**Responsible applicant:** Elena Groppa, PhD
**Project title:** Interactions between vessels and fibro/adipogenic progenitors (FAPs) in skeletal muscle regeneration.

## 1. State of general research

### 1.1 Skeletal muscle regeneration

Taking advantage of the regenerative potential of stem and precursors cells, skeletal muscle is renewed in response to injury, disease or aging, via either complete or partial regeneration [1, 2]. When regeneration does not fully occur, reparative processes entailing an overproduction of stromal components promote the continuity of tissue at the expense of its normal structure and function, causing "reparative disorders", such as for example fibrosis [2]. Fibrosis is primarily caused by myofibroblasts derived from the differentiation of resident fibroblasts in response to extracellular triggers, such as Transforming Growth Factor β-1 (TGF-β1), Wnt, Jagged (Jag)/Notch, and Hedgehog, and consists in the deposition of extracellular matrix (ECM) [3-5]. Fibrosis can be associated to other two types of reparative disorders, i.e. adipocyte infiltration and heterotopic ossification [2]. The astonishing regenerative capacity of skeletal muscle makes it an ideal regenerative/degenerative model system to understand and harness the cellular and molecular mechanisms that lead to tissue repair [1, 2]. This knowledge has the potential to impact the treatment of a large number of patients with muscle diseases that cause weakness or destruction of the muscles [1].
The usual sequence of regenerative events that occurs in skeletal muscle includes: 1) sealing the damage site 2) inflammation 3) angiogenesis 4) activation of satellites cells and proliferation of myoblasts with migration to the injury site 5) differentiation of the myoblasts and fusion to form myotubes followed by myotubes maturation and innervation [6].

#### 1.1.1 Cell types involved in skeletal muscle regeneration

##### 1.1.1.1 Satellite cells

Satellite cells are the primary stem cells in adult skeletal muscle and responsible for postnatal muscle growth, hypertrophy, and regeneration. In mature muscle, most satellite cells are in a quiescent state, but they activate and begin proliferating in response to extrinsic signals. Following activation, a subset of satellite cell progeny returns to the quiescent state during the process of self-renewal that can occur by either asymmetric cell division or stochastic cell fate model [7, 8]. Quiescent satellite cells are located between the basal lamina and sarcolemma of myofibers and identified by the expression of paired box transcription factor Pax7 [9]. The commitment of satellite cells to mature postmitotic myofibers occurs through the loss of Pax7 and Myf5 myogenic precursor markers, and the expression of myotube markers such as for example Desmin, Myosin Heavy Chain (MyHC), and α-actinin [1]. Expanding myoblasts interact with inflammatory and stromal cells through paracrine interaction that are important in regulating their activity. Given this, inflammatory and stromal cells are called "accessory cells" that are not tissue-specific, instead, they are involved in biological processes such as inflammation and fibrosis that are common to many regenerative environments [10].

##### 1.1.1.2 Inflammatory cells: neutrophils and monocytes/macrophages

Muscle damage is followed by inflammation that consists in the invasion of muscle by specific myeloid cell populations through the bloodstream, mainly neutrophils and monocytes/macrophages (MO/MP) [10]. Neutrophils appear to the injured site within 2 hours in response to chemotactic growth factors and fragment of the ECM, and they rapidly decline after 24 hours [6]. After reaching the site of damage, activated neutrophils undergo a respiratory burst and degranulation to rapidly secrete free radicals and proteases that target the cellular debris or ECM for degradation. Moreover, neutrophils release pro-inflammatory cytokines that further promote recruitment and/or proliferation of macrophages, potentiating their influence on regeneration and repair [10]. MO/MP enrollment covers a key role in skeletal muscle regeneration, in that it favors the growth of myogenic precursor cells (mpc) and rescues myoblasts and myotubes from apoptotsis. MO/MP promote mpc proliferation by soluble factors, including Insulin-like Growth Factor (IGF) I and II, Hepatocyte Growth Factor (HGF), Fibroblast Growth Factors (FGFs), Platelet Derived Growth Factor-BB (PDGF-BB), Epidermal Growth Factor (EGF), and Interleukin-6 (IL-6), and mpc survival by direct contacts [11, 12]. In addition, MO/MP show a phenotypic plasticity during skeletal muscle regeneration proceeding through different activation states [13, 14]. Within 24 hours after damage, pro-inflammatory and phagocytic MO/MP type M1 are recruited to injured fibers reaching the peak at 2-3 days. These MO/MP express Interleukin-β1 (IL-β1) and Tumor Necrosis Factor-α (TNF-α), do not increase in number, and favor mpc proliferation. At 3-4 days after damage, MO/MP M1 switch to anti-inflammatory and pro-angiogenic M2 and secrete TGF-β and Interleukin-10 (IL-10), proliferate, and promote mpc differentiation into myotubes [15].

Phagocytosis of mpc debris is crucial for the change of the MO/MP phenotype, therefore for entailing a proper regeneration of the muscle [13]. Consistently, impaired MO/MP recruitment is deleterious for muscle repair and causes considerable accumulation of adipocytes between regenerated fibers [16].

#### 1.1.1.3 Non-myogenic mesenchymal stem cells

Initially, the exclusion of dye Hoechst 33342 was applied to define the side population (SP) in skeletal muscle [17], which includes non-satellite skeletal muscle-resident stem cells that have myogenic potential in vivo. Following, several groups sought to characterize these non-satellite cells with specific markers, thereby identifying an heterogeneity of profiles [9]. In the 2010, two groups discovered a subset of tissue-resident mesenchymal progenitors with bipotent fibro/adipogenic potential (FAP), whose fingerprint is given by $CD45^-/CD31^-/\alpha$ integrin$7^-/Sca1^+/CD34^+/PDGFR\alpha^+$ pattern [18, 19]. These progenitors are placed in the muscle interstitium and closed to myofibre-associated blood vessels. The fate of these cells is largely dependent on the muscle environment, in that they support myogenic differentiation after injury with notexin (NTX), a myotoxin that acts on terminally differentiated myofibers, whereas they form adipocytes upon treatment with glycerol, a model of adipocyte infiltration (*Fig.1*). FAPs contribute in a paracrine fashion to skeletal muscle repair by secreting pro-myogenic signals such as IGF-1, IL-6, Wnt1, Wnt3A, and Wnt5A. In healthy regenerating muscles, FAPs are recruited during inflammation and proliferate reaching the peak at 3-4 days after tissue injury (expansion phase), and then they return to pre-damage values (extinction phase).

