate restriction in exercise, and limitation in daily life activities; 3 = same as 2, plus fixed muscle weakness, with or without wasting and severe limitations on exercise and most daily life activities”.

STATISTICAL ANALYSIS

In order to decrease the risk of statistical type I error, we used the nonparametric Friedman test (instead of a repeated-measures ANOVA) to compare within subjects the mean values of all the variables measured at the different time points. All statistical tests were performed using the Social Sciences package (SPSS, 2010, IBM SPSS Statistics 19 Core System User’s Guide; SPSS Inc., Chicago, IL). Also to avoid type I error, *post hoc* pairwise comparisons were only performed when a significant time effect was found. Significance was set at $\alpha = 0.05$ and results are expressed as means \pm standard error of the mean (SEM).

RESULTS

ADHERENCE AND SIDE EFFECTS

Adherence to training was 100% in 5 patients and 84% in the 2 remaining patients (with reasons for missing sessions being independent from the training itself, i.e., viral respiratory infection in 1 patient and household or children care tasks in the other one). No major contraindication was noted during the training or strength assessment sessions other than the usual muscle discomfort and soreness associated with resistance exercise in non-habituated people (especially during the initial sessions). We only had to interrupt a given set of repetitions due to muscle stiffness on 5 occasions (2 patients). No episode of myoglobinuria (i.e., no occurrence of “dark urine”) was reported, which is consistent with the fact that serum CK activity levels showed no major increases above the reference limits for this population (see below).

The training program had a significant impact on total and lower extremities’ lean mass ($P < 0.05$ for the time effect), with mean values increasing with training and decreasing with detraining (Table 2). In contrast, body fat mass remained essentially unchanged ($P > 0.05$). The increase in total or lower extremities’ lean mass from pre- to post-training averaged +855 g (95% confidence interval (CI): 30, 1679) and +547 g (95% CI: 116, 978), respectively, with all patients showing an increase with training, except one patient (i.e., patient B, 33 years, severity class 3). Detraining resulted in a significant decrease in total ($-1,222$ g (95%CI: $-2,585$, 340)) and lower extremities’ lean mass compared to post-training (-521 g (95%CI: -846 , -197)). Detraining values of total and lower extremities’ lean mass were not significantly different from pre-training values.

The results of upper body (bench press) or lower body muscle strength (half-squat), expressed as the highest value of average muscle power (W, Figure 2) or force (N) in the concentric-propulsive phase of repetitions (Figure 3), or as the load (kg) eliciting such values (Figure 4), showed the following overall pattern: consistent increase (e.g., bench press: +52 W (95% CI: 13, 91); half-squat: +173 W (95% CI: 96, 251)) over the 4-month training period (such that post-training values were significantly higher than pre-training values), and a decline after detraining. Detraining resulted in a significant loss compared to post-training, but strength after detraining was still significantly greater than at pre-training. Importantly, all the participants, with no exception, showed a clear gain in muscle strength after the 4-month training period.

No significant time effect ($P > 0.05$) was noted for baseline or post-strength assessment values of serum CK activity (Figure 5), which remained essentially within the range values reported in our laboratory for McArdle patients (and well below the upper limit value of 95% CI (3,387 U·L⁻¹)), indicating that the training program did not induce major increases in CK-emia. Further, an increasing trend in CK levels was noted in the 1st month of training that was reversed thereafter, which reflected a positive adaptation to the program.

The clinical course of the disease is shown in Figure 6. All patients changed to a lower severity class with training, such that none of them belonged to the highest disease severity category (class 3) after training and, as such, did not have fixed muscle

