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Genome-wide Expression Analysis and EMX2 Gene Expression in Embryonic Myoblasts Committed to Diverse Skeletal Muscle Fiber Type Fates

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Abstract

Background—Primary skeletal muscle fibers form during embryonic development and are characterized as fast or slow fibers based on contractile protein gene expression. Different avian primary muscle fiber types arise from myoblast lineages committed to formation of diverse fiber types. To understand the basis of embryonic muscle fiber type diversity and the distinct myoblast lineages that generate this diversity, gene expression analyses were conducted on differentiated muscle fiber types and their respective myoblast precursor lineages.

Results—Embryonic fast muscle fibers preferentially expressed 718 genes, and embryonic fast/slow muscle fibers differentially expressed 799 genes. Fast and fast/slow myoblast lineages displayed appreciable diversity in their gene expression profiles, indicating diversity of precursor myoblasts. Several genes, including the transcriptional regulator EMX2, were differentially expressed in both fast/slow myoblasts and muscle fibers versus fast myoblasts and muscle fibers. EMX2 was localized to nuclei of fast/slow myoblasts and muscle fibers and was not detected in fast lineage cells. Furthermore, EMX2 overexpression and knockdown studies indicated that EMX2 is a positive transcriptional regulator of the slow myosin heavy chain 2 (MyHC2) gene promoter activity in fast/slow muscle fibers.

Conclusions—These results indicate the presence of distinct molecular signatures that characterize diverse embryonic myoblast lineages before differentiation.

Keywords

Myoblast; Lineage; Fiber Type; Transcription; Gene Expression

Introduction

Adult and developing vertebrate musculature is composed of muscle fibers that vary in contractile and metabolic characteristics. These types of muscle fibers are often categorized as fast or slow, based principally upon the contractile properties and expression of the myosin heavy chain (MyHC) genes that determines the fiber type specific contractile characteristics in both adult and developing muscle (Reiser, et al., 1985; Reiser, et al., 1988).

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In avian species, muscle fibers are defined as fast, fast/slow, or slow based on expression of genes encoding MyHCs with corresponding ATPase activities. Nearly all avian muscle fibers express one or more fast MyHC isoform genes throughout development and in the adult (Bandman, et al., 1982). Three slow MyHC isoform genes are expressed in chicken development and in the adult. The slow MyHC3 gene is expressed transiently during skeletal muscle development and becomes restricted to the atria as development proceeds (Wang, et al., 1996). Slow MyHC1 and slow MyHC2 are the predominant slow MyHC isoforms in skeletal muscle. The slow MyHC1 gene is expressed in nearly all slow muscle fibers, many of which also express the slow MyHC2 gene. Slow MyHC2 gene expression is restricted to slow muscle fibers and is most characteristic of the slow muscle fiber phenotype (Page, et al., 1992). Therefore, expression of the slow MyHC2 gene defines those avian muscle fibers that are most distinct from fibers that express exclusively fast MyHC isoform genes.

Vertebrate skeletal muscle fiber formation occurs in three distinct stages. The embryonic phase generates primary muscle fibers from the differentiation of embryonic myoblasts. These primary muscle fibers establish the basic anatomic structure of each muscle and presage the general contractile and metabolic characteristics of the muscle as a whole. The following fetal phase of myogenesis yields secondary muscle fibers from fetal myoblasts. Lastly, the adult stage of myogenesis is partly characterized by the presence of mitotically quiescent satellite cells (Stockdale, 1992). Diversity in avian muscle fiber types is readily detectable throughout development at each phase of myogenesis with fiber type specific expression of MyHC isoform genes, including the slow MyHC2 gene (Page et al., 1992).

Although both primary and secondary muscle fibers display similar phenotypic diversity in MyHC gene expression, the mechanisms that control their myogenic precursors and ultimate diversification of fiber types within each phase are quite different (Hutcheson, et al., 2009). The embryonic and fetal myoblast populations that give rise to primary and secondary muscle fibers, respectively, display developmental stage specific differences in response to proliferative cues, differentiation and fusion properties, and morphology (Biressi, et al., 2007a). Moreover, embryonic and fetal myoblasts have unique patterns of genome-wide gene expression, including expression of Nuclear Factor IX (Nfix) that activates expression of fetal stage specific myogenic genes and suppresses embryonic stage specific genes (Biressi, et al., 2007b; Messina, et al., 2010).

Most research on the mechanisms that control muscle fiber types has focused on regulation in adult muscle in response to altered activity, electrical stimulation, and innervation. A number of transcriptional regulators and signaling molecules have been implicated in control of adult skeletal muscle fiber phenotypes. These factors include calcineurin, Nuclear Factor of Activated T cells (NFAT; Calabria, et al., 2009), Myocyte Enhancer Factor (MEF2; Liu, et al., 2005), MusTRD/GTF3 (Calvo, et al., 2001; Polly, et al., 2003) and PGC1α (Lin, et al., 2002). Expression patterns of the Myogenic Regulatory Factors (e.g. MyoD, myogenin) have also been associated with different adult muscle fiber types (Hughes, et al., 1993). The majority of these signaling proteins and transcriptional regulators function in response to activity states and innervation patterns in the adult.

Additional research has focused on the appearance of fiber type diversity during secondary myogenesis. Broadly similar to the regulatory mechanisms in adult muscle, the repertoire of contractile and metabolic genes expressed in diverse fiber types at fetal stages is determined in large part by the specific motor neuron input and activity status of the muscle (Schiaffino, et al., 2007). For example, cross-reinnervation of fast and slow contracting muscles with the accompanying neural input induces a switch in expression of fiber type specific genes and corresponding contractile characteristics of the muscle (Roy, et al., 1996). Yet, restrictions

to secondary fiber type diversification and plasticity in response to altered activity and innervation in both mammalian and avian species have been shown by different laboratories (Condon, et al., 1990; DiMario, et al., 1997).

The cellular mechanisms that regulate muscle fiber type diversification during embryonic muscle development are less well understood. Cell autonomous, lineage-dependent differentiation of myoblasts into diverse muscle fiber types in the absence of functional innervation has been reported in avian, rodent, cat, and zebrafish model systems in vivo and in vitro (Page, et al., 1992; Miller and Stockdale, 1986a; Condon, et al., 1990; Roy, et al., 2008; Devoto, et al., 1996). In addition, primary muscle fibers continue to express fiber type specific genes after surgical or functional denervation (Crow and Stockdale, 1986; Fredette and Landmesser, 1991). Furthermore, clonal analysis of embryonic avian myoblasts has shown that individual myoblasts are committed to the formation of specific muscle fiber types both in vitro and in vivo (Miller and Stockdale, 1986b: DiMario, et al., 1993). Therefore initial diversity in muscle fiber types arises from intrinsic embryonic myoblast commitment within specific myoblast lineages.

Only a few clues regarding the transcriptional regulation of embryonic muscle fiber type formation have been garnered. These have been primarily derived from studies in zebrafish, mouse, and avian model systems. Interestingly, many of the signaling and transcriptional regulators that control fiber type specific gene expression in adult and/or fetal stages do not appear to be operative at earlier stages of development. For example, calcineurin is required for the maintenance of adult slow muscle fibers (most of which are derived from fetal myoblasts) in the mouse, but is not required for generation of embryonic slow muscle fibers (Oh, et al., 2005). Similarly, diversification of embryonic muscle fiber types from distinct avian myoblast lineages occurs independently of NFAT and MEF2 transcription factor activities, which are required for expression of muscle fiber type specific genes at later stages of avian and mammalian development (Theobald and DiMario, 2011; Jiang, et al., 2004; Olson and Williams, 2000).

Several factors have been identified that regulate muscle fiber type development in embryonic stages. Six1 and Six4 homeodomain proteins are required for normal hypaxial muscle development and full activation of the fast muscle fiber phenotype in mouse myotomal muscle (Grifone, et al., 2004; Grifone, et al., 2005; Niro, et al., 2010). Six1/Six4 deficient embryos display altered fiber type specific gene expression at fetal (ED18.5) stages of development (Richard, et al., 2011). In zebrafish, Hedgehog signaling induces expression of the *u-boot (ubo)* gene which encodes the transcription factor Blimp1/PRDM1 (Baxendale, et al., 2004). PRDM1 activates the slow muscle fiber phenotype and represses the fast muscle fiber phenotype in the developing zebrafish myotome (Liew, et al., 2008). PRDM1 also represses Sox6 gene expression during zebrafish myotome development (von Hofsten, et al., 2008). Interestingly, Sox6 gene expression during fetal (E15.5) mouse muscle development contributes to development of fast muscle fibers by repression of the slow fiber phenotype. Sox6 knockout mice display increased slow muscle fibers, indicating that Sox6 functions as a transcriptional repressor of the slow fiber phenotype (Hagiwara, et al., 2007).

EMX1 and EMX2 are vertebrate homologs of the Drosophila empty spiracles (ems) gene. In Drosophila, ems functions as a gap homeobox gene and is required for normal anterior (head) structure specification and development of posterior spiracles (Walldorf and Gehring, 1992). In vertebrates, EMX2 is expressed in a wide variety of developing tissues and is involved in diverse developmental events. It is expressed in the developing cerebral cortex and olfactory bulbs of mice at E9.5 (Simeone, et al., 1992). EMX2 promotes neurogenesis and may contribute to correct neuronal pathfinding by direct transcriptional activation of the teneurin-1 gene (Brancaccio, et al., 2010; Beckmann, et al., 2011). EMX2 is also required

for normal development of the mouse urogenital system (Miyamoto, et al., 1997) and hair cell development in the inner ear (Holley, et al., 2010). In vertebrate limb development, EMX2 is required for scapula and ilium formation (Pellegrini, et al. 2001; Malashichev, et al., 2008).

Results

Genome-wide Gene Expression Analysis of Differentiated Fast and Fast/Slow Myogenic Cell Lineages

Embryonic avian myoblasts, isolated from developing limbs during primary muscle fiber formation, are stably committed to the formation of specific muscle fiber types in vitro and in vivo (Miller and Stockdale, 1986a,b; DiMario et al., 1993). For this study, multiple clonal populations of myoblasts were expanded and each clonal population was characterized for its differentiation into muscle fibers that expressed either fast MyHC genes or both fast MyHC and slow MyHC2 genes. Differentiated muscle fibers in vitro formed from clonal myoblasts were immunostained with monoclonal antibodies F59 and S58 to detect fast MyHCs and slow MyHC2, respectively. We have previously reported aggregate data regarding numbers of types of myoblast clones, the similar fusion indices of fast and fast/slow myoblasts, and expression of fast MyHC and slow MyHC2 genes in differentiated clonal cultures (Theobald and DiMario, 2011). For genome-wide gene expression analysis, five fast myogenic clones and four fast/slow myogenic clones were used. The expression of fast MyHC and slow MyHC2 genes in muscle fibers from each clone is shown in Supplement Figure 1A. Myotubes derived from myoblasts committed to the fast fiber fate expressed fast MyHC gene(s) and did not express the slow MyHC2 gene. Myotubes derived from fast/slow myoblasts immunostained with both F59 and S58 antibodies, indicating expression of both fast MyHC gene(s) and the slow MyHC2 gene. Fast and fast/slow myoblast clonal populations selected for gene expression analysis had similar average fusion indices (Supp Fig 1B). RNA was isolated from differentiated muscle fiber cultures of each clonal population. RNAs from the five fast muscle fiber cultures were pooled, as were RNAs from the four fast/slow clonal muscle fiber cultures, to reduce any relative clonal variations (Kendziorski, et al., 2005).

Pooled samples were hybridized to the Affymetrix GeneChip Chicken Genome Array that allows for determination of expression levels of 28,000 transcripts. To validate the results from the microarray hybridization relative to the differentiated phenotypes of the fast and fast/slow muscle fiber clonal populations, expression levels of myosin and myosin-associated protein genes were evaluated (Tables 1 and 2). Genes typically associated with fast muscle fiber types were expressed in differentiated cultures of both fast and fast/slow myoblast types. This is evident by the relative expression levels of fast fiber associated genes in both fast and fast/slow muscle fibers. On average, fast fiber associated genes were expressed 1.32 times greater in fast/slow muscle fibers compared to fast muscle fibers (Table 1). Since all muscle fibers derived from both fast and fast/slow myogenic clones express a fast MyHC gene(s), it is reasonable to anticipate that fast muscle fiber associated genes would be expressed and represented in both fast and fast/slow myogenic clone samples used for microarray analysis. Indeed, the microarray data does not indicate a difference in expression of fast fiber associated genes. In contrast, expression of slow muscle fiber associated genes was on average 6.45 times greater in muscle fibers derived from fast/slow myoblasts versus fast myoblasts (Table 2). Therefore, the microarray analysis identified differential gene expression supporting the existence of myogenic cell clones that differentiate into distinct fast versus fast/slow muscle fiber types.