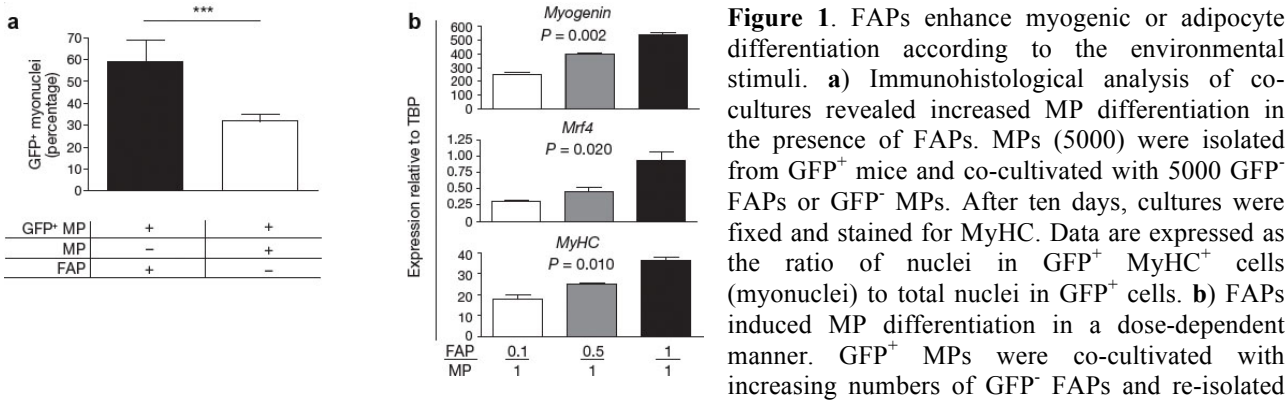


**Figure 1**. FAPs enhance myogenic or adipocyte differentiation according to the environmental stimuli. **a**) Immunohistological analysis of co-cultures revealed increased MP differentiation in the presence of FAPs. MPs (5000) were isolated from $GFP^+$ mice and co-cultivated with 5000 $GFP^-$ FAPs or $GFP^-$ MPs. After ten days, cultures were fixed and stained for MyHC. Data are expressed as the ratio of nuclei in $GFP^+$ $MyHC^+$ cells (myonuclei) to total nuclei in $GFP^+$ cells. **b**) FAPs induced MP differentiation in a dose-dependent manner. $GFP^+$ MPs were co-cultivated with increasing numbers of $GFP^-$ FAPs and re-isolated by FACS after ten days. The expressions of markers of myogenic differentiation were analyzed by qRT-PCR; *P* values were determined using ANOVA. **c)** FAPs from a donor ubiquitously expressing membrane-bound human alkaline phosphatase (hAP) were transplanted in skeletal muscle that was previously injected with glycerol to induce adipocytic infiltration ($n = 4$). FAPs efficiently engrafted and gave rise to differentiated adipocytes. All scale bars, 50 µm.

## 1.2 Skeletal muscle regeneration and angiogenesis

### 1.2.1 Angiogenesis

Angiogenesis is the formation of new capillary branches from preexisting blood vessels and occurs in development following vasculogenesis, which is the de novo formation of the initial vascular plexus in the embryo [20]. In adult life, with the exception of the ovary, the endometrium, and the placenta, vessels are quiescent, although endothelial cells retain high plasticity to recognize and respond to angiogenic signals [21]. The maintenance of endothelial quiescence is controlled by co-existence of endogenous negative regulators and pro-angiogenic factors in different tissues [21, 22]. However, in certain conditions, such as wound healing, inflammation, or pathological situations, positive angiogenic factors prevail and the endothelium is induced to form new vessels. Angiogenesis comprises two phases, i.e. an activation phase that is the initiation and progression of the angiogenic process, and a resolution phase during which vessels newly formed become mature and stable [21].

#### 1.2.1.1 Vascular activation

In a healthy adult, quiescent endothelial cells form a monolayer of cells sealed by junctional molecules. Here, endothelial cells have long half-life because they are protected against insults by the action of maintenance signals, such as Angiopoietin-1 (Ang-1) and low doses of Vascular Endothelial Growth Factor (VEGF), secreted by pericytes that are tightly associated with the endothelium into the basement membrane (BM) [22]. However, metabolic and hemodynamic changes may disturb quiescent vessels and activate endothelial cells, which start a cascade of events that give rise to new capillaries [22, 23]. In general, angiogenic factors released by the tissue in response to hypoxia induce sprouting angiogenesis, occurring through a tip ($Dll4^+$) and stalk ($Notch1^+$) endothelium pattern, whereas high levels of shear stress lead to intussusception angiogenesis [23]. Signaling involved in angiogenic activation of endothelium occurs via extracellular signals, which are mainly secreted paracrine factors, frequently ligands of surface transmembrane receptors, and extracellular matrix components that usually bind to integrins and specialized receptors. The main transmembrane receptors that transduce angiogenic signals are tyrosine kinase receptors (RTK) and tyrosine-kinase-associated receptors [24], such as VEGF-Receptor 1 and 2 (VEGF-R1, -R2), TGFβ-Receptor (TGFβ-R), Tie2, and EphB4 that bind VEGF, Ang-1 and -2, TGF-β and Bone Morphogenetic Proteins (BMPs), and EphrinB2, respectively. Among these signaling pathways, VEGF is the master regulator of vascular growth both in development and disease and, upon expression as a single factor, is capable of initiating the cascade of events leading from endothelial activation to the generation of new functional and stable vascular networks [25].