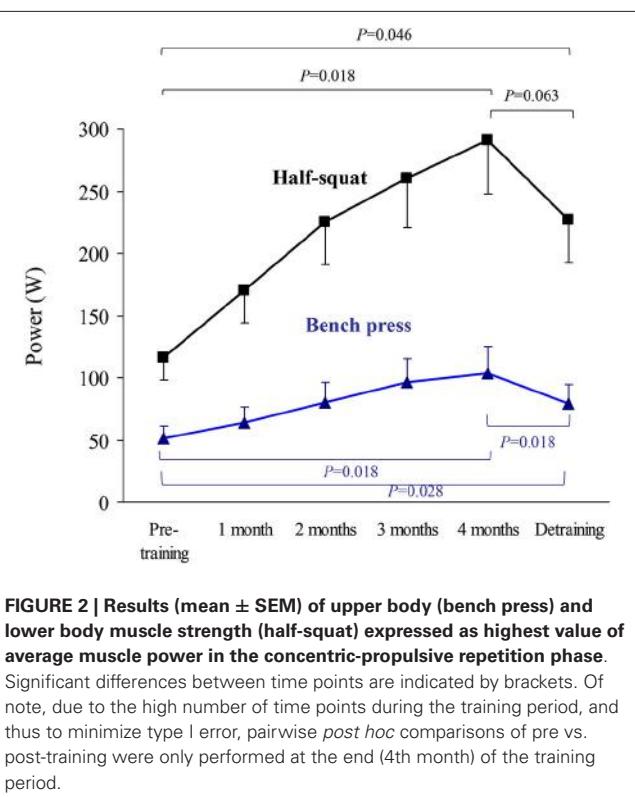


FIGURE 2 | Results (mean \pm SEM) of upper body (bench press) and lower body muscle strength (half-squat) expressed as highest value of average muscle power in the concentric-propulsive repetition phase.

Significant differences between time points are indicated by brackets. Of note, due to the high number of time points during the training period, and thus to minimize type I error, pairwise *post hoc* comparisons of pre vs. post-training were only performed at the end (4th month) of the training period.

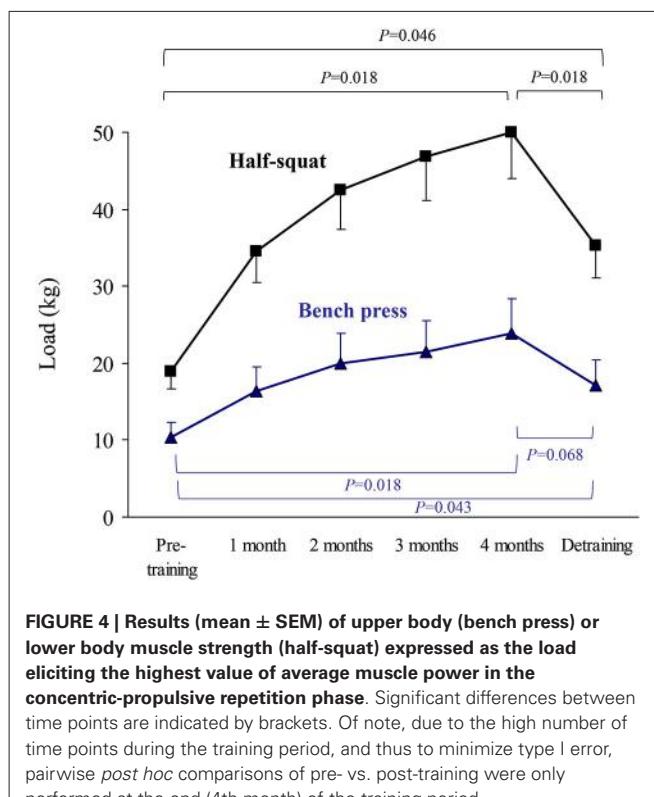


FIGURE 4 | Results (mean \pm SEM) of upper body (bench press) or lower body muscle strength (half-squat) expressed as the load eliciting the highest value of average muscle power in the concentric-propulsive repetition phase.

Significant differences between time points are indicated by brackets. Of note, due to the high number of time points during the training period, and thus to minimize type I error, pairwise *post hoc* comparisons of pre- vs. post-training were only performed at the end (4th month) of the training period.