Embryonic muscle fibers formed from fast and fast/slow myoblast clonal populations exhibited differences in gene expression in a variety of cellular functions. Fast muscle fibers

exhibited increased expression of 718 genes, and fast/slow fibers had increased expression of 799 genes. Relative gene expression levels of two fold or greater were included in the data shown in Figure 1. Biological functions of differentially expressed genes were assigned by GO annotation and/or Entrez Gene and Expasy Proteomics Servers. Functional gene categories include metabolism, transcription, signal transduction, etc. Of those genes that were differentially expressed in fast versus fast/slow embryonic muscle fibers, 23.1% and 23.5% of them were genes associated with metabolic function in fast and fast/slow muscle fibers, respectively. Genes associated with transcriptional regulation in fast versus fast/slow muscle fibers comprised 7.2% and 10.4%, respectively, of differentially expressed genes. Signal transduction genes in fast versus fast/slow muscle fibers accounted for 7.4% and 10.3%, respectively, of differentially expressed genes.

Table 3 lists genes of known identity differentially expressed in fast versus fast/slow muscle fibers. Transcriptional regulatory genes differentially expressed in fast fibers included several helix-loop-helix (HLH) regulatory genes (e.g. ID1, ID2, BHLHB2), interferon regulatory genes (e.g. IFRD1 and IRF10), and homeodomain protein genes (e.g. HoxA10 and NKX-6.1). A complete list of the fast muscle fiber identified gene profile is included in Supplement Table 1.

Table 4 lists genes of known identity differentially expressed in fast/slow versus fast embryonic muscle fibers. Transcriptional regulatory genes expressed in fast/slow muscle fibers included several Hox genes (e.g. HoxA7, Meis2, MEOX2), Nuclear Factor of Activated T Cells (NFATC3), peroxisome proliferator-activated receptor genes (PPARA and PPARG), and zinc finger protein genes (e.g. ZEB1 and Sp3). A complete list of identified genes differentially expressed in fast/slow versus fast embryonic muscle fibers is included in Supplement Table 2.

Eight genes were selected for verification of relative expression levels by quantitative RT-PCR. Relative expression of four genes differentially expressed in fast muscle fibers (DACH1, FHL2, FoxC2, and Sox8) and four genes expressed in fast/slow muscle fibers (EYA4, Foxo1A, NFIB, and PPARA) were quantitated (Figure 2). Differentially expressed genes in fast or fast/slow muscle fibers identified by microarray analyses were differentially expressed by 2.3 to 3.8 fold. The qRT-PCR results validated the microarray analyses.

Fast and fast/slow primary embryonic muscle fibers are derived from myoblasts committed to the fast and fast/slow myogenic cell lineages, respectively. To investigate the basis for differential commitment of fast and fast/slow myoblast lineages to specific embryonic fiber type formation, genome-wide gene expression analysis was conducted on undifferentiated fast and fast/slow myoblasts. Five fast and four fast/slow clonal myoblast populations were pooled according to fiber type commitment (i.e. fast versus fast/slow) and expression of the chicken genome was interrogated. Fast myoblasts differentially expressed 303 genes (Figure 3), and 12% of these genes were associated with transcriptional regulation. Genes encoding the transcription factors BTF3 and PITX2 were among the genes in this functional group expressed in fast myoblasts (Table 5). Genes associated with signal transduction accounted for 16% of genes differentially expressed in fast myoblasts and included FGF13 and GDF10. Transport function was associated with 8% of fast myoblast genes, and 23% were associated with metabolic function. The complete list of genes differentially expressed in fast myoblasts is included in Supplement Table 3. Fast/slow myoblasts differentially expressed 380 genes (Table 6). Genes associated with transcriptional regulation (e.g. MEOX2 and HoxD8) and signal transduction (e.g. FGF4 and IGFBP5) accounted for 10% and 12% of these genes, respectively (Figure 3). Transport and metabolic functions were associated with 7% and 18%, respectively, of genes differentially expressed in fast/slow myoblasts versus fast myoblasts. The complete list of genes differentially expressed in fast/

slow myoblasts is included in Supplement Table 4. Collectively, these results indicate that fast and fast/slow myoblasts express unique subsets of genes and further indicate that fast and fast/slow myoblasts are distinct cell types.

A subset of genes was differentially expressed in both fast myoblasts and fast myotubes (Table 7). Of the 15 genes expressed before and after fast myogenic cell clone differentiation, 6 genes were associated with signal transduction and included FGF13 and FGFR3. Three genes were associated with metabolic function. Similarly, three genes encoded proteins of the cytoskeleton. Lastly, only 2 genes were associated with adhesion function, and 1 gene was identified with structural function. No genes encoding transcriptional regulators were identified as genes differentially expressed in both fast myoblasts and myotubes.

A total of 51 genes were identified as differentially expressed in both fast/slow myoblasts and myotubes versus the fast myogenic cell lineage (Table 8). Genes associated with metabolic function comprised the largest category (41%). Representative genes included SOD2 and SOD3. Signal transduction genes comprised approximately 25% of these genes. Transcriptional regulatory genes accounted for one-third of genes differentially expressed in both fast/slow myoblasts and myotubes versus fast myoblasts and myotubes. Representative genes in this group included TSHZ2, TSHZ3, PPARA, and EMX2.

EMX2 Expression in Fast and Fast/Slow Myogenic Clones

The gene encoding the transcriptional regulator EMX2 was identified in the microarray analysis as a gene that was expressed in both fast/slow myoblasts and muscle fibers. To verify that the gene encoding EMX2 was differentially expressed in fast/slow versus fast myogenic cell lineages, RT-PCR was conducted using two fast myoblast clones and two fast/slow myoblast clones (Figure 4). Expression of the EMX2 gene was identified in both fast/slow myoblast clones, and no significant levels of EMX2 gene expression were detected in fast myoblast clones. Similarly, EMX2 cDNA was amplified from RNAs obtained from differentiated cultures of the two fast/slow myogenic clones (Figure 4). EMX2 gene expression was not detected in differentiated cultures of fast myogenic clones. The product of RT-PCR amplification using the EMX2-specific primers was verified as EMX2 cDNA by DNA sequencing.

To detect EMX2 protein in embryonic myoblasts, myoblast clones differentially committed to the formation of fast and fast/slow primary muscle fibers were fixed and incubated with EMX2 antibody (Figure 5). EMX2 was detected in myoblasts that differentiate into fast/slow primary muscle fibers. EMX2 was predominantly localized to nuclei in these cells. EMX2 was not readily detected in myoblasts committed to the formation of fast primary muscle fibers. Similarly, EMX2 protein was detected in muscle fibers derived from fast/slow myogenic clones and was not readily detected in differentiated cultures of fast myogenic clones (Figure 5).

EMX2 is a Positive Regulator of Slow MyHC2 Promoter Activity

To determine whether expression of EMX2 contributes to the embryonic fast/slow muscle fiber phenotype, the effect of EMX2 expression on slow muscle fiber type specific gene promoter activity was measured. The slow MyHC2 gene promoter is regulated by distinct molecular mechanisms in fast/slow embryonic versus fast/slow fetal muscle fibers. The slow MyHC2 promoter in fast/slow fetal muscle fibers is regulated by an innervation and stimulation-dependent transcriptional mechanism involving MEF2, NFAT, and the proximal 1.43kb promoter (Jiang, et al., 2004). However this promoter region does not confer muscle fiber type specific slow MyHC2 gene expression in embryonic muscle fibers. An additional

~4kb of upstream DNA contained within the promoter-reporter construct, 6150SM2Luc, confers this fiber specificity (Theobald and DiMario, 2011).

Fast and fast/slow myoblast clones were transiently transfected with 6150SM2Luc. Myoblasts were also co-transfected with the EMX2 expression construct, CMVEMX2, or the empty plasmid vector DNA. Myoblasts were allowed to differentiate for 4 days and promoter activities were then measured (Figure 6A). Luciferase activities from the promoterless pGL3Basic vector were unaffected by co-transfection of CMVEMX2. We have previously shown that the slow MyHC2 promoter is specifically activated in fast/slow versus fast embryonic muscle fibers (Theobald and DiMario, 2011). Forced expression of EMX2 in embryonic fast/slow muscle fibers further increased, by approximately 2 fold, slow MyHC2 promoter activity. Interestingly, expression of EMX2 in fast muscle fibers further reduced residual slow MyHC2 promoter activity.

To further investigate the role of EMX2 as a positive regulator of slow MyHC2 gene expression, EMX2 gene expression was knocked down by transfection of EMX2-specific siRNAs. Fast/slow myoblasts were transfected with 6150SM2Luc and either control siRNAs of scrambled nucleotide sequence or EMX2-specific siRNAs. After myogenic differentiation, EMX2 gene expression was assessed by RT-PCR, and slow MyHC2 promoter activities were measured. EMX2-specific siRNAs effectively reduced EMX2 gene expression by 83.3% (Figure 6B). Furthermore, EMX2 siRNAs reduced slow MyHC2 promoter activity in fast/slow myotubes by 41% (Figure 6C). The EMX2 overexpression and knockdown studies indicate that EMX2 functions as a positive regulator of slow MyHC2 gene transcription.

Discussion

Skeletal muscle fiber type diversity arises through different mechanisms at specific developmental stages. Numerous studies in a variety of model systems have demonstrated that skeletal muscle fiber type is dependent on specific neural input or stimulation patterns. However, the studies on muscle fiber type regulation have typically focused on muscle fibers derived from fetal stages of development. Few studies have focused on the mechanism of muscle fiber type diversification during embryonic formation of primary muscle fibers from embryonic myoblasts. Clonal analysis studies, both in vitro and in vivo, have demonstrated that embryonic myoblasts are stably committed to the formation of distinct muscle fiber types and that this commitment is independent of neural input (Miller and Stockdale, 1986a,b; DiMario, et al., 1993). These distinct myoblast cell lineages differentiate into muscle fibers expressing either fast MyHC genes or both fast and slow MyHC genes. The basis of differential expression of fast versus slow fiber type specific genes in embryonic and fetal muscle fibers is also different. For example, slow MyHC2 gene expression in innervated or stimulated fetal avian muscle fibers derived from myoblasts of slow muscle origin is dependent on NFAT transcriptional activity (Jiang, et al., 2004; Crew, et al., 2010). However, slow MyHC2 gene expression in embryonic muscle fibers is not regulated by NFAT in a fiber type specific manner (Theobald and DiMario, 2011).

To investigate the nature of the differences that define fast versus fast/slow embryonic avian muscle fiber types, gene expression profiles of differentiated cultures of fast and fast/slow clonal myoblasts were generated. Fast and fast/slow embryonic muscle fibers displayed a wide array of genes that were differentially expressed. Microarray analysis identified differential expression of 718 genes in fast muscle fibers and 799 genes in fast/slow muscle fibers. The divergent gene expression profiles of fast versus fast/slow embryonic muscle fibers indicate that the muscle fiber diversification extends beyond expression of different

myosin genes. The fast and fast/slow muscle fibers displayed significant heterogeneity in gene expression within multiple cellular processes and functions. Of the genes assigned definitive functions, the largest gene categories included metabolism, transport, signal transduction, and transcription.

To developmentally link fast and fast/slow embryonic muscle fibers as distinct differentiated cells to distinct myoblast cell lineages, additional gene expression profiling was conducted. Similar to differentiated fast and fast/slow muscle fibers, the myoblasts committed to formation of these fast and fast/slow muscle fibers also displayed significant heterogeneity in gene expression. Fast myoblasts differentially expressed 303 genes relative to fast/slow myoblasts. Conversely, fast/slow myoblasts differentially expressed 380 genes. Transcriptional regulators accounted for 12% and 10% of these genes, respectively. This heterogeneity in expression of genes that control transcription as well as other cellular functions such as metabolism, transport, and signal transduction further substantiates the existence of inherent differences between myoblast lineages committed to the differentiation of diverse muscle fiber types.