#### 1.2.1.2 Vascular maturation and stabilization

To become functional, newly formed vessels must mature in term of vessel wall and network connectivity [26, 27]. In regard to the vessel wall, a fundamental feature of vascular maturation is the recruitment of mural cells, pericytes in capillaries and vascular smooth muscle cells in arteries and veins. Concerning the vessel network, vascular maturation means an optimal capillary remodeling into a hierarchically branched mesh that responds to local tissue needs [27]. Several lines of evidence from developmental and adult models have demonstrated that PDGF-BB/PFGFRβ pathway has a key role in recruiting pericytes [28].
After maturation, vessels undergo stabilization through the onset of blood flow, the integration of mural cells into the vascular wall, and the deposition of perivascular ECM, in particular the vascular BM [29, 30]. This phase defines the transition from an actively growing vascular bed to a quiescent, fully formed, and functional network that is independent of pro-angiogenic factor stimulus withdrawal [30].

### 1.2.2 Angiogenesis in skeletal muscle regeneration

Muscle regeneration involves the coordination of myogenesis and revascularization to restore proper muscle function [31]. Communication between myogenic and angiogenic cells seems plausible based on the number of growth factors produced by endothelial cells to promote mpc growth such as for example IGF-1, HGF, bFGF, PDGF-BB, and VEGF [32]. In particular, the delivery of VEGF was shown to promote the growth of myogenic fibers in both cardiotoxin-injured and dystrophic muscles [33, 34], through the activation of VEGF signaling in mpc that express VEGF receptors [35, 36]. Conversely, VEGF-pathway blockade by soluble receptor sFlt1 leads to myotube hypotrophy and inhibits myogenic differentiation [36-38]. On the other hand, mpc trigger angiogenesis enhancing the expression of VEGF [39, 40], which, therefore, plays a key role in the bidirectional interplay between myogenesis and angiogenesis [41].
During tissue regeneration, the endothelium communicates also with the inflammatory population to trigger the cascade of events that leads to tissue repair. After tissue damage and inflammation stimulation, endothelial barrier defenestrates leading to increased vascular permeability and extravasation of fluid [42, 43]. Preformed P-selectin is translocated to the luminal surface of the vascular wall, adhesion molecules such as E-selectin, ICAM-1, and VCAM-1 are expressed, and chemokines are expressed and released. This favors the binding and transmigration of neutrophils through the endothelium into the tissue. Then, changes in the pattern of adhesion molecules and secretion of monocyte chemoattractant protein-1 (MCP-1) by the endothelium, promote the selective recruitment of monocyte populations [44, 45]. Upon reaching the site of injury, MO/MP contributes to the restoration of tissue VEGF levels and the dynamic process of capillary formation [46].
Taking advantage of its own stemness features, the perivascular component seems to be directly involved in tissue regeneration/degeneration rather then acting through paracrine signaling [47-50]. Birbrair and co-workers observed that $NG2^+/Nestin^+$ pericyte subpopulation participates to muscle regeneration differentiating into myofibers, whereas $NG2^+/Nestin^-$ subset becomes fibroblast/adipocyte and causes fat accumulation. Interestingly, the latter subtype expressed Sca-1, CD34, and PDGFRα suggesting a correlation between pericytes and the FAPs previously described. This observation is in agreement with the work of Dulauroy

and colleagues who demonstrated that ADAM12$^{+}$ cells are reprogrammed during vascular wall development and are re-induced by injury as a sub-population of PDGFRα$^{+}$ perivascular progenitors (expressing neuro-glial 2 proteoglycan, NG2, and PFGFRβ pericyte-markers) with a specific pro-fibrotic fate and function [51]. Consistently, several other studies shed light on the source of the fibrogenic myofibroblast, and highlighted the central role of pericytes in the origins of fibrosis [5, 52].

## 2. State of own research

During my PhD project I acquired extensive experience with myoblast-mediated delivery of angiogenic genes to skeletal muscle [53]. This method is based on the retroviral transduction of syngeneic myoblasts and provides robust expression of the transgene that is stable over time. Taking advantage of such cell-based ex-vivo approach to gene delivery, we previously found that VEGF can induce either normal capillaries or aberrant angioma-like structures depending strictly on its dose in the microenvironment around each producing cell in vivo, and not on the total amount [54]. However, stimulation of pericyte recruitment by co-expression of PDGF-BB could increase the number of pericytes enrolled to the area of neo-angiogenesis and prevent the aberrant angiogenic phenotype induced by high VEGF levels [55]. In term of vascular stability, VEGF-sustained delivery of 4 weeks was required to maintain newly induced normal capillaries upon VEGF-signaling abrogation, whereas aberrant vascular structures never became VEGF-independent [54].

The aim of my project was to further investigate the cellular and molecular mechanisms regulating 1) the switch between normal and aberrant angiogenesis and 2) the achievement of vascular stabilization in the presence of increasing VEGF doses, in order to identify novel and potentially more specific molecular targets to improve both the safety and the efficacy of VEGF-based strategies for therapeutic angiogenesis [56].