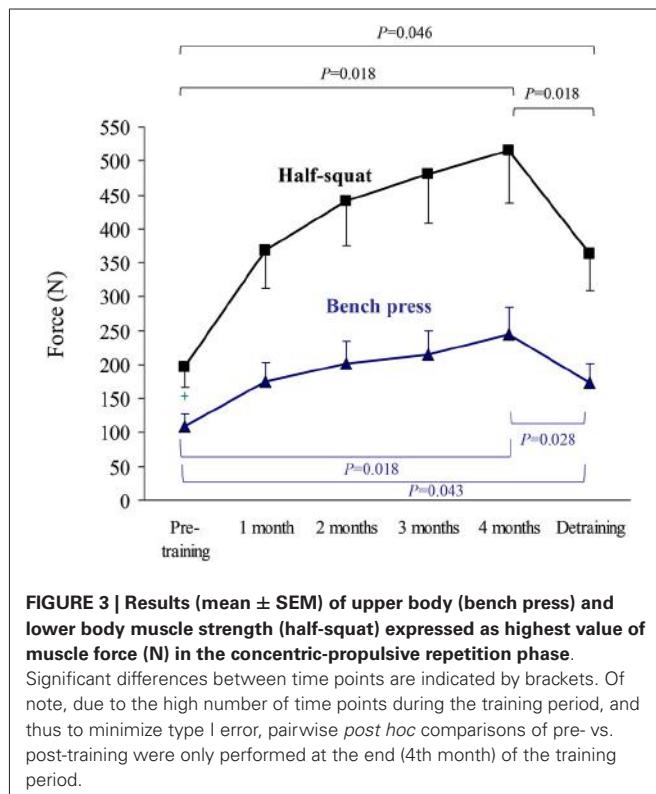


FIGURE 3 | Results (mean \pm SEM) of upper body (bench press) and lower body muscle strength (half-squat) expressed as highest value of muscle force (N) in the concentric-propulsive repetition phase.

Significant differences between time points are indicated by brackets. Of note, due to the high number of time points during the training period, and thus to minimize type I error, pairwise *post hoc* comparisons of pre- vs. post-training were only performed at the end (4th month) of the training period.

weakness anymore. Further, 2 patients moved to class 0, which is essentially symptom-free. Most of the clinical improvements

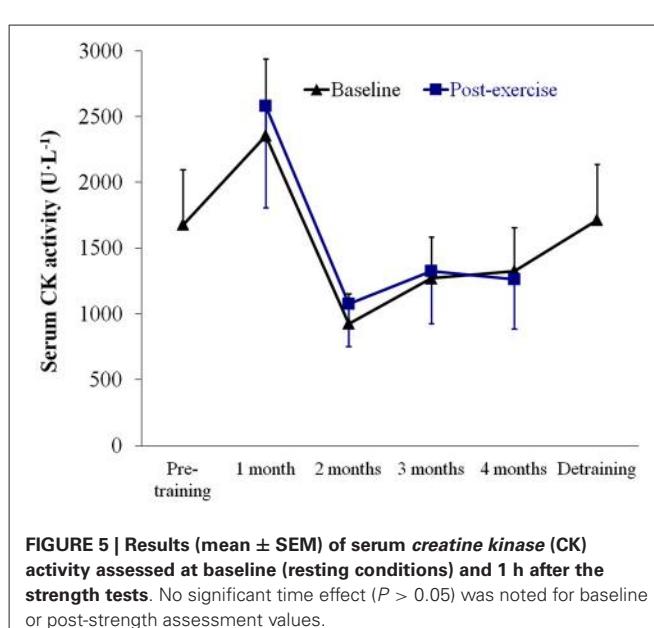
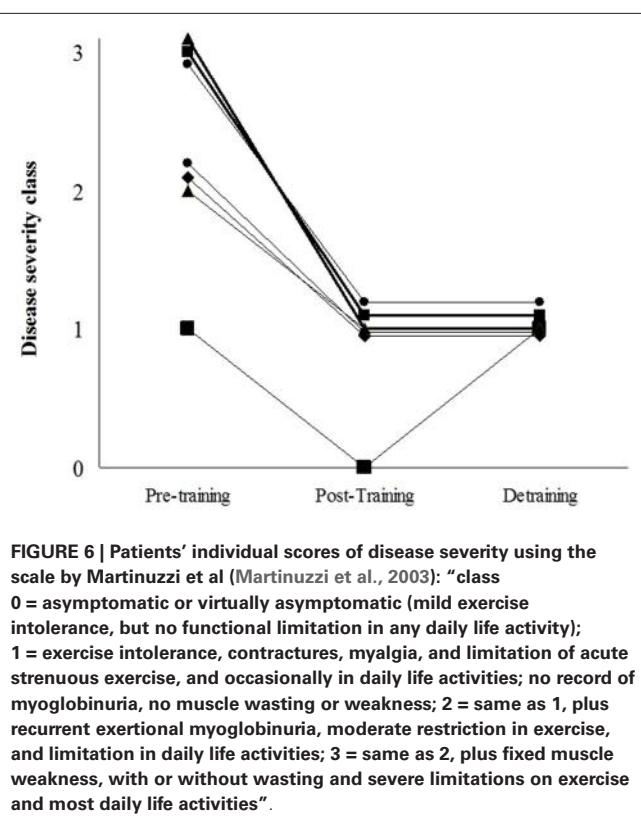