Comparative analysis of the gene expression profiles of the distinct myoblast types in relation to their corresponding differentiated muscle fiber type was also conducted. Within the embryonic fast myogenic lineage, 15 genes were differentially expressed in both myoblasts and muscle fibers, compared to the fast/slow myogenic lineage. Genes functionally related to cell metabolism and signal transduction were expressed in both fast myoblasts and muscle fibers. Interestingly, no genes of known transcriptional regulators were identified in the shared fast myoblast and fast/slow muscle fiber expression profiles. In contrast, 51 genes were differentially expressed in both myoblasts and muscle fibers of the fast/slow myogenic lineage. Genes associated with metabolic function, transport, and signal transduction were identified. Importantly, 17 genes encoding transcriptional regulators were identified as differentially expressed genes in both fast/slow myoblasts and muscle fibers.

Gene expression profiling of embryonic myoblasts committed to the fast/slow muscle fiber fate as well as profiling of fast/slow muscle fibers themselves identified the transcriptional regulator EMX2 as a gene expressed in fast/slow versus fast myogenic cells. Expression of EMX2 in fast/slow myoblasts and muscle fibers was verified by RT-PCR and immunodetection. This is the first known evidence of expression of EMX2 in skeletal muscle cells.

The EMX2 gene was overexpressed in fast and fast/slow muscle fibers to determine the effect on activity of the slow MyHC2 promoter. Forced EMX2 expression significantly increased slow MyHC2 promoter activity in fast/slow muscle fibers. Therefore, EMX2 is a positive regulator in the differentiation of the fast/slow embryonic myogenic lineage. Furthermore, since EMX2 gene expression occurs in both embryonic fast/slow myoblasts and muscle fibers, it is a marker of this myogenic lineage. The role of EMX2 in lineage determination has also been described in development of the central nervous system. In mammalian cerebral cortex, EMX2 functions as a molecular determinant of CNS precursor cell fate (Heins, et al., 2001). Forced expression of EMX2 in embryonic chick telencephalon resulted in a shift of cell specification toward neuroepithelial identity (von Frowein, et al., 2006). It has been reported that EMX2 gene expression is regulated by developmental signaling pathways such as the β-catenin pathway in developing limbs (Hill, et al., 2006). However, expression of the EMX2 gene can also be cell-autonomous (Nakagawa, et al., 1996). The results reported here demonstrate cell autonomous expression of the EMX2 gene in both embryonic fast/slow myoblasts and muscle fibers.

As an autonomously expressed transcriptional regulator in fast/slow myoblasts and muscle fibers, it is reasonable to hypothesize that EMX2 orchestrates the molecular mechanism of myogenic lineage commitment to embryonic fiber type formation as a singular regulatory factor. As such, it may be anticipated that EMX2 gene expression would drive re-specification of the fast myoblast lineage to the fate of fast/slow muscle fibers when forcibly expressed in the fast myogenic lineage. This hypothesis is supported by increased slow MyHC2 promoter activity in fast/slow muscle fibers overexpressing the EMX2 gene. However, to date, we have not been able to demonstrate that EMX2 gene expression in fast myoblasts and muscle fibers results in a fast to fast/slow lineage re-specification or fiber type transition. There are several possibilities to account for these observations. EMX2 may function as a transcriptional regulator that further distinguishes lineage commitment and/or expression of fiber type specific genes, such as the slow MyHC2 gene. These possible outcomes are not necessarily the same, and additional research is required to completely define the role of EMX2 gene expression in these processes. Nevertheless, our studies suggest that EMX2 gene expression does contribute to molecular and phenotypic distinctions between fast and fast/slow muscle fibers by enhancement of slow MyHC2 promoter activity in fast/slow muscle fibers. Another function for EMX2 gene expression may be more directly related to myogenic fiber type lineage commitment. EMX2 may participate in embryonic myoblast commitment of specific fiber type formation but require other transcription factors, either as direct co-regulators within a transcriptional complex or as other transcription factors simultaneously expressed. Further research is required to elucidate these possible mechanisms.

Experimental Procedures

Cell Culture

Embryonic myoblasts were incubated in cell culture medium consisting of 10% horse serum (Hyclone), 5% chick embryo extract, supplemented with 1.32mM $CaCl_2$, 2mM glutamine and 1X antibiotic/antimycotic (Invitrogen) in Ham's F-10 basal medium (Sigma) mixed with an equal volume of the same medium conditioned by incubation for 2 days in cultures of fully differentiated ED13 chicken myotubes. These cells were prepared as previously described (O'Neill and Stockdale, 1972).

Immunostaining and Fusion Indices

To detect MyHC isoforms, myotubes were immunostained with monoclonal antibodies F59 and S58 for fast MyHCs and slow MyHC2, respectively, as previously described (Crow and Stockdale, 1986; Theobald and DiMario, 2011). Texas Red-conjugated anti-mouse IgG (Vector Labs) and fluorescein-conjugated anti-mouse IgA (Southern Biotech) were used to detect F59 and S58 primary antibodies, respectively. EMX2 was detected using an EMX2 antibody (Sigma). Cells were washed with phosphate buffered saline (PBS) and fixed in 3.7% formaldehyde, 0.1% NP-40 in PBS for 10 minutes. Cells were washed with PBS and then incubated in blocking solution (5% horse serum, 2% bovine serum albumin in PBS) for 1 hour at room temperature. Cells were then incubated in EMX2 antibody, diluted 1:100 in blocking solution, for 1 hour at room temperature. Cells were washed as before and then incubated in FITC-conjugated anti-rabbit IgG (Vector Labs), diluted 1:200 in PBS, for 1 hour at room temperature. Cells were then washed as before and viewed by fluorescence microscopy.

To determine fusion indices, differentiated myotube cultures were immunostained with F59 monoclonal antibody to detect all myotubes. All nuclei were stained with 1.2uM 4′6-diamidino-2-phenylindole (DAPI) in PBS. The ratio of myotube nuclei to all nuclei within a

microscopic field was determined. Four to six random fields were counted for each myogenic clone. A minimum of 1,000 nuclei was counted for each clone.

Microarray Analysis

Total RNAs were extracted using RNA-STAT 60 reagent (Tel-Test, Inc) from 5 fast myogenic clones and 4 fast/slow myogenic clones. RNAs were obtained from clonal myoblasts and myotubes. An equal amount of RNA (1ug) from each clone was used to generate a pooled sample for the fast and fast/slow myoblasts and myotubes (total 4 samples). RNA integrity was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The microarray chips used in this study were the GeneChip Chicken Genome Arraychips (Affy part #900590). Briefly, the Chicken Genome Array contains comprehensive coverage of 32,773 transcripts corresponding to over 28,000 chicken genes. Microarray analysis was performed essentially as described (McCarthy, et al., 2007). The pooled RNA samples were used to synthesize cDNAs that were then used as templates to generate biotinylated cRNAs. cRNA was fragmented and hybridized to the Chicken Genome Array chip, washed, scanned and intensity values for each probe set condensed using the GC-RMA algorithm. A total of 4 chips were processed in this manner, and the data files will be available at Gene Expression Omnibus (www.ncbi.nih.gov/geo). A custom-written MATLAB routine (The MathWorks, Inc., Natick, MA) was used to scrub the data by removing probe sets that were considered “not-expressed” in both fast and fast/slow clones. Our criteria for this was to remove all probe sets in which the intensity value for both fast and fast/slow clones was <350. If one of the two samples or both of the samples had a probe set intensity above 350, the probe set was kept in the dataset for analysis. The analysis for differential expression in the dataset compared the fast sample versus the fast/slow sample or the fast/slow sample versus the fast sample. Those probe sets that were expressed ≥ 2-fold higher were kept in the analysis.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

For quantitative real-time PCR, RNA was extracted as above. cDNA synthesis and amplification was conducted using Full Velocity or Brilliant II SYBR Green QRT-PCR Master Mix Kit (Stratagene) and MJ Research Opticon 2 DNA Engine. Gene expression levels were determined using the comparative Ct method. For semiquantitative RT-PCR, total RNA was extracted using RNA-STAT 60 reagent (Tel-Test, Inc.). EMX2 gene specific product was reverse transcribed and amplified using Access RT-PCR reagents (Promega) and the following oligonucleotides: 5′-CCCAAGCGCTGTTTCACCATCG-3′ and 3′-ATCGTCCGACGTGACGTCGATTTCTT-5′. Quail GAPDH RNA was reverse transcribed and amplified using the following DNA primers: Forward Primer: 5′-CGCCATCACTATCTTCCAGGAGC-3′; Reverse Primer: 5′-GCCAAAGTTGTCATGGATGACC-3′. PCR products were resolved in a 1.2% agarose gel. Identities of the amplified products were verified by DNA sequencing.

Promoter Activity Analysis

Either the slow MyHC2 promoter-reporter DNA (6150SM2Luc) or a promoterless pGL3Basic luciferase DNA construct (Promega) (3ug) was transfected into myogenic cell clones in 35mm cell culture plates using Lipofectamine 2000 (Invitrogen). pRL-SV40 (Promega) (2μg) containing Renilla luciferase was co-transfected to normalize for variations in transfection efficiencies. Either the pCMVTAG empty vector (Stratagene) or CMVEMX2 expression construct (1ug/plate) was co-transfected. Cells were transfected in cell culture medium containing the DNAs and without antibiotic for 5 hours at 37°C in a 5% CO_2 incubator. Transfection medium was then replaced with normal cell culture medium. Five days following transfection, luciferase activities were measured using the Dual-Glo Luciferase Assay (Promega).

For EMX2 knockdown, fast/slow myoblasts in 35mm cell culture plates were co-transfected with 6150SM2Luc (3ug) and the following EMX2-specific siRNAs and their reverse compliment oligonucleotides (100pM): 5′-AAACUCAGGUAAAAGUAUGGUdTdT-3′, 5′-AAGGGAUCCCUCCACCUUCUAdTdT-3′, 5′-AAGGACAAAGUUCAAGCGGCAdTdT-3′. Control siRNAs were designed by randomization of each EMX2-specific siRNA nucleotide sequence. pRL-SV40 (2μg) was co-transfected to normalize for variations in transfection efficiencies. Myoblasts were allowed to differentiate for 5 days before promoter activity was measured.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key findings

Embryonic myoblasts are comprised of distinct myogenic cell lineages, each with unique signatures of gene expression and muscle fiber type formation.

EMX2 gene expression is associated with the fast/slow embryonic myoblast lineage.

EMX2 gene expression is required for normal slow MyHC2 promoter activity in myotubes derived from the fast/slow embryonic myoblast lineage.

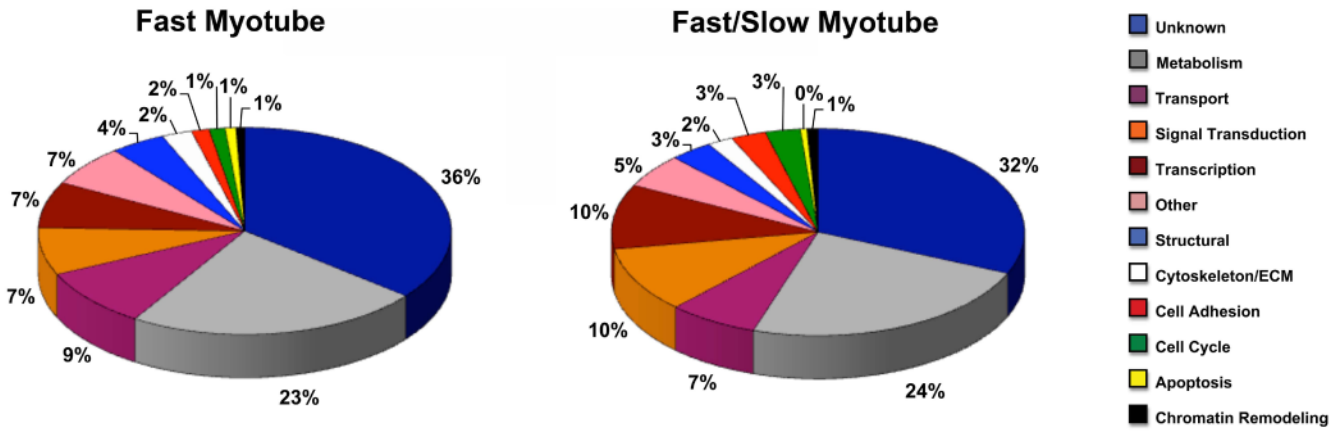

Fig. 1.

Relative distribution of genes differentially expressed in fast versus fast/slow myotubes based on function. Genes expressed more than two-fold in fast or fast/slow myotubes were included in the analysis. Gene functions were assigned by GO annotation and Entrez Gene and Expasy Proteomic Servers. Pie charts represent the percentages of genes assigned particular functions (refer to color legend) for genes differentially expressed in fast myotubes (718 total genes) and fast/slow myotubes (799 total genes) from multiple myogenic clones.