The point 1) aimed to identify the role of the specific endothelium-pericyte signaling pathways such as TGF-β1/TGFβR, Angs/Tie2 and ephrinB2/EphB4 in the VEGF dose-dependent transition between normal and aberrant angiogenesis. A monoclonal population of transduced myoblasts, expressing a moderate VEGF dose that induces only normal angiogenesis, was further transduced to secrete soluble blockers of the TGF-β1/TGFβ-R, Tie2/Angs or EphB4/ephrinB2 pathways (LAP, sTie2Fc and sEphB4, respectively) and implanted into mouse skeletal muscles. After 2 weeks, neither TGF-β1 nor Ang signaling blockade altered the normal angiogenesis induced by low VEGF levels. However, inhibition of ephrinB2/EphB4 signaling caused normal vessels to grow into aberrant angioma-like structures, despite low VEGF expression, similar to the previously described effects of blocking pericyte recruitment [55]. Conversely, gain-of-function (GOF) experiments, performed by systematic treatment with an ephrinB2-Fc fusion protein, showed that stimulation of EphB4 signaling completely prevented aberrant angiogenesis induced by high VEGF levels and yielded a physiological network of normal and mature capillaries. Analysis of the initial stage of vascular induction by low VEGF (3 and 4 days) showed that EphB4 signaling inhibition: a) did not interfere with pericyte recruitment, contrary to similarly aberrant structures induced by high VEGF alone; b) caused an enlargement of initially formed vessels, similar to those induced by high VEGF alone; and c) increased the proliferation rate of endothelial cells. On the other hand, GOF of EphB4: 1) accelerated remodeling of mother vessels in pericyte-covered capillary networks, resembling the phenotype previously observed by VEGF and PDGF co-expression; and 2) decreased the proliferation rate of endothelial cells. *In conclusion, these results suggest that: 1) ephrinB2/EphB4 signaling plays a key role in controlling the switch between normal and aberrant angiogenesis by increasing VEGF doses, independently of pericytes recruitment; and 2) is a promising molecular target to modulate the side effects of VEGF gene delivery and improve both its safety and efficacy.*

I am currently performing some experiments in order to: 1) understand how ephrinB2/EphB4 signaling directly or indirectly control endothelial proliferation in the switch between normal and aberrant angiogenesis induced by VEGF overexpression 2) test EphrinB2 in combined therapy to prompt normalization of VEGF-induced aberrant angiogenesis by using a therapeutically relevant gene therapy approach, like adenovirus vectors. Following, we are going to submit this work.

The point 2) aimed to define whether the stabilization of newly formed vessels is regulated by the dose of VEGF over-expression in a therapeutic setting and to investigate the underlying cellular and molecular mechanisms. By implanting myoblast clones expressing specific VEGF levels in skeletal muscle, and subsequently abrogating VEGF signaling by systemic treatment with recombinant VEGF-Trap after 2 and 3 weeks, we found that VEGF impaired vascular stabilization in a dose-dependent fashion. In fact, 35% and 50% of normal vessels induced by low VEGF levels already stabilized by 2 and 3 weeks, respectively. Instead, normal angiogenesis induced by medium VEGF levels completely regressed by 2 weeks and only 10% stabilized by 3 weeks. Aberrant structures caused by high VEGF levels never become VEGF-independent. Further, all normal capillaries induced by low and medium VEGF doses displayed similar perfusion and normal pericytes coverage, despite different stabilization rates. However, gene expression

analysis on the injected muscles, revealed a correlation between decreasing stabilization rates at the different VEGF doses and tissue expression of TGF-β1 and Sema3A, which can promote vessel stabilization by recruiting bone marrow-derived myeloid cells, defined by the expression of both the monocyte marker CD11b and the VEGF co-receptor Neuropilin-1 (NP-1) and named therefore Neuropilin-Expressing Monocytes (NEM). To elucidate the underlying mechanisms, we found that: 1) VEGF down-regulated Sema3A expression by endothelial cells in vitro; 2) NEM recruitment in injected muscles was reduced with increasing VEGF doses; 3) Sema3A expression in Flow Activated Cell Sorting (FACS)-purified endothelial cells from injected muscles was down-regulated with increasing VEGF doses; 4) FACS-purified NEM expressed TGF-β1, but its level per cell was not affected by VEGF dose; 5) however, in vitro stimulation of endothelial cells with TGF-β1 up-regulated Sema3A expression dose-dependently, providing the basis for a positive feedback loop between Sema3A, NEM recruitment, TGF-β1, and further Sema3A expression in conditions of low VEGF. *Taken together, these data suggest a model in which $VEGF_{164}$, expressed within a range of doses that induce only normal angiogenesis, negatively regulates vascular stabilization by inhibiting the Sema3A/NEM/TGF-β1 axis, rather than directly acting on the endothelium-pericyte crosstalk.* We have just submitted this work (see annexed *Groppa et al. submitted*).

I think that the study and work achieved to structure and develop my PhD project provide me with an ideal set of skills for leading the proposed project described in the Section 3 in Dr Rossi's group. In particular I will capitalize on: 1) my strong background in the field of vascular biology that is clearly crucial in tissue regeneration; 2) my awareness of the importance of studying complex biological processes as a unique whole where several cellular and molecular players act a role; 3) my experience and creative thinking in designing in vitro and in vivo loss-of-function (LOF) and GOF experiments that aim to dissect cellular and molecular mechanisms; 4) my knowledge into a variety of techniques such as histological and gene expression analysis, in vitro and in vivo work, FACS, ex-vivo cell purification; 5) my soft skills and capacity to work both independently as well as in team.

## 3. Detailed research plan

One of the goals of Dr Rossi's research consists in understanding the mechanisms of skeletal muscle regeneration in a model that describes myogenic cells as direct contributors to tissue repair, and non-myogenic cells from other developmental sources as important accessories. As described in the Section 1.1.1.3, FAPs cover a key role in skeletal muscle regeneration acting in a paracrine manner on muscle progenitors. In addition, FAPs interact with macrophages whose switch from M1 to M2 dictates FAP clearance or development to fibroblasts/adipocytes (*Lemos et al., manuscript submitted, Fig.2*). However, no one has investigated the interplay between FAPs and vessels that is likely based on the tight association of vessels and FAP in skeletal muscles, and the described bidirectional signaling between endothelium and mesenchymal stem cells (MSC) [57, 58]. Given this, we are interested in studying the regulatory relationship between vascular remodeling and FAP expansion and clearance during skeletal muscle regeneration, through the identification of signaling pathways that regulate their crosstalk. Our long-term goal is to assess the role of such crosstalk in modulating overall regeneration efficiency. To achieve this, we specifically aim to: 1. Characterize the vascular process, i.e. activation and resolution phases, within skeletal muscle regeneration; and 2. Investigate regulatory interactions between the vascular unit (endothelial cells and pericytes) and FAPs during skeletal muscle regeneration by dissecting the role of specific candidate signaling pathways.