FIGURE 5 | Results (mean \pm SEM) of serum creatine kinase (CK) activity assessed at baseline (resting conditions) and 1 h after the strength tests. No significant time effect ($P > 0.05$) was noted for baseline or post-strength assessment values.

(except for one patient) were retained at detraining, that is, all patients were classed as class 1 for disease severity.

DISCUSSION

The main, novel findings of our study were threefold. First, the training program was feasible, free of noticeable adverse effects (with no episode of myoglobinuria and with CK-emia remaining



within safe limits) and well tolerated by the patients. This is especially relevant when considering that ~60% of them originally belonged to the highest severity class and thus had fixed muscle weakness and limitations during daily living activities such as house-hold tasks. Second, the training program significantly increased total and lower extremities' lean mass, with all the patients except one showing an improvement in these variables. This increase in lean mass was reflected in the consistent increase (>twofold) of upper and lower body strength over the 4-month training period, with *all* the patients showing an improvement. Importantly, training-induced gains were not totally lost with detraining. Finally, the program had direct clinical benefits, because clinical severity decreased with training. Notably, after training, all the patients moved to severity classes 0–1, that is, they became virtually asymptomatic (class 0) or at most had occasional limitations in daily life activities (class 1). In addition, the improvements in clinical course were essentially retained at detraining, which is consistent with the fact that previous gains in muscle strength were not completely lost after detraining. To date, we are not aware of any therapeutic intervention showing such practical benefits in McArdle patients, and arguably of any other lifestyle intervention able to induce such clinical improvement in genetic neuromuscular disorders. No significant beneficial effects have been reported in McArdle patients receiving branched chain amino acids (MacLean et al., 1998), depot glucagon (Day and Mastaglia, 1985), dantrolene sodium (Poels et al., 1990), verapamil (Lane et al., 1986), vitamin B6 (Phoenix et al., 1998) (except in one recent case report (Sato et al., 2012)), or high-dose oral ribose (Steele et al., 1996). More controversial are the

effects of creatine supplementation: low-dose supplementation ($60 \text{ mg kg}^{-1} \cdot \text{day}^{-1}$ for 4 weeks) attenuated muscle complaints in 5 out of the 9 McArdle patients (Vorgerd et al., 2000) but higher doses ($150 \text{ mg kg}^{-1} \cdot \text{day}^{-1}$) actually exacerbated exercise-induced myalgia for unknown reasons (Vorgerd et al., 2002). A 12-week treatment with the angiotensin converting enzyme (ACE) inhibitor ramipril (2.5 mg day^{-1}) attenuated disability in McArdle patients, but the effect was more marked in those harboring the D/D genotype of the insertion(I)/deletion(D) polymorphism in the ACE gene (Martinuzzi et al., 2008). A short-term trial (10 days) with a “read through” compound able to synthesize full proteins from transcripts containing premature termination stop codons (i.e., gentamicin) failed to normalize 31P magnetic resonance spectroscopy indicators of *myophosphorylase* deficiency in the muscle of McArdle patients (Schroers et al., 2006).