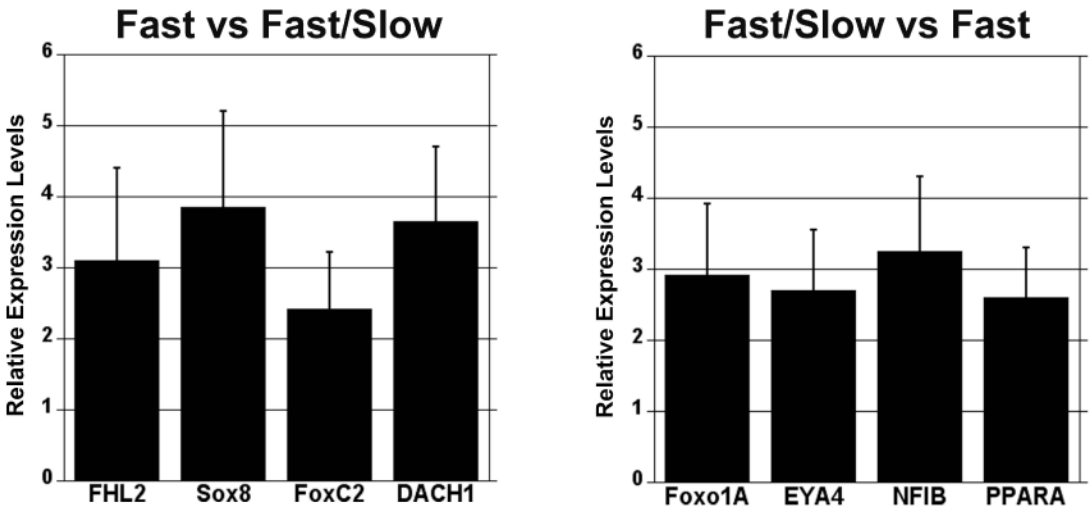

Fig. 2.

Quantitative RT-PCR of select genes. Expression levels of 8 genes was determined by qRT-PCR. Four genes (FHL2, Sox8, FoxC2, and DACH1) were selected from the list of fast myotube associated genes generated from the microarray analysis. Similarly, four genes (Foxo1A, EYA4, NFIB, and PPARA) were selected from the list of fast/slow myotube associated genes. Bars represent relative expression levels of genes. For example, FHL2 is expressed approximately 3 fold higher in fast muscle fibers versus fast/slow fibers.

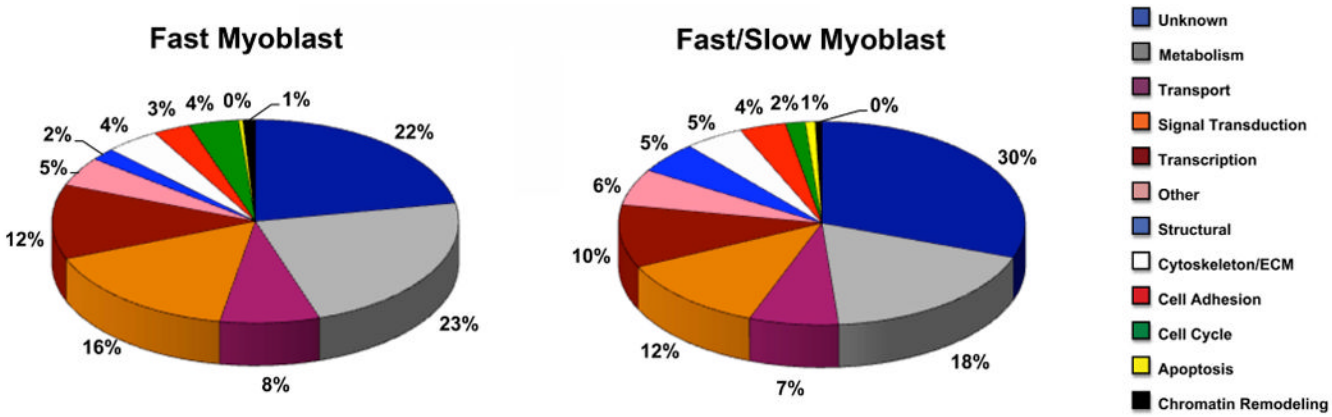

Fig. 3.

Relative distribution of genes differentially expressed in fast versus fast/slow myoblasts. Genes expressed more than two-fold in fast or fast/slow myoblasts were included in the analysis. Gene functions were assigned by GO annotation and Entrez Gene and Expasy Proteomic Servers. Pie charts represent the percentages of genes assigned particular functions (refer to color legend) for genes differentially expressed in fast myoblasts (303 total genes) and fast/slow myoblasts (380 total genes) from multiple myogenic clones.

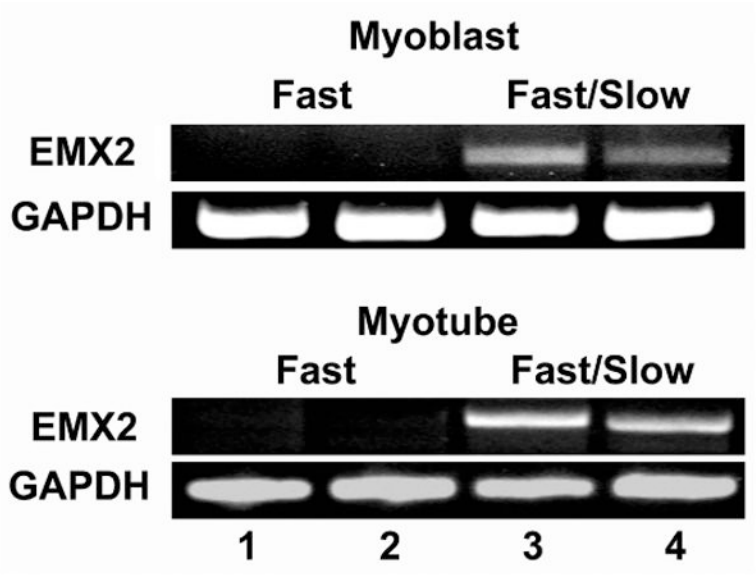

Fig. 4.

RT-PCR amplification of EMX2 cDNA. RNAs from cultures of undifferentiated myoblasts and differentiated muscle fibers from two fast (Lanes 1 and 2) and two fast/slow (Lanes 3 and 4) myogenic clones were reverse transcribed and amplified using EMX2-specific primers. EMX2 RNA was detected in fast/slow myoblasts and myotubes, but not in fast myoblasts or myotubes. GAPDH cDNA was amplified as a control for all samples.

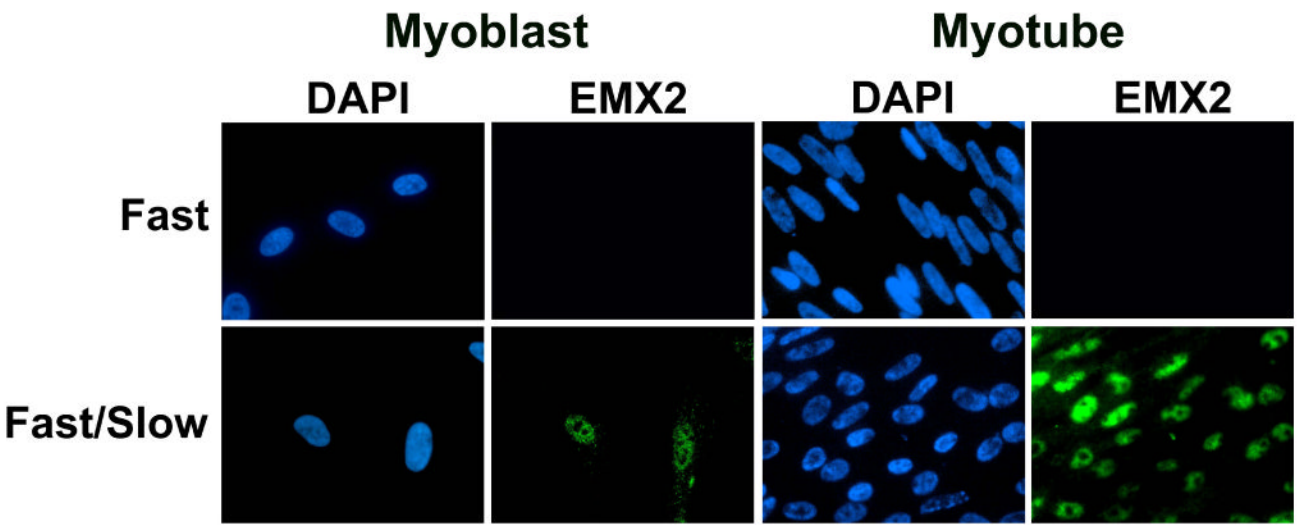

Fig. 5.

Immunodetection of EMX2 protein. Myoblasts and myotubes from fast and fast/slow myogenic clones were immunostained using an EMX2 antibody followed by a FITC-conjugated secondary antibody. EMX2 protein was detected in myoblasts and myotubes of fast/slow myogenic cell origin. EMX2 protein was primarily associated with nuclei. EMX2 was not readily detected in fast myoblasts or myotubes. DAPI staining located all nuclei.

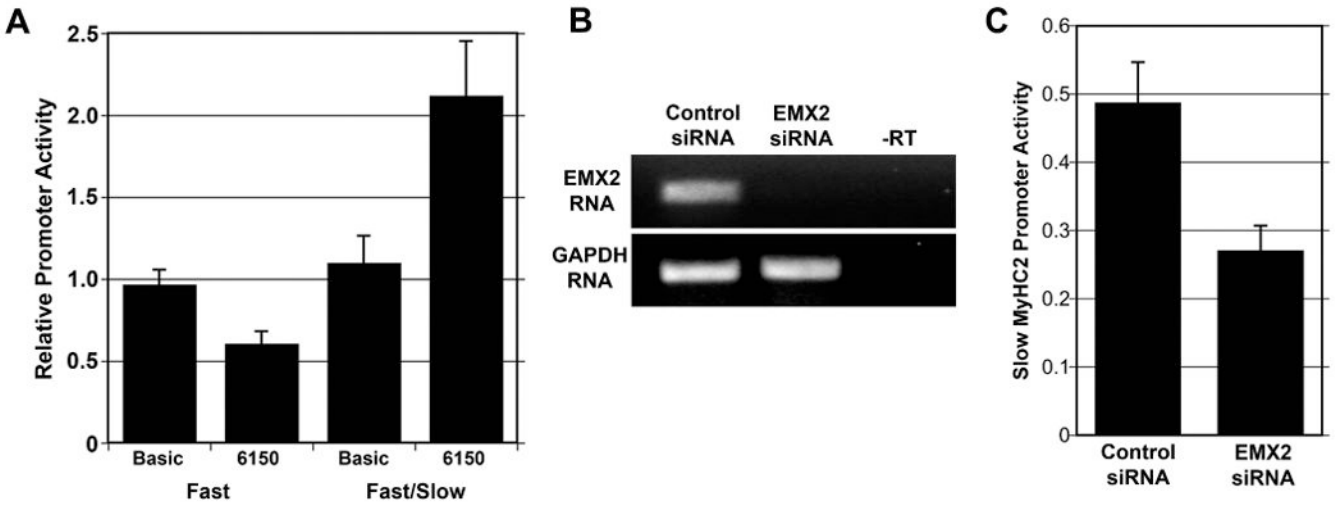

Fig. 6.

EMX2 gene expression regulates slow MyHC2 promoter activity. **A:** Fast and fast/slow myoblast clones were transiently co-transfected with the full-length slow MyHC2 promoter-luciferase DNA construct, 61050SM2Luc, and the EMX2 expression construct, CMVEMX2 (+EMX2), or empty vector (-EMX2). Alternatively, promoterless pGL3Basic (Basic) was also co-transfected with or without CMVEMX2. Bars are mean fold activation of slow MyHC2 promoter activities by EMX2 expression as measured by luciferase activities and normalized by Renilla luciferase activities from co-transfection of pRLSV40 (mean ± S.E.M.). EMX2 expression significantly increased 6150SM2Luc activity in fast/slow muscle fibers ($n = 27$; $p<0.01$) and significantly repressed activity in fast muscle fibers ($n = 10$; $p<0.01$). p values were determined by two-tailed Student's T test. **B:** Transfection of EMX2 siRNAs reduced EMX2 gene expression as determined by RT-PCR. Myoblasts were transfected with EMX2-specific siRNAs (EMX2 siRNA) or siRNAs containing scrambled EMX2 nucleotide sequence (Control siRNA; see Experimental Procedures). RNA was prepared from differentiated myotubes. RNA samples from myotubes transfected with control siRNAs were similarly processed, but without reverse transcriptase (-RT) to access genomic DNA contamination. A representative RT-PCR analysis is shown (n=3). **C:** Transfection of EMX2 siRNAs versus control siRNAs significantly reduced slow MyHC2 promoter activity in fast/slow myotubes (mean ± S.E.M, $p<0.01$ as determined by one-tailed Student's T test).