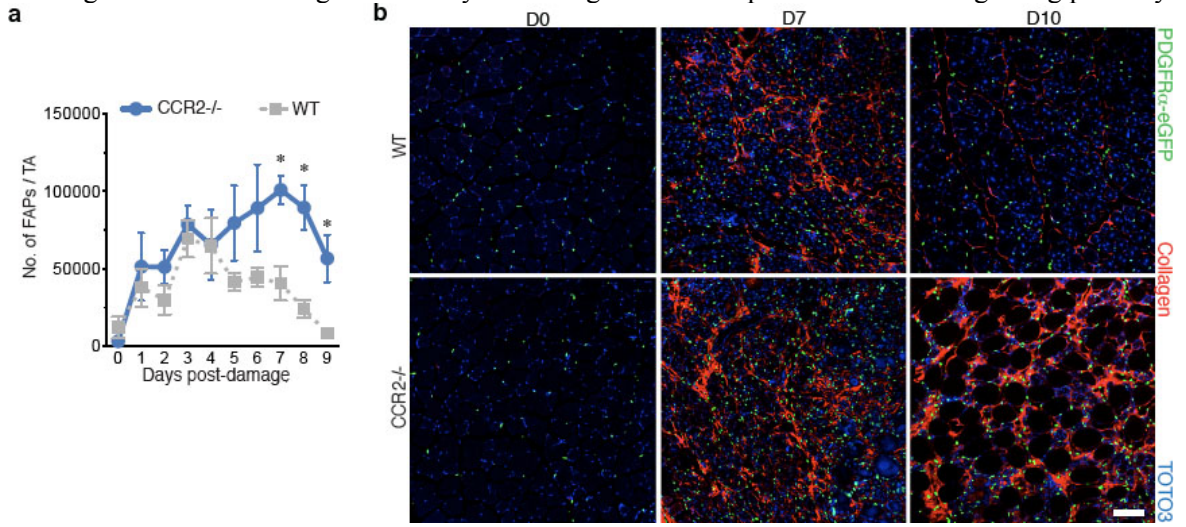


**Figure 2**. **a)** FAP clearance is impaired during skeletal muscle regeneration in absence of M1 monocyte/macrophage

infiltration, i.e. in CCR2-/- mice. Quantification by flow cytometry of the total number of FAPs per tibialis anterior (TA) in CCR2-/- mice at the indicated time points following NTX injection. Mean values ± standard deviation are shown. *$P$<0.05; $n$ = 4 mice per time point. Data from WT mice (Fig 1a) is shown for comparison purposes. **b**) Increased FAP numbers and collagen deposition in damaged CCR2$^{-/-}$ muscle. Representative images of TA sections from PDGFRα -H2B::EGFP and PDGFRα::H2B-eGFP/CCR2-/- mice at the indicated time points following damage. Scale bar, 20μm.

### 3.1 Aim 1. To characterize the vascular process, i.e. activation and resolution phases, within skeletal muscle regeneration

Hypothesis

*The patterns of vascular activation and maturation and FAP expansion and clearance are coupled during muscle regeneration.*

FAPs contribute to skeletal muscle regeneration through an expansion and clearance phase that occurs at 3-4 days and between 4 and 7 days after damage, respectively [18]. Angiogenesis also begins early (3 days) during muscle regeneration, a notion supported by the reported endogenous pattern of angiogenesis-related factors [31, 59]. The acute inflammation resulting after tissue damage destabilizes endothelial junctions increasing vessel permeability that allows plasma components and inflammatory cells to exit the bloodstream, and leads to endothelial cell-activation [42]. Subsequent vessel maturation involving adjacent pericytes and smooth muscle cells alters microcirculatory properties such as permeability [60]. However, the sequence of changes in endothelium-gene expression, morphology, and functionality (vascular perfusion and permeability) of newly induced vessels during skeletal muscle regeneration have not been fully described yet. Such descriptive work is key to establish correlations between the various phases of angiogenesis and the activity of other cellular components of the regenerative process such as FAPs, myogenic cells, and inflammatory cells, and will lead to the formulation of hypotheses on the molecular mechanisms underlying such correlations, which will be functionally tested in the next aim.

Experimental procedure:

The activation and maturation/stabilization phases of angiogenesis will be defined by ex-vivo gene expression analysis on endothelial cells freshly isolated from tibialis anterior (TA) and gastrocnemius (GC) muscles collected from healthy mice treated with saline solution or the myotoxin NTX [18]. This analysis will take advantage of a core service for transcriptome analysis by next generation sequencing recently established in the Rossi laboratory, which has optimized techniques to obtain high quality libraries from FACS-sorted cells. Pathway analysis (Ingenuity) will be used to interpret these results, focusing on the expression of genes known to be part of the activation signature of signaling pathways involved in angiogenesis. In addition, a candidate gene approach will be taken, in which particular attention will be given to: Ang-2, VEGF, Dll4, MPC-1and the receptors Tie2, VEGF-R, and Notch-1, for the activation phase, and PDGF-B, EphrinB2, TGF-β1, Sema3A and their receptors PDGFRβ, EphB4, TGFβ-R, and NP-1 for the maturation/stabilization one. These analyses will be performed at 1, 3, 5, 7, 10, 14, and 28 days after the tissue damage.

The morphology of vascular structures newly induced during muscle regeneration will be assessed by immunofluorescence staining of fresh-frozen sections of TA and GC muscles collected from healthy mice treated with saline solution or NTX, and analyzed by standard or confocal microscopy [18, 55]. Antibodies that specifically recognize EC (PECAM), smooth muscle cells (α-smooth muscle actin, α-SMA), pericytes (NG2) and vascular basement membranes (laminin and collagen type IV) will be used. The maturation index of the induced vessels will be calculated as the percentage of endothelial structures associated with pericytes [61]. Notably, pericytes will be unequivocally defined as perivascular cells displaying a typical dendritic morphology, expressing NG2, but not α-SMA, and embedded in the vessel basal lamina [55].