This is the first report on the feasibility and functional and clinical effects of a resistance training program in adult McArdle patients. The data are novel and we believe that assessing the applicability of this type of program in adults with McArdle disease is of medical interest because this type of exercise should form part of the routine exercise prescription to maintain and improve health and functional status in most, if not all population groups (Garber et al., 2011). Resistance training is also gaining growing attention for its effectiveness in attenuating aging sarcopenia as well as the status of muscle weakness and atrophy that accompanies most chronic conditions, such as neuromuscular disorders (Sanchis-Gomar et al., 2014). This is an important consideration also in McArdle disease because the baseline values of muscle mass and strength of the study participants were quite low compared to the general population. For instance, the mean total lean mass (~42 kg) of the 5 female patients (age range: 34–48 years) at the start of the program, was only ~6% higher than the values recently reported in old, sedentary Spanish women (mean age 75 years) (Gómez-Cabello et al., 2013a), and the values of upper body lean mass were even lower (~−11%) in the same McArdle patients compared to the old sedentary Spanish women (Gómez-Cabello et al., 2013b). The latter finding is consistent with the fact that 4 of the female patients had fixed muscle weakness affecting mostly proximal muscles. As for the muscle strength values, recent research with the same test and equipment for strength assessment used here showed that young male adults with a mean age comparable to that of the 2 studied male patients had average values of peak power during the bench press test (at 30 kg) of ~320 W (vs. only 140–188 W at the same load in our male patients) (Sanchez-Medina et al., 2010).

Concerns are frequently raised by clinicians as to the potential risks of exercise, particularly weight lifting, in McArdle patients. However, serum CK activity did not increase with resistance training (and in fact tended to decrease with training after the 1st month, indicating a good muscle tissue adaptation to the program) and we recorded no incidence of myoglobinuria. This indicates that the weight training program was well tolerated. On the other hand, it must be kept in mind that muscle damage, or at least some degree of it, as indicated by high serum CK activity, is a necessary physiological

stimulus for muscle to be repaired and adaptative hypertrophy to occur (Clarkson and Hubal, 2002). Our results are consistent with those of a previous case report study from our laboratory showing the functional benefits of resistance training in an adolescent with McArdle disease (García-Benítez et al., 2013). Muscle mass was not measured in this case study and thus it could not be determined to what extent muscle strength gains were due to neuromuscular adaptations only or to the occurrence of some degree of muscle hypertrophy, whereas here we showed that exercise-induced hypertrophy is an attainable goal in McArdle patients. The finding that both muscle mass and strength increased in these patients is of clinical relevance because low muscle mass and poor muscle strength are highly prevalent among westerners and are important risk factors for disability and potentially mortality in individuals as they age (especially if combined with high adiposity), whereas high muscle mass and strength are associated with a healthier cardiometabolic phenotype (Kalyani et al., 2012). With regards to this, besides the problem of the low muscle mass/strength levels shown here, recent research from our group has indicated that McArdle patients have an overall unfavorable cardiometabolic profile (Munguía-Izquierdo et al., 2014). On the other hand, the fact that gains in muscle strength were not totally lost after detraining is in overall agreement with previous research in healthy adults (Mujika and Padilla, 2001) or in chronic disease populations (Herrero et al., 2006) showing that, at least compared to muscle oxidative capacity, muscular strength suffers a more limited decrease after relatively short periods of detraining. This phenomenon is due, at least partly, to the fact that gains in neuromuscular performance (i.e., motor unit recruitment) can be relatively retained during periods of detraining (Mujika and Padilla, 2001).

Our study is not without limitations. First, we did not assess a control group of McArdle patients receiving no exercise intervention, although the quasi-experimental design we used (where each subject acted as their own control) might overcome, at least partly, this limitation. It would have been interesting to compare the effects of the present resistance intervention with other types of exercise programs (e.g., stair climbing, brisk walking) in McArdle patients. On the other hand, assessing healthy controls performing the same weight training intervention might have allowed us to determine if the ability to gain muscle mass and strength is limited (or not) in McArdle patients compared to non-patients. Finally, further research might determine if adding protein or creatine supplements to the current training program might contribute to maximize the gains in patients' muscle mass.

In summary, if appropriate training guidelines are followed (i.e., qualified instruction, competent supervision, and appropriate progression of the volume and intensity of training as we did here), regular participation in a strength training program has the potential to improve the muscle strength and mass, as well as the clinical status, of McArdle patients. While keeping in mind the need for large sample intervention studies (which might not be easily feasible in rare diseases as this one), our preliminary data suggest that supervised resistance training is feasible in

McArdle patients and has medical benefits, i.e., increased muscle strength and force and attenuation of clinical severity. Thus, we believe that the statement that "exercise is medicine" also applies to a disease which has been traditionally considered to be the paradigm of exercise intolerance, especially with regard to weight lifting.

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