Table 1

Expression of Fast Muscle Fiber Associated Genes

Gene Symbol	Probe Set ID	Fold Change	Gene Title/Comments
TPM1	Gga4108.4.S1.s.at	1.25	tropomyosin 1 alpha
	Gga4108.1.S2.at	0.28	
	Gga 4108.4.S1.x.at	1.50	
	Gga4108.1.S1.at	0.90	
	GgaAffx20738.1.S1.s.at	0.54	
TNNT3	Gga4090.6.S1.a at	2.90	troponin T type 3
	Gga4090.1.S1.a.at	2.15	
TNNI2	Gga 700.1.S1.at	1.73	troponin I type 2
MYBPC2	Gga4986.1.S1.at	0.69	myosin binding protein C
MYL1	Gga18909.1.S1.s.at	1.53	myosin light chain 1
	Gga18909.1.S1.a.at	0.98	
	Gga4835.1.S1.a.at	1.44	

Table 2

Expression of Fast/Slow Muscle Fiber Associated Genes

Gene Symbol	Probe Set ID	Fold Change	Gene Title/Comments
TPM3	Gga4975.1.S1.a.at	2.35	tropomyosin 3
TNNI1	Gga6340.2.S1.a.at	2.14	troponin I type 1
TNNC1	Gga3041.1.S1.at	1.49	troponin C type 1
STNT	GgaAffx21770.S1.s.at	1.59	slow troponin T
MYBPC1	Gga3063.1.S1.at	3.09	myosin binding protein C1
	Gga10173.1.S1.at	54.93	
	Gga10173.1.S1.s.at	8.59	
	GgaAffx8106.1.S1.s.at	0.39	
MYL2	Gga841.1.S1.at	1.83	myosin light chain 2
MYL3	Gga4198.2.S1.a.at	2.32	myosin light chain 3 slow
SM1	Gga16803.1.S1.s.at	0.79	slow myosin heavy chain 1
MYO1C	GgaAffx11931.1.S1.s.at	1.09	myosin 1C
MYH7	GgaAffx11330.1.S1.at	0.53	myosin heavy chain 7
MYH7B	Gga103.1.S1.at	2.20	myosin heavy chain 7B
AMHC1	Gga5315.1.S1.s.at	13.46	atrial myosin heavy chain 1

Table 3

Genes Preferentially Expressed in Fast Myotubes

Gene Symbol	Probe Set ID	Fold Change	Gene Title/Comments
Apoptosis			
BAG3	GgaAffx.12756.1.S1_at	2.53	BCL2-associated athanogene 3
CABC1	Gga.6127.1.S1_at	2.63	chaperone, ABC1 activity of bc1 complex homolog (S. pombe)
MCL1	Gga.16560.2.S1_s_at	2.35	myeloid cell leukemia sequence 1 (BCL2-related)
TNFRSF6B	Gga.5386.1.S1_at	4.22	tumor necrosis factor receptor superfamily, member 6b, decoy
Cell Adhesion			
ADRM1	Gga.4135.2.S1_a_at	2.37	adhesion regulating molecule 1
ITGB3	Gga.1039.1.S1_at	6.89	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)
K-CAM	Gga.728.1.S1_a_at	4.24	B-cadherin
NCAM1	GgaAffx.22381.3.S1_at	2.16	neural cell adhesion molecule 1
Cell Cycle			
ANAPC2	Gga.7685.3.S1_a_at	3.35	anaphase promoting complex subunit 2
CDT1	Gga.7249.1.S1_at	2.64	chromatin licensing and DNA replication factor 1
Chromatin Remodelling			
SMARCD1	GgaAffx.3872.1.S1_at	2.03	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 1
SMARCE1	GgaAffx.11797.1 .S1_at	2.35	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily e, member 1
Cytoskeleton			
CAPZB	Gga.4050.2.S1_a_at	2.22	capping protein (actin filament) muscle Z-line, beta
DCTN4	GgaAffx.2799.1.S1_at	2.51	dynactin 4 (p62)
HIP1	GgaAffx.22557.1.S1_s_at	2.04	huntingtin interacting protein 1
EMILIN3	GgaAffx.2369.1.S1_at	2.36	elastin microfibril interfacer 3
MGP	Gga.540.1.S1_at	8.37	matrix Gla protein
TUFT1	Gga.14691.1.S1_at	4.61	tuftelin 1
Metabolism			
ACOT7	Gga.5995.1.S1_at	2.16	acyl-CoA thioesterase 7
ASCC3L1	Gga.9209.1.S1_at	2.96	activating signal cointegrator 1 complex subunit 3-like 1
AYTL2	Gga. 16935.1.S1_at	2.05	acyltransferase like 2
B4GALT2	Gga.2424.2.S1_a_at	2.70	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 2
CKB	Gga.2722.1.S1_a_at	3.48	creatine kinase, brain
CREB3L1	GgaAffx.5291.1.S1_at	2.38	cAMP responsive element binding protein 3-like 1
FBP1	Gga.5139.1.S1_at	3.77	fructose-1,6-bisphosphatase 1
FOXRED1	Gga.18113.1.S1_at	2.33	FAD-dependent oxidoreductase domain containing 1
GALE	Gga. 9722.1.S1_at	2.02	UDP-galactose-4-epimerase
GALNS	GgaAffx.21893.2.S1_s_at	2.24	galactosamine (N-acetyl)-6-sulfate sulfatase (Morquio syndrome, mucopolysaccharidosis type IVA)
GALNT5	GgaAffx.7959.1.S1_at	2.62	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 5 (GalNAc-T5)

Gene Symbol	Probe Set ID	Fold Change	Gene Title/Comments
GALT	GgaAffx.1454.1.S1_at	2.55	galactose-1-phosphate uridylyltransferase
GCAT	Gga. 16744.1.S1_at	2.78	glycine C-acetyltransferase (2-amino-3-ketobutyrate coenzyme A ligase)
GCK	Gga.12945.1.S1_at	3.04	glucokinase
GOT2	Gga.4425.1.S2_at	2.60	glutamic-oxaloacetic transaminase 2, mitochondrial (aspartate aminotransferase 2)
GPD2	Gga.11036.1.S1_s_at	2.94	glycerol-3-phosphate dehydrogenase 2 (mitochondrial)
GPI	GgaAffx.11394.1.S1_s_at	2.35	glucose phosphate isomerase
GRHPR	Gga. 7241.1.S1_at	2.42	glyoxylate reductase/hydroxypyruvate reductase
GSS	Gga. 5371.1.S1_at	2.10	glutathione synthetase
HMGCL	Gga. 2537.1. S1_at	2.22	3-hydroxymethyl-3-methylglutaryl-Coenzyme A lyase
LIPT1	Gga.11145.1.S1_at	2.09	lipoyltransferase 1
NDOR1	GgaAffx.5614.1.S1_at	3.48	NADPH dependent diflavin oxidoreductase 1
NOX4	GgaAffx.25209.3.S1_s_at	2.56	NADPH oxidase 4
PFKL	Gga.2810.2.S1_at	2.29	phosphofructokinase, liver
PFKM	Gga.2810.1.S1_at	7.17	phosphofructokinase, muscle
PI4K2A	GgaAffx.3835.1.S1_at	4.87	phosphatidylinositol 4-kinase type 2 alpha
PIP5K1C	GgaAffx.515.2.S1_s_at	4.07	phosphatidylinositol-4-phosphate 5-kinase, type I, gamma
PKM2	Gga.4299.1.S1_at	2.09	pyruvate kinase, muscle
PYGL	GgaAffx.12722.1.S1_s_at	3.42	liver glycogen phosphorylase
RRM2B	GgaAffx.10231.1.S1_at	2.03	ribonucleotide reductase M2 B (TP53 inducible)
SARDH	GgaAffx.1837.1.S1_s_at	2.02	sarcosine dehydrogenase
TPI1	Gga.4148.1.S1_at	2.26	triosephosphate isomerase 1
UROD	GgaAffx.6433.3.S1_s_at	2.18	uroporphyrinogen decarboxylase
Signal Transduction			
BMP10	Gga.9509.1.S1_at	4.58	bone morphogenetic protein 10
CHRM4	GgaAffx.5277.1.S1_at	3.37	cholinergic receptor, muscarinic 4
DDR2	Gga.1162.1.S1_at	2.62	discoidin domain receptor family, member 2
EPHB3	Gga. 3053.1. S1_at	4.33	EPH receptor B3
FBXW4	GgaAffx.22338.1.S1_at	2.24	F-box and WD repeat domain containing 4
FGD3	GgaAffx.26456.1.S1_s_at	2.25	FYVE, RhoGEF and PH domain containing 3
FGF13	Gga.2685.1.S2_at	6.65	fibroblast growth factor 13
FGFR3	Gga. 16413.1.A1_a_at	8.42	fibroblast growth factor receptor 3
GPR88	GgaAffx.26462.1.S1_at	2.20	G protein-coupled receptor 88
GRK6	Gga.19304.1.S1_s_at	2.71	G protein-coupled receptor kinase 6
HGS	Gga.7570.1.S1_at	2.73	hepatocyte growth factor-regulated tyrosine kinase substrate
MAP2K1IP1	Gga.4355.2.S1_s_at	2.55	mitogen-activated protein kinase kinase 1 interacting protein 1
PPP2R2B	GgaAffx.4722.1.S1_s_at	2.24	protein phosphatase 2 (formerly 2A), regulatory subunit B, beta isoform
PRKAB2	GgaAffx.1098.1.S1_s_at	2.91	protein kinase, AMP-activated, beta 2 non-catalytic subunit
RAP2A	GgaAffx.10815.1.S1_at	2.33	RAP2A, member of RAS oncogene family
RERG	GgaAffx.8303.1.S1_at	2.71	RAS-like, estrogen-regulated, growth inhibitor

Gene Symbol	Probe Set ID	Fold Change	Gene Title/Comments
RHOC	Gga. 17535.1.S1_at	2.10	ras homolog gene family, member C
Structural			
MYL2	Gga.839.1.S1_at	2.27	Myosin light chain 2 (LC2f)
MYL	Gga.840.2.S1_a_at	2.28	Myosin alkali light chain mRNA, complete cds, clone pG17-1
ACTG2	Gga.644.1.S1_at	5.37	actin, gamma 2, smooth muscle, enteric
MYL3	Gga.4198.2.S1_a_at	2.32	myosin, light chain 3, alkali; ventricular, skeletal, slow
MYL4	Gga.2698.1.S1_at	3.97	myosin, light chain 4, alkali; atrial, embryonic
SYNC1	GgaAffx.2198.1.S1_at	3.50	syncoilin, intermediate filament 1
TLN1	Gga.4319.1.S1_at	2.31	talin 1
TNNC2	Gga.823.1.S1_at	5.57	troponin C type 2 (fast)
TNNT3	Gga.4090.6.S1_a_at	2.90	troponin T type 3 (skeletal, fast)
TPM1	Gga.4108.5.S1_x_at	2.72	tropomyosin 1 (alpha)
TPM3	Gga.4975.1.S1_a_at	2.35	tropomyosin 3
Transcription			
BHLHB2	GgaAffx.22522.1.S1_at	2.34	basic helix-loop-helix domain containing, class B, 2
CBFB	Gga.17908.1.S1_s_at	2.23	core-binding factor, beta subunit
CEBPB	Gga.4285.1.S1_at	2.05	CCAAT/enhancer binding protein (C/EBP), beta
DACH1	Gga.79.1.S1_at	5.13	dachshund homolog 1 (Drosophila)
ELK4	GgaAffx.26765.1.S1_at	2.70	ELK4, ETS-domain protein (SRF accessory protein 1)
ETV5	Gga.447.1.S1_at	9.23	ets variant gene 5 (ets-related molecule)
FHL2	Gga.3108.1.S1_at	2.80	four and a half LIM domains 2
FOXC2	Gga.469.1.S1_at	5.45	forkhead box C2 (MFH-1, mesenchyme forkhead 1)
HES1	Gga. 3754.2. S1_at	2.61	hairy and enhancer of split 1, (Drosophila)
HOXA10	Gga. 10332.1.S1_at	3.56	Homeobox A10
ID1	Gga.892.1.S1_at	2.64	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein
ID2	Gga.3125.1.S2_at	2.67	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein
IFRD1	GgaAffx.21710.1.S1_s_at	2.17	interferon-related developmental regulator 1
IRF10	Gga.158.1.S1_a_at	15.34	interferon regulatory factor 10
MED16	GgaAffx.25352.1.S1_s_at	2.83	mediator complex subunit 16
MITF	Gga.275.1.S1_at	2.82	microphthalmia-associated transcription factor
MIZF	Gga.7048.1.S1_at	2.52	MBD2-interacting zinc finger
NKX-6.1	Gga.4083.1.S1_at	2.22	homeodomain protein
SOX8	Gga.4309.1.S1_at	2.17	SRY (sex determining region Y)-box 8
Transport			
ABCA3	GgaAffx.25344.4.S1_s_at	2.13	ATP-binding cassette, sub-family A (ABC1), member 3
AE2	Gga. 1335.1. S1_at	2.20	AE2-1 anion exchanger
ATP1B1	Gga.3301.1.S1_at	5.10	ATPase, Na+/K+ transporting, beta 1 polypeptide
ATP6V0A1	Gga. 4672.1.S1_at	2.09	ATPase, H+ transporting, lysosomal V0 subunit A1
CACNA1G	GgaAffx.4763.7.S1_at	2.76	calcium channel, voltage-dependent, T type, alpha 1G subunit