The establishment of functional blood flow in newly induced vascular structures will be assessed by intravenous injection of fluorescently labeled tomato lectin, which binds to the luminal endothelial surface of vessels only if they are connected to the systemic circulation. An amount of 50 μg of fluorescein isothiocyanate (FITC)-labeled *Lycopersicon esculentum* lectin in 50 μl (Vector Laboratories) will be injected into the femoral vein and allowed to circulate for 4 minutes before perfusion of 1% paraformaldehyde and collection of TA and GC leg muscles, which will be processed as previously described [62].

The vascular leakage will be quantified by injecting intravenously the Evans Blue dye, which binds to plasma proteins and therefore is used as a tracer for their extravasation [63]. After 4 hours, the animals will be sacrificed by fixative perfusion to clear intravascular dye, the tissue will be harvested and weighed and the extravasated dye will be extracted with formamide and quantified with a spectrophotometer as previously reported [54]. *In aggregate, these analyses will allow us to establish the precise temporal sequence of changes taking place in the vascular compartment during muscle regeneration, and to start building temporal*

*correlations with the behavior of other cell types involved, the mapping of which has been a focus of the Rossi lab for years.*

### 3.2 Aim 2. To investigate the potential crosstalk between the vascular unit and FAPs during skeletal muscle regeneration by dissecting the role of specific signaling pathways

Hypothesis

*Inhibition/activation of specific signaling pathways involved in vessel cells-FAPs crosstalk will influence the process of skeletal muscle regeneration.*

As mentioned above, a deeper knowledge of the temporal correlation between specific functional characteristics of the different cellular players involved in regeneration, combined with a map of transcriptional changes in the same cells built by deep sequencing of highly purified subsets (already ongoing in the Rossi laboratory for myogenic and mesenchymal cells), should allow the formulation of innovative hypotheses concerning the molecular mechanisms involved in coordinating the roles of these different cell types. These hypotheses will be tested as they are generated using methods similar to those I used during my PhD. However, some very promising hypotheses in regard to vessel cells-FAPs crosstalk can already be formulated based on published results and will be initially followed. Specifically:

a) As described in the Section 1.2.1.1, Angs/Tie2 signaling is important in angiogenesis [64]. Ang-2 is expressed by endothelial cells only upon stimuli like hypoxia, shear stress, and VEGF, or in some pathological conditions, and activates Tie2-expressing endothelial cells during the early stages of newly induced angiogenesis. Ang-1 is expressed by periendothelial cells, fibroblasts, and other types of non-vascular normal and tumour cells, is present in the blood of healthy people, but is up-regulated in angiogenesis where it promotes vascular maturation and reduces vascular leakage [44, 64]. Interestingly, Tie2 has been identified as a novel marker of tissue-resident stem cell population that exhibits FAP phenotype and properties. $Tie2^+$, $PDGFR\alpha^+$, $Sca\text{-}1^+$ cells reside in the interstitium of skeletal muscle and other tissues, are associated with blood vessels, and have multilineage developmental potential [65]. Moreover, Ang-1 was found to: 1) be expressed by the SP cellular fraction [66]; 2) influence MSC differentiation [67]; 3) regulate tissue response in wound healing [68]. Given this, Ang/Tie2 is an excellent candidate to regulate the vessels-FAPs interplay in skeletal muscle regeneration.

b) Both endothelial cells and FAPs express Wnt ligands and receptors [18, 66, 69]. Wnt-β catenin signaling in the endothelium promotes proliferation, survival, and capillary-like networks [70], and in mesenchymal progenitors regulates the balance between adipogenic vs osteogenic lineage [2]. The findings that endothelial cells can inhibit adipogenesis in mesenchymal progenitors via Wnt pathway [58], and conversely, mesenchymal cells promote angiogenesis under hypoxia condition by Wnt4 expression [71], let us to hypothesize that angiogenesis and FAPs are associated via Wnt signaling during skeletal muscle regeneration.

Experimental plan (a)

We will address the activity of Angs/Tie2 signaling on FAPs in vitro by treating FACS sorted FAPs with Ang-1 or Ang-2 recombinant proteins (500ng/mL, R&D systems), or endothelial or mural cell-conditioned medium, without/with the addition of Tie2 competitor, Tie2-Fc (4 μg/mL, R&D systems) in the absence/presence of chondrogenic, osteogenic, and adipogenic induction medium [67, 72]. Upon treatment, we will analyze FAP proliferation and differentiation capacity, as previously reported [67]. For these assays, murine microvascular endothelial and pericyte-like C3H/10T1/2 cells commercially available (from Cell Biologics and European Collection of Cell Cultures, respectively) will be used [73].

To investigate the role of vascular-derived Ang-2 and Ang-1 on FAP expansion and extinction during muscle regeneration, we will block Ang/Tie2 signaling in transgenic-inducible mice treated with saline solution or NTX and analyze the tissue. In particular, to investigate the activity of endothelium-derived Ang-2 on FAPs, we will use a mouse line expressing tamoxifen (ICN)-inducible Cre-recombinase (Cre-ERT2) under the regulation of the vascular endothelial cadherin promoter (VECad), which will be bred to $Ang2^{lox}$ animals, thereby generating VECad-CreERT2/$Ang2^{lox}$. Tamoxifen will be injected intraperitoneally to delete endothelial Ang-2 expression prior to the induction of tissue damage. Similarly, to address the role of Ang-1, we will use an inducible whole-body knockout of Ang1, Rosatet/tetOCre/$Ang1^{lox}$, which deletes Ang1 upon administration of doxycycline, provided to the animals in the drinking water [68]. In addition, to study the function of Ang-1 by FAPs on angiogenesis and FAP fate during muscle regeneration, the $Ang1^{lox}$ strain will be bred with FAP specific deleters, e.g. the commercially available PDGFRα-CreERT2 strain. In all the latter experimental designs, FAP proliferation in vivo will be studied by administrating 5-ethynyl-2'-deoxyuridine **(**EdU) in drinking water (0.8 g/l in 2% sucrose) and by intraperitoneal injection (100 mg/kg). For flow cytometric analysis, cells will be stained for surface markers as previously described [18]. Following,