Gene Symbol	Probe Set ID	Fold Change	Gene Title/Comments
IPO13	GgaAffx.12959.1.S1_at	2.15	importin 13
PITPNC1	GgaAffx.25933.1.S1_at	2.08	phosphatidylinositol transfer protein, cytoplasmic 1
SCAMP4	Gga. 17554.1.S1_at	3.10	secretory carrier membrane protein 4
SLC1A6	GgaAffx.26346.2.S1_s_at	2.33	solute carrier family 1 (high affinity aspartate/glutamate transporter), member 6
SLC37A2	GgaAffx.25722.2.S1_s _at	6.73	solute carrier family 37 (glycerol-3-phosphate transporter), member 2
TMC6	GgaAffx.4503.1.S1_at	2.93	transmembrane channel-like 6
XPO5	GgaAffx.23205.1 .S1_s_at	3.87	exportin 5

Table 4

Genes Preferentially Expressed in Fast/Slow Myotubes

Gene Symbol	Probe Set ID	Fold Change	Gene Title/Comments
Apoptosis			
API5	GgaAffx. 11374.1. S1_at	2.61	apoptosis inhibitor 5
BFAR	GgaAffx. 25742.1.S1_at	2.02	bifunctional apoptosis regulator
Cell Adhesion			
CD164	Gga.7158.1.S1_at	2.15	CD164 molecule, sialomucin
CDH2	GgaAffx.21844.1.S1_s_at	2.08	cadherin 2, type 1, N-cadherin (neuronal)
FN1	Gga.9772.1.S1_s_at	2.12	fibronectin 1
ITGA1	Gga.566.1.S1_at	3.27	integrin, alpha 1
ITGA6	Gga.2967.1.S1_at	4.20	integrin, alpha 6
SDC1	Gga.6597.1.S1_at	2.09	syndecan 1
THBS2	GgaAffx.21822.1.S1_s_at	2.19	thrombospondin 2
TJP1	Gga.20045.1.S1_s_at	2.64	tight junction protein 1 (zona occludens 1)
Cell Cycle			
CCAR1	GgaAffx.11996.1.S1_s_at	2.07	cell division cycle and apoptosis regulator 1
CCND1	Gga.3039.1.S1_at	2.04	cyclin D1
CENP-N	GgaAffx.8595.2.S1_s_at	2.05	centromere protein N
GSPT1	Gga.9336.1.S1_at	2.20	G1 to S phase transition 1
SPIN1	Gga.4322.1.S1_at	2.63	spindlin 1
Chromatin Remodeling			
ARID1B	GgaAffx.24250.1.S1_s_at	3.32	AT rich interactive domain 1B (SWI1-like)
ATRX	GgaAffx.22386.2.S1_s_at	3.43	alpha thalassemia/mental retardation syndrome X-linked
BAZ1A	Gga.19082.1.S1_s_at	4.11	bromodomain adjacent to zinc finger domain, 1A
SMARCA1	Gga.2597.1.S1_at	3.29	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 1
SMARCA5	GgaAffx. 11920.1.S1_s_at	2.71	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5
Cytoskeleton and ECM			
CKAP4	GgaAffx.8020.1.S1_at	3.09	cytoskeleton-associated protein 4 (p63)
EML4	GgaAffx.23072.2.S1_s_at	6.74	echinoderm microtubule associated protein like 4
NEXN	Gga.13445.1.S1_s_at	2.36	nexilin
TIMP4	GgaAffx. 26374.1. S1_at	2.72	TIMP metallopeptidase inhibitor 4
Metabolism			
AACS	GgaAffx.1857.1.S1_s_at	2.18	acetoacetyl-CoA synthetase
AGA	GgaAffx.12577.1.S1_at	7.58	aspartylglucosaminidase
AGPS	Gga.5897.1.S1_at	4.02	alkylglycerone phosphate synthase
ALDH1L2	GgaAffx.8040.1.S1_s_at	2.97	aldehyde dehydrogenase 1 family, member L2
AMPD3	GgaAffx. 26558.1. S1_at	3.15	adenosine monophosphate deaminase (isoform E)
ARSJ	GgaAffx. 23739.1. S1_at	2.60	arylsulfatase family, member J

Gene Symbol	Probe Set ID	Fold Change	Gene Title/Comments
BHMT	GgaAffx.2789.1.S1_at	6.35	betaine-homocysteine methyltransferase
CASK	Gga.7689.2.S1_x_at	6.03	calcium/calmodulin-dependent serine protein kinase (MAGUK family)
CDO1	Gga.6921.1.S1_a_at	2.16	cysteine dioxygenase, type I
CPT1A	GgaAffx.20100.1.S1_at	10.00	carnitine palmitoyltransferase 1A (liver)
DDX21	Gga.5656.1.S1_a_at	2.32	DEAD (Asp-Glu-Ala-Asp) box polypeptide 21
GALC	GgaAffx.6700.1.S1_s_at	4.81	galactosylceramidase
GFPT2	GgaAffx.8765.3.S1_s_at	2.02	glutamine-fructose-6-phosphate transaminase 2
GNPDA2	GgaAffx.9013.1.S1_at	2.02	glucosamine-6-phosphate deaminase 2
GNPTAB	GgaAffx.23993.3.S1_s_at	2.02	N-acetylglucosamine-1-phosphate transferase, alpha and beta subunits
GPD1L	GgaAffx. 7282.1.S1_at	2.39	glycerol-3-phosphate dehydrogenase 1-like
GSTT1	Gga.2437.1.S1_at	2.90	glutathione S-transferase theta 1
IDE	GgaAffx.8525.8.S1_s_at	3.25	insulin-degrading enzyme
IDI1	Gga.8851.2.S1_a_at	2.27	isopentenyl-diphosphate delta isomerase 1
ME1	Gga.1132.1.S1_at	2.90	malic enzyme 1, NADP(+)-dependent, cytosolic
PGM5	GgaAffx.9522.1.S1_at	4.38	phosphoglucomutase 5
SOD2	Gga.937.1.S1_at	6.63	superoxide dismutase 2, mitochondrial
SOD3	Gga.1128.2.S1_a_at	3.11	superoxide dismutase 3, extracellular
Signal Transduction			
CALM2 /// RCJMB04_24e7	Gga.4454.2.S1_s_at	2.01	calmodulin 2 (phosphorylase kinase, delta) /// calmodulin 1 (phosphorylase kinase, delta)
CAMK2G	Gga.17610.1.S1_at	2.22	calcium/calmodulin-dependent protein kinase (CaM kinase) II gamma
AGTR1	Gga.632.1.S1_at	5.72	angiotensin II receptor, type 1
ARFGEF2	GgaAffx.26281.3.S1_s_at	3.30	ADP-ribosylation factor guanine nucleotide-exchange factor 2 (brefeldin A-inhibited)
ARHGAP21	Gga.2743.1.S1_at	3.03	Rho GTPase activating protein 21
ASCC3	GgaAffx.9843.1.S1_s_at	13.31	activating signal cointegrator 1 complex subunit 3
EPHA3	Gga.805.1.S1_at	12.94	EPH receptor A3
EPHB1	Gga.694.1.S1_at	2.51	EPH receptor B1
ERBB2IP	GgaAffx.24516.2.S1_s_at	3.09	erbb2 interacting protein
GDAP2	Gga.12508.1.S1_at	2.63	ganglioside induced differentiation associated protein 2
GRM7	GgaAffx. 5262.1.S1_at	2.75	glutamate receptor, metabotropic 7
IL1R1	Gga.846.1.S1_at	4.50	interleukin 1 receptor, type I
INPP5F	Gga. 13374.1.S1_at	2.30	inositol polyphosphate-5-phosphatase F
LTBP1	GgaAffx.6607.2.S1_s_at	3.32	latent transforming growth factor beta binding protein 1
MAPK9	Gga.3651.1.S1_at	2.63	mitogen-activated protein kinase 9
PDE3A	GgaAffx.24123.1.S1_at	3.01	phosphodiesterase 3A, cGMP-inhibited
PDGFD	Gga.9675.1.S1_at	5.61	platelet derived growth factor D
PIK3C2A	GgaAffx.26752.1.S1_s_at	2.83	phosphoinositide-3-kinase, class 2, alpha polypeptide
PIK3CA	GgaAffx. 5619.1.S1_at	2.62	phosphoinositide-3-kinase, catalytic, alpha polypeptide

Gene Symbol	Probe Set ID	Fold Change	Gene Title/Comments
PKIA	Gga.3155.1.S1_at	2.94	protein kinase (cAMP-dependent, catalytic) inhibitor alpha
PLCD1	Gga.12980.1.S1_s_at	2.84	phospholipase C, delta 1
PRKD3	GgaAffx.6712.2.S1_s_at	2.51	protein kinase D3
RCAN1	Gga.5465.1.S1_at	3.80	regulator of calcineurin 1
RGS9BP	Gga.9490.1.S1_at	2.69	regulator of G protein signaling 9 binding protein
SGSM2	GgaAffx.3595.1.S1_s_at	3.65	small G protein signaling modulator 2
TOB1	Gga.1160.1.S1_at	13.60	transducer of ERBB2, 1
WISP1	Gga. 7551.1.S1_at	3.16	WNT1 inducible signaling pathway protein 1
Structural			
COL1A2	Gga.3607.1.S1_a _at	3.67	collagen, type I, alpha 2
DMD	Gga.718.2.S1_a_at	2.09	dystrophin
MYO1B	GgaAffx.22337.2.S1_s_at	2.25	myosin IB
MYOM3	GgaAffx.2577.2.S1_s_at	5.50	myomesin family, member 3
TMOD3	GgaAffx.11704.1.S1_s_at	2.52	tropomodulin 3 (ubiquitous)
TTC8	GgaAffx. 6738.1.S1_at	2.68	tetratricopeptide repeat domain 8
TUBB	Gga.4579.1.S1_x_at	4.10	tubulin, beta
Transcription			
BCLAF1	GgaAffx.24308.2.S1_s_at	2.13	BCL2-associated transcription factor 1
BRD1	GgaAffx. 22617.1.S1_at	2.28	bromodomain containing 1
BRMS1L	GgaAffx.11818.1.S1_s_at	2.29	breast cancer metastasis-suppressor 1-like
EBF1	Gga.276.1.S1_at	2.79	early B-cell factor 1
EMX2	Gga. 7683.1.S1_at	4.95	empty spiracles homeobox 2
EYA4	Gga.420.1.S1_s_at	3.19	eyes absent homolog 4 (Drosophila)
EZH2	Gga.20057.1.S1_s_at	2.26	enhancer of zeste homolog 2 (Drosophila)
FHL5	Gga.10208.1.S1_a_at	2.69	four and a half LIM domains 5
FOXO1A	Gga.3406.1.S1_at	2.21	forkhead box O1A
FOXO3	Gga.19700.1.S1_at	2.35	forkhead box O3
HOXA7	Gga.5122.1.S1_at	2.02	homeobox A7
HOXD8	Gga.3187.1.S1_at	5.80	homeobox D8
JAZF1	Gga.7912.1.S1_at	2.35	JAZF zinc finger 1
LHX9	Gga.2348.1.S1_a_at	6.63	LIM homeobox 9
MEIS2	Gga.4046.1.S1_at	2.91	Meis homeobox 2
MEOX2	Gga.90.1.S1_at	3.95	mesenchyme homeobox 2
NFATC3	Gga.19337.1.S1_s_at	2.14	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3
NFIB	Gga.17307.1.S1_at	3.92	nuclear factor I/B
PITX1	Gga.13903.1.S1_at	3.83	paired-like homeodomain 1
PPARA	Gga.4006.2.S1_a_at	3.03	peroxisome proliferator-activated receptor alpha
PPARG	Gga.3858.2.S1_a_at	2.16	peroxisome proliferator-activated receptor gamma
R3HDM1	GgaAffx.23837.4.S1_s_at	2.10	R3H domain containing 1

Gene Symbol	Probe Set ID	Fold Change	Gene Title/Comments
RAB8B	Gga.13026.1.S1_at	2.30	RAB8B, member RAS oncogene family
RAI14	Gga.12606.1.S1_s_at	3.75	retinoic acid induced 14
RARB	Gga.2668.2.S1_at	2.54	retinoic acid receptor, beta
RREB1	Gga.1491.1.S1_at	2.45	ras responsive element binding protein 1
SMARCD3	GgaAffx.8276.3.S1_s_at	3.03	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 3
SP3	Gga.2337.1.S1_s_at	2.03	Sp3 transcription factor
TBPL1	Gga.4434.1.S1_at	2.95	TBP-like 1
TFDP1	Gga.3952.1.S1_at	5.16	transcription factor Dp-1
TSHZ3	Gga.15899.1.S1_at	3.52	teashirt zinc finger homeobox 3
YAF2	Gga.1754.1.S1_s_at	2.51	YY1 associated factor 2
ZEB1	Gga.3548.1.S1_at	2.22	zinc finger E-box binding homeobox 1
ZFHX4	GgaAffx.9993.1.S1_at	4.29	zinc finger homeobox 4
ZMYND11	GgaAffx.21984.1.S1_at	3.07	zinc finger, MYND domain containing 11
Transport			
ATP6AP1	GgaAffx.5549.1.S1_at	2.17	ATPase, H+ transporting, lysosomal accessory protein 1
BBS5	Gga.19986.1.S1_at	2.13	Bardet-Biedl syndrome 5
BIN1	GgaAffx.11745.1.S1_s_at	2.13	bridging integrator 1
CAST	GgaAffx.9300.1.S1_at	2.20	calpastatin
COLEC12	Gga.10960.1.S1_at	2.82	collectin sub-family member 12
CYB5	GgaAffx.21828.1.S1_s_at	3.58	cytochrome b-5
FTD	Gga.20.1.S2_at	9.22	ferritoid
KPNA3	Gga.1482.1.S1_at	2.68	karyopherin alpha 3 (importin alpha 4)
OPTN	Gga.4189.1.S1_s_at	2.45	optineurin
RBP7	Gga.9386.1.S1_at	3.12	retinol binding protein 7, cellular
SCFD1	GgaAffx.6231.1.S1_s_at	2.11	sec1 family domain containing 1
SCP2	Gga.3425.1.S1_at	2.86	sterol carrier protein 2
SLC22A16	GgaAffx.24590.1.S1_s_at	5.21	solute carrier family 22 (organic cation transporter), member 16
SLC25A36	GgaAffx.3298.1.S1_s_at	2.56	solute carrier family 25, member 36
SLC30A1	Gga.10012.1.S1_s_at	2.60	solute carrier family 30 (zinc transporter), member 1
SLC45A4	Gga.5046.1.A1_s_at	2.30	Solute carrier family 45, member 4
SNX2	GgaAffx. 3339.1.S1_at	3.30	sorting nexin 2
SRP54	Gga.1375.3.S1_s_at	2.57	signal recognition particle 54kDa
STX16	GgaAffx.12300.1.S1_s_at	2.05	syntaxin 16
SYTL2	GgaAffx.8937.1.S1_at	4.55	synaptotagmin-like 2
TMED5	Gga.3703.1.S1_s_at	2.14	transmembrane emp24 protein transport domain containing 5

Table 5

Genes Preferentially Expressed in Fast Myoblasts

Gene Symbol	Probe Set ID	Fold Change	Gene Title/Comments
Cell Adhesion			
ALCAM	Gga.2734.1.S2_at	7.70	activated leukocyte cell adhesion molecule
ANKK1	GgaAffx.22381.3.S1_s_at	2.65	ankyrin repeat and kinase domain containing 1
ITGB3	Gga.1039.1.S1_at	2.31	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)
Cell Cycle			
CCNF	GgaAffx.22831.1.S1_at	2.18	cyclin F
CDKN2C	GgaAffx. 6661.1.S1_at	2.49	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)
CKS2	Gga.1958.1.S1_a_at	3.34	CDC28 protein kinase regulatory subunit 2
GINS1	Gga.12208.1.S1_a_at	3.12	GINS complex subunit 1 (Psf1 homolog)
KNTC1	GgaAffx.26234.1.S1_s_at	2.01	kinetochore associated 1
SEPT2	GgaAffx.3632.1.S1_at	2.32	septin 2
Chromatin Remodeling			
SUZ12	Gga.19626.1.S1_s_at	2.11	suppressor of zeste 12 homolog (Drosophila)
Cytoskeleton			
AFAP1	Gga.185.1.S1_a_at	2.33	actin filament associated protein 1
DCTN4	GgaAffx.2799.1.S1_at	3.04	dynactin 4 (p62)
DYNLL2	Gga.17308.1.S1_s_at	2.07	dynein, light chain, LC8-type 2
KIF26A	GgaAffx.23603.1.S1_s_at	2.05	kinesin family member 26A
MAP4	GgaAffx.21343.1.S1_s_at	2.04	microtubule-associated protein 4
Metabolism			
AER61	GgaAffx.8545.1.S1_s_at	2.00	glycosyltransferase
ASPH	Gga.11883.4.S1_s_at	2.35	Aspartate beta-hydroxylase
B4GALT6	GgaAffx.9633.1.S1_s_at	3.60	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 6
COMT	Gga.7199.1.S1_s_at	3.35	catechol-O-methyltransferase
DHFR	GgaAffx.11934.1.S1_s_at	2.07	dihydrofolate reductase
GLT25D2	Gga.3249.1.S1_at	2.49	glycosyltransferase 25 domain containing 2
HEXB	Gga.9970.1.S1_at	2.40	hexosaminidase B (beta polypeptide)
HMGCR	GgaAffx.12414.1.S1_s_at	2.20	3-hydroxy-3-methylglutaryl-Coenzyme A reductase
MAN1A1	Gga.20070.1.S1_at	3.38	mannosidase, alpha, class 1A, member 1
NADK	GgaAffx.907.1.S1_at	2.20	NAD kinase
NAT13	GgaAffx.9403.1.S1_s_at	2.17	N-acetyltransferase 13
PCMT1	Gga. 16623.2.S1_a_at	2.08	protein-L-isoaspartate (D-aspartate) O-methyltransferase
PMPCB	Gga.7638.1.A1_at	2.28	peptidase (mitochondrial processing) beta
PTPN2	Gga.1107.1 .S1_at	2.15	protein tyrosine phosphatase, non-receptor type 2
ROR1	Gga.9476.1.S1_at	2.56	receptor tyrosine kinase-like orphan receptor 1
SENP8	GgaAffx.1329.1.S1_at	2.43	SUMO/sentrin specific peptidase family member 8
ST3GAL1	Gga. 3672.1.S1_at	3.26	ST3 beta-galactoside alpha-2,3-sialyltransferase 1

Gene Symbol	Probe Set ID	Fold Change	Gene Title/Comments
ST8SIA2	Gga.19493.2.S1_s_at	4.92	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 2
TXNDC10	GgaAffx.24266.1 .S1_at	2.21	thioredoxin domain containing 10
Signal Transduction			
ADCYAP1R1	GgaAffx. 3269.1. S1_at	12.84	adenylate cyclase activating polypeptide 1 (pituitary) receptor type I
ARHGAP12	GgaAffx.4536.1.S1_s_at	2.39	Rho GTPase activating protein 12
BMPR1A	Gga.755.1.S1_at	2.27	bone morphogenetic protein receptor, type IA
DKK3	Gga.3573.2.S1_a_at	4.44	dickkopf homolog 3 (Xenopus laevis)
EDN1	GgaAffx.8070.1.S1_at	5.40	endothelin 1
EPHB3	Gga.3053.1.S1_at	2.03	EPH receptor B3
FGF13	GgaAffx.21832.1.S1_s_at	7.36	fibroblast growth factor 13
FGFR3	Gga.16413.1.A1_a_at	5.02	fibroblast growth factor receptor 3
FLT1	Gga.150.2.S1_a_at	2.72	fms-related tyrosine kinase 1
FRZB	Gga.4955.1.S1_at	3.08	frizzled-related protein
GDF10	GgaAffx.3720.1.S1_at	8.39	growth differentiation factor 10
GFRA1	Gga.588.1.S1_at	3.21	GDNF family receptor alpha 1
GPR23	Gga.11466.2.S1_a_at	6.08	G protein-coupled receptor 23
IL1R1	Gga.846.1.S1_at	2.32	interleukin 1 receptor, type I
ITPR3	GgaAffx.1993.5.S1_s_at	2.37	inositol 1,4,5-triphosphate receptor, type 3
KREMEN1	GgaAffx.3631.1.S1_at	2.67	kringle containing transmembrane protein 1
MRAS	Gga.5500.2.S1_a_at	2.22	muscle RAS oncogene homolog
RASL11B	Gga.12911.1.S1_at	2.16	RAS-like, family 11, member B
RGS3	Gga.8344.2.A1_a_at	3.47	regulator of G-protein signalling 3
RPS6KA1	Gga.9321.1.S1_at	7.40	ribosomal protein S6 kinase, 90kDa, polypeptide 1
SOCS1	Gga.10606.1.S1_at	2.74	suppressor of cytokine signaling 1
Structural			
CTXN1	GgaAffx.210.1.S1_at	2.84	cortexin 1
FBLN2	GgaAffx.3200.1.S1_s_at	2.18	fibulin 2
TNNT2	Gga.4984.1.S1_at	3.51	troponin T type 2 (cardiac)
Transcription			
BRD8	GgaAffx.9060.2.S1_s_at	2.02	bromodomain containing 8
BTF3	Gga.11922.1.S1_at	2.40	basic transcription factor 3
E2F1	Gga.3213.1.S1_at	2.06	E2F transcription factor 1
EGR1	GgaAffx.11738.1.S1_s_at	2.71	early growth response 1
FOXP1	GgaAffx.4846.4.S1_s_at	2.87	forkhead box P1
HOXA11	Gga.957.1.S1_at	2.06	homeobox A11
PITX2	Gga.3398.2.S1_a_at	4.47	paired-like homeodomain 2
SNAI1	Gga.3851.1.S1_at	2.01	snail homolog 1 (Drosophila)
TCF12	Gga.4007.3.S1_a_at	2.03	transcription factor 12 (HTF4, helix-loop-helix transcription factors 4)
ZBTB41	GgaAffx.25447.1.S1_at	2.31	zinc finger and BTB domain containing 41

Gene Symbol	Probe Set ID	Fold Change	Gene Title/Comments
Transport			
ATP2B1	GgaAffx.23508.1.S1_at	2.17	ATPase, Ca++ transporting, plasma membrane 1
KCNK1	Gga.4356.1.S1_at	2.25	potassium channel, subfamily K, member 1
SLC39A10	GgaAffx.22358.1.S1_s_at	2.51	solute carrier family 39 (zinc transporter), member 10
SNX30	Gga.11940.1.S1_at	2.27	sorting nexin family member 30
VLDLR	Gga.679.1.S1_at	3.64	very low density lipoprotein receptor