samples will be surface-stained, fixed, stained for EdU using commercial kits, and analyzed by FACS. To characterize tissue regeneration, FAP differentiation, and angiogenesis in vivo, animals will be perfused transcardially and tissues will be processed for cryosectioning using standard methods. Immunostaining will be performed using monoclonal antibodies against markers for newly induced regenerating myofibers (emMyHC), FAP differentiation (perilipin, osterix, and α-SMA), and angiogenesis (CD31, α-SMA, NG2, and laminin). In addition, the different cell subsets will be sorted and subjected to new generation RNA sequencing to assess changes in gene expression. To achieve this experimental plan, VE-cadherin-CreERT2 mice will be provided by Dr Iruela Arispe (UCLA, Los Angeles) and Rosatet/tetOCre/Ang1$^{lox}$ and Ang1$^{lox}$ strains by Dr Quaggin (University of Toronto, Toronto). Taking advantage of the outstanding expertise of the host institution, Ang2$^{lox}$ mice will be generated by flanking a critical portion of Ang2 gene with loxP sites oriented in the same direction (cis arrangement), so that Cre recombinase mediates a deletion of the floxed segment, thereby preventing Ang2 expression [74].

To further understand the contribution of Ang/Tie2 pathway in tissue repair, we will trigger Tie2 signaling in C57BL/6 healthy animals (> 8 weeks) treated with saline solution, NTX, or glycerol by 1) electroporation of Ang-2, Ang-1 or empty plasmids that are on the market (OriGene); and 2) systemic treatment with Comp-Ang1 (AdipoGen), a soluble, stable, and potent Ang-1 variant that contains a minimal coiled-coil domain of cartilage oligomeric matrix protein enough for oligomerization [67, 72, 75]. The collected tissues will be analyzed as described above.

Experimental plan (b)

We will study the activation of Wnt signaling in endothelial cells and FAPs during skeletal muscle regeneration, by analyzing RNA-seq data sets for the expression of Wnt-downstream targets, such as for example Axin2, in endothelial cells and FAPs derived from mice treated with saline solution or NTX [76]. We will also assess the expression of Wnt ligands and receptors that will be further validated at the protein level by CyTOF®2 mass cytometry, which allows multi-parametric analysis of up to 40 protein markers on cells labeled with stable heavy metal isotopes using state-of-the-art time-of-flight atomic mass cytometry technology.

Based on the previous results, we will select a group of Wnt signaling molecules that may associate endothelial cells and FAPs (e.g. Fzd4 receptor by the endothelium and Wnt4 ligand by FAP) and investigate in vitro their activity on endothelium proliferation and FAP proliferation and differentiation in the absence/presence of chondrogenic, osteogenic, and adipogenic induction medium. Co-culture of endothelial cells and FAPs in absence/presence of Wnt competitor, such as soluble Wnt receptor (sFRP) [77], will be performed to investigate the possible paracrine activity by the two cell types through the selected Wnt signaling.

To address the role of Wnt in the interplay between angiogenesis and FAP during skeletal muscle regeneration, we will perform LOF and GOF experiments led by the Wnt ligands/receptors identified in the previous investigations. The signaling depletion will be performed in C57BL/6 healthy animals treated with saline solution or NTX by 1) direct intramuscular injection of Wnt inhibitors like sFRP, Dickkopf related protein 1 (DKK1) (R&D systems), or control solution into the muscle surrounding the injury site [77]; and 2) silencing (by siRNA) of canonical Wnt signal transducer [71] in FAPs collected from C57BL/6-CMV-β actin-EGFP transgenic mice and transplanted into syngeneic GFP$^-$ recipients. GOF of Wnt-pathway will be achieved in C57BL/6 healthy mice treated with saline solution, NTX, or glycerol by direct intramuscular injection of Wnt-recombinant proteins or control solution into the muscle surrounding the injury site [77]. After performing LOF and GOF, we will collect and analyze the tissues as explained in the Section a). The strain of C57BL/6-CMV-β actin-EGFP is available in the Rossi lab.

Besides these studies, we will consider other potential signaling regulating vessels and FAPs crosstalk, derived from the design of intercellular networks. These interactive maps will be generated with data derived from next generation RNA sequencing of the mRNA transcriptome of endothelial cells and FAPs collected at different stages of the tissue repair process.

## 4. Importance of the project

Tissue regeneration in response to injury, disease, or aging, can either fully restore the damaged area or cause a process of excessive and persistent ECM deposition, named fibrosis, that replace the functional tissue [2]. In skeletal muscle, fibrosis is most often associated with the muscular dystrophies, a clinically and molecularly heterogeneous group of diseases. Phenotypically, these diseases are characterized by inflammation of the muscle tissue and skeletal-muscle wasting, which compromises patient mobility so that affected people become confined to a wheelchair. In the most severe cases, such as Duchenne muscular dystrophy (DMD, caused by the lack of the dystrophin protein), muscle loss and fibrosis also cause

premature death through respiratory and cardiac failure [78]. It is considered that fibrosis, not only exacerbates the loss of skeletal muscle function in DMD patients, but also impairs the feasibility and efficacy of cell- and gene- therapies for replacement of defective genes [79]. Hence, the comprehension of the mechanisms by which fibrosis is caused appear crucial to apply modifications to the muscle environment for halting or attenuating the progression of the disease as well as for improving stem cell engraftment and gene delivery in otherwise untreatable patients [4].

It is now recognized that muscle regeneration is led by myogenic cells that directly contribute to tissue repair, but also by non-myogenic cells from other developmental sources, which act as pivotal accessory players. Following this model, it becomes evident that a full understanding of the mechanisms regulating tissue regeneration requires the study of integrated cellular interactions rather than one-sided investigations exclusively focused on myogenic cells [10]. In this perspective, Dr Rossi and his colleagues elucidated the role of FAPs as quiescent mesenchymal stem cells that reside in skeletal tissue and, upon tissue damage, proliferate leading to myofybers formation in a paracrine fashion or differentiating in fibroblasts/adipocytes according to the environmental stimuli [18]. When normal skeletal-muscle repair occurs, FAP activation is followed by a clearance phase that is regulated by specific factors released by monocytes/macrophages (*Lemos et al., manuscript submitted*). Also angiogenesis has been described to be involved in the process of tissue repair via bidirectional signaling with both myogenic precursors and inflammatory cells (see Section 1.2.2).

Taking advantage of my knowledge in vascular biology and Dr Rossi's expertise in muscle tissue regeneration, we aim to dissect the potential interplay between vessel cells and FAPs in order to further understanding the complex process that guides muscle regeneration. The acquired knowledge will allow us to identify novel targets that may be harnessed for preventing fibrosis, thereby increasing the success of the new cell- and gene-based therapies applied for DMD treatment.

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## 5. Timetable

| Aim | Activity | Year 1 | | | | Year 2 | | | | Year 3 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Q1 | Q2 | Q3 | Q4 | Q1 | Q2 | Q3 | Q4 | Q1 | Q2 | Q3 | Q4 |
| ***1.Characterization of the vascular process during skeletal muscle regeneration*** | Study gene expression on FACS sorted-endothelial cells | **X** | **X** | **X** | | | | | | | | | |
| | Analyze vessel morphology | **X** | **X** | **X** | | | | | | | | | |
| | Analyze vessel functionality | **X** | **X** | **X** | | | | | | | | | |
| ***2a.Study Ang/Tie2 signaling in vessel cells-FAPs crosstalk during skeletal tissue repair*** | Study activity of Ang/Tie2 signaling in vessel cells-FAPs crosstalk in vitro | | | **X** | **X** | | | | | | | | |
| | Generate $Ang2^{lox}$ strain | **X** | **X** | **X** | **X** | **X** | **X** | | | | | | |
| | Breed $Ang2^{lox}$ and VECad-CreERT mice | | | | | | | **X** | **X** | **X** | **X** | **X** | **X** |
| | Characterize muscle regeneration in VECad-CreERT/$Ang2^{lox}$ | | | | | | | | | **X** | **X** | **X** | **X** |
| | Breed $Ang1^{lox}$ and PDGFRα-CreERT2 mice | | | **X** | **X** | **X** | **X** | **X** | **X** | | | | |
| | Characterize muscle regeneration in PDGFRα-CreERT/$Ang1^{lox}$ | | | | | **X** | **X** | **X** | **X** | | | | |
| | Characterize muscle regeneration in Rosatet/tetOCre/$Ang1^{lox}$ | | | | | **X** | **X** | **X** | **X** | | | | |
| | Characterize muscle regeneration upon GOF of Tie2 signaling by Ang-1 and Ang-2 in C57BL/6 animals | | | | | | | | | **X** | **X** | **X** | **X** |
| ***2b.Study Wnt signaling in vessel cells-FAPs crosstalk during skeletal tissue repair*** | RNA-seq and CyTOF®2 analysis of Wnt-signaling in FACS-sorted FAPs and endothelial cells | | | **X** | **X** | **X** | **X** | | | | | | |
| | Study activity of Wnt-signaling in vessel cells-FAPs crosstalk in vitro | | | | | | **X** | **X** | **X** | **X** | | | |
| | Characterize skeletal muscle regeneration upon LOF of Wnt-signaling in C57BL/6 animals | | | | | | | | | **X** | **X** | **X** | **X** |
| | Characterize skeletal muscle regeneration upon GOF of Wnt-signaling in C57BL/6 animals | | | | | | | | | **X** | **X** | **X** | **X** |

## 6. Reason for the choice of research institution

I am asking the SNF Postdoc Early Mobility Fellowship to have the opportunity to work in Dr Rossi's group, at the University of British Columbia in the Department of Medical Genetics (Vancouver, Canada), for the following reasons 1) the interest to introduce my vascular background into another research field, which I have always desired to delve, i.e. tissue regeneration; 2) the wish to work with Dr Rossi, an excellent worldwide known scientist who was trained in some of the most famous universities in the world, e.g. Heidelberg- and Standford-University. His broad scientific knowledge, which spreads through skeletal muscle regeneration, central nervous system-related diseases, and epigenetic, and his manner to live science at the same time as a pleasure and as a mission, has fascinated me. Taken together, these considerations make me feeling that by having him as mentor, I will learn to ask and develop innovative scientific questions and improve my soft skills, which are important requirements for further pursing my scientific career, in both academy or company context; 3) the international, open, and outstanding scientific environment with whom I could interact during my visit in Dr Rossi's laboratory last year, in December 2013; 4) the availability of a number of core facilities enabling me to address almost any relevant scientific question; 5) the national and

international network led by Dr Rossi, which bridges his research group and several other ones inside and outside Canada.

**7. Relevance for personal career development**

I think that the fellowship as Postdoc in Dr Rossi's laboratory could offer me: 1) the opportunity to match my background in vascular biology with a different scientific topic, i.e. muscle regeneration, thereby developing a new research line at the interface of these two mature fields for my future career; 2) the possibility to gain my knowledge into a variety of in vivo models, for example the generation of transgenic mice, and innovative technologies like the CyTOF®2 mass cytometry; 3) the occasion to know and interact with a new scientific environment, such as the Canadian research network, which could help me to unveil new ways to approach and do science and establish future collaborations between Canada and Europe.

**8. Planned publications**

The results of the proposed research are planned to be published in peer-reviewed international journals and will provide 1) a characterization of the vascular process during skeletal muscle regeneration 2) identification of the possible crosstalk between vessel cells and FAPs in the process of tissue repair, and dissection of the underlying regulating mechanisms.