Table 6

Genes Preferentially Expressed in Fast/Slow Myoblasts

Gene Symbol	Probe Set ID	Fold Change	Gene Title/Comments
Cell Adhesion			
ITGA1	Gga.566.1.S1_at	10.65	integrin, alpha 1
LAMA2	Gga.8352.1.S1_at	2.81	similar to laminin alpha 2 subunit precursor; laminin M
RELN	Gga.496.1.S1_at	5.44	extracellular reelin
THBS2	Gga.1686.1.S1_s_at	2.50	thrombospondin 2
TNC	GgaAffx.26374.1.S1_at	2.87	tenascin
Cell Cycle			
CCNG2	Gga.15984.1.S1_at	5.98	cyclin G2
CDC42	Gga.4438.1.S1_at	2.74	cell division cycle 42
Chromatin Remodeling			
SMARCA1	GgaAffx.4778.1.S1_s_at	2.15	similar to possible global transcription activator SNF2L1
Cytoskeleton			
DCN	Gga.1719.1.S1_at	3.87	decorin
FBLN5	Gga.10096.1.S1_at	3.99	fibulin 5
KRT75	Gga.17686.1.S1_at	6.24	type II alpha keratin IIB
MAP1LC3C	Gga.3183.1.S1_a_at	8.28	microtubule-associated protein 1 light chain 3 gamma
NEFM	Gga.4179.1.S1_at	9.43	neurofilament 3
SDC2	Gga. 4675.1. S1_at	2.13	syndecan 2
Metabolism			
CAMK2D	GgaAffx.12207.1.S1_at	2.39	calcium/calmodulin-dependent protein kinase IID
CARS	GgaAffx.21941.1.S1_at	4.10	cysteinyl-tRNA synthetase
CDO1	Gga.6921.1.S1_a_at	5.14	similar to cysteine dioxygenase
DPYD	GgaAffx.3458.1.S1_s_at	6.38	dihydropyrimidine dehydrogenase
DPYSL3	Gga.9493.1.S1_at	10.32	dihydropyrimidinase-like 3
DUSP1	Gga.4120.1.S1_at	2.34	dual specificity phosphatase 1
DUSP5	Gga.19025.1.S1_at	2.64	dual specificity phosphatase 5
FAP	GgaAffx.23453.2.S1_s_at	3.78	fibroblast activation protein, alpha
FECH	Gga.166.1.S1_at	2.91	ferrochelatase
FUT8	GgaAffx.13151.1.S1_at	2.91	fucosyltransferase 8
GALNTL4	Gga.11756.1.S1_at	10.49	N-acetylgalactosaminyltransferase-like 4
GFPT2	GgaAffx.8765.2.S1_at	3.05	similar to glutamine:fructose-6-phosphate amidotransferase 2
GSTK1	Gga.14517.1.S1_s_at	3.26	glutathione S-transferase kappa 1
GSTT1	Gga. 2437.1. S1_at	2.76	glutathione S-transferase theta 1
HAS2	Gga.329.1.S1_at	2.44	hyaluronan synthase 2
LYCAT	Gga.7898.1.S1_at	4.02	lysocardiolipin acyltransferase
MAN2C1	GgaAffx.1059.1.S1_s_at	2.05	similar to alpha-mannosidase 2C1
ME1	Gga.1132.1.S1_at	3.32	malic enzyme 1, NADP(+)-dependent, cytosolic

Gene Symbol	Probe Set ID	Fold Change	Gene Title/Comments
MOXD1	Gga.969.1.S1_at	2.43	monooxygenase, DBH-like 1
MTRR	GgaAffx.24101.1.S1_at	2.04	similar to methionine synthase reductase isoform 2
PAPSS1	GgaAffx.23250.1.S1_s_at	2.07	3′-phosphoadenosine 5′-phosphosufate synthase 1
PGM5	GgaAffx.9522.1.S1_at	3.55	similar to phosphoglucomutase 5
PLK2	Gga.10660.2.S1_at	2.21	similar to polo-like kinase 2
PPAP2B	GgaAffx.23330.1.S1_at	4.09	similar to phosphatidic acid phosphatase type 2B
SOD2	Gga.4220.1.S1_a_at	3.22	superoxide dismutase 2, mitochondrial
SOD3	Gga.19934.1.S1_at	3.03	superoxide dismutase 3, extracellular
SULT1B1	Gga.735.1.S1_at	4.44	sulfotransferase family, cytosolic, 1B, member 1
UPP1	Gga.18724.1 .S1_s_at	3.71	uridine phosphorylase 1
Signal Transduction			
CXCL14	GgaAffx.21581.1.S1_s_at	4.66	chemokine ligand 14
DGKH	GgaAffx.10860.2.S1_s_at	2.16	similar to A-kinase anchor protein 11
DKK1	Gga.897.1.S1_at	4.03	Dikkopf homolog 1
EPHA3	Gga.805.1.S1_at	22.05	EPH receptor A3
FGF3	Gga.2701.1.S1_at	4.25	fibroblast growth factor 3
FGF4	GgaAffx.4716.1.S1_at	49.16	fibroblast growth factor 4
GTPBP4	Gga.9844.1.S1_s_at	2.49	GTP binding protein 4
IGF2R	Gga.3597.1.S1_at	2.08	insulin-like growth factor 2 receptor
IGFBP2	Gga.759.1.S1_at	3.13	insulin-like growth factor receptor binding protein 2
IGFBP5	Gga. 9364.1.S1_at	4.19	insulin-like growth factor binding protein 5
IL6	Gga.2769.1.S1_at	2.07	interleukin 6
IL8	Gga.826.1.S1_s_at	3.87	interleukin 8
LSP1	Gga.16589.1.S1_at	10.23	lymphocyte-specific protein 1
LTBP1	GgaAffx.6607.2.S1_s_at	2.65	similar to latent transforming growth factor beta binding protein 1
MAPK13	GgaAffx.549.1.S1_at	2.58	mitogen-activated protein kinase 14
NRG1	Gga.135.3.S1_a_at	8.07	neuregulin 1
PDE3A	GgaAffx.24123.1.S1_at	4.09	similar to cyclic nucleotide phosphodiesterase PDE3A
PDGFD	Gga. 9675.1.S1_at	4.07	platelet derived growth factor D
PPP2R3A	GgaAffx.23502.1.S1_at	2.86	similar to alpha isoform of regulatory subunit B, protein phosphatase 2, isoform 1
RHOJ	Gga.12598.1.S1_at	3.05	ras homolog gene family, member J
SH3BGR	Gga.11787.2.S1_s_at	3.38	SH3 domain binding glutamic acid-rich protein
TGFB3	GgaAffx.21766.1.S1_s_at	2.86	transforming growth factor beta 3
VEGFC	Gga.10930.1.S1_at	2.11	similar to vascular endothelial growth factor C
WNT9A	GgaAffx.21279.1.S1_at	2.99	wingless-type MMTV integration site family , member 9A
ZIC1	Gga.11492.1.S1_at	2.84	zic family member 1
Structural			
ACTA1	Gga. 5962.1.S1_at	2.32	A-actin
ACTN2	Gga.4843.2.S1_a_at	4.36	actinin, alpha 2

Gene Symbol	Probe Set ID	Fold Change	Gene Title/Comments
MYH6	Gga. 2617.1. S1_at	3.06	myosin, heavy polypeptide 6
MYL3	Gga.4198.2.S1_a_at	3.16	myosin, light polypeptide 3, alkali; skeletal slow
MYOM2	Gga.4216.1.S1_at	9.40	myomesin (M-protein) 2
SHROOM3	Gga.15872.1.S1_s_at	2.48	similar to shroom-related protein
TNNC2	Gga. 1722.1.S1_at	2.42	troponin C type 2
TNNI1	Gga.3818.1.S1_at	2.27	troponin I type 1
Transcription			
EBF1	Gga.276.2.S1_a_at	12.03	early B-cell factor 1
EMX2	Gga. 7683.1. S1_at	5.35	empty spiracles homolog 2
EYA2	Gga.1839.1.S1_at	3.75	eyes absent homolog 2
EYA4	GgaAffx.24324.1.S1_at	12.58	eyes absent homolog 4
FOXO1A	Gga. 3406.1. S1_at	2.49	forkhead box 01A
FOXP2	GgaAffx.5942.1.S1_at	33.57	forkhead box P2
HEY2	GgaAffx.9430.1.S1_at	2.26	similar to hairy/enhancer-of-split related
HOXD8	Gga.3187.1.S1_at	6.63	homeobox D8
ID4	Gga.2070.2.S1_a_at	3.54	inhibitor of DNA binding 4
KLF3	Gga. 12232.1.S1_at	2.33	Krüppel-like factor 3
LHX9	Gga.2348.1.S1_a_at	10.27	LIM homeobox 9
MEOX2	Gga.90.1.S1_at	16.35	mesenchme homeobox 2
NFE2L2	Gga. 3659.1. S1_at	2.05	nuclear factor (erythroid-derived 2)-like 2
NFIB	Gga.17307.1.S1_at	3.49	nuclear factor I/B
PPARA	Gga.4006.1.S1_at	2.46	peroxisome proliferative activated receptor, alpha
PRRX1	Gga. 1546.1. S1_at	5.35	paired related homeobox 1
SOX4	Gga.937.1.S1_at	2.24	SRY (sex determining region Y)-box 4
Transport			
ATP6V0D1	Gga.7507.1.S1_at	2.06	ATPase, H+ transporting
ATP6V1G1	Gga.4824.1.S1_at	10.86	similar to ATP6V1G1-prov protein
CYB5A	GgaAffx.21828.1.S1_s_at	2.26	cytochrome B-5
FABP4	Gga.4939.1.S1_s_at	20.36	fatty acid binding protein 4
FTD	Gga.20.1.S2_at	3.29	ferritoid
NXT2	GgaAffx.11867.1.S1_s_at	2.49	nuclear transport factor 2-like export factor 2
ORMDL1	GgaAffx.25485.1.S1_at	2.32	solute carrier family 40 (iron-regulated transporter), member 1
RBP4	Gga.4126.1.S1_at	2.28	retinol binding protein 4, plasma
RBP7	Gga.9386.1.S1_at	3.97	retinol binding protein 7, cellular

Table 7

Genes Expressed in Both Fast Myoblasts and Myotubes

Gene Symbol	Gene Title/Comments
Cell Adhesion	
ITGB3	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)
PODXL	podocalyxin-like
Cytoskeleton	
DCTN4	dynactin 4 (p62)
MGP	matrix Gla protein
SMTN	smoothelin
Metabolism	
CTSD	cathepsin D
GALNT5	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 5 (GalNAc-T5)
GPD2	glycerol-3-phosphate dehydrogenase 2 (mitochondrial)
Signal Transduction	
CCNDBP1	cyclin D-type binding-protein 1
DKK3	dickkopf homolog 3 (Xenopus laevis)
EPHB3	EPH receptor B3
FGF13	fibroblast growth factor 13
FGFR3	fibroblast growth factor receptor 3
FZD2	frizzled homolog 2 (Drosophila)
Structural	
FBLN2	fibulin 2

Table 8

Genes Expressed in Both Fast Myoblasts and Myotubes

Gene Symbol	Gene Title/Comments
Cell Adhesion	
ARVCF	armadillo repeat gene
FMN1	formin
ITGA1	integrin, alpha 1
POSTN	periostin, osteoblast specific factor
THBS2	thrombospondin 2
WTIP	
Cell Cycle	
CCNG2	cyclin G2
PPP3CA	protein phosphatase 3, catalytic subunit, alpha isoform
Chromatin Remodeling	
SMARCA1	similar to possible global transcription activator SNF2L1
Cytoskeleton	
SPARC	
TIMP4	
Metabolism	
ANXA1	calcium-dependent membrane binding protein annexin 1
CARS	cysteinyl-tRNA synthetase
CASK	similar to CASK
CDO1	similar to cysteine dioxygenase
CRISPLD1	cysteine-rich secretory protein LCCL domain containing 1
CRISPLD2	
FAP	fibroblast activation protein, alpha
GFPT2	similar to glutamine:fructose-6-phosphate amidotransferase 2
GSTT1	glutathione S-transferase theta 1
HAS2	hyaluronan synthase 2
HTRA3	HtrA serine peptidase 3
ME1	malic enzyme 1, NADP(+)-dependent, cytosolic
MTRF1	mitochondrial translational release factor 1
PGM5	similar to phosphoglucomutase 5
PLK2	similar to polo-like kinase 2
PRSS35	protease, serine, 35
PXDN	
RDH10	similar to retinol dehydrogenase 10
SOD2	superoxide dismutase 2, mitochondrial
SOD3	superoxide dismutase 3, extracellular
UPP1	uridine phosphorylase 1

Gene Symbol	Gene Title/Comments
Signal Transduction	
CAMSAP1L1	
DENND2A	DENN/MADD domain containing 2A
EPHA3	EPH receptor A3
ITSN1	intersectin 1
LTBP1	latent transforming growth factor beta binding protein
MYO10	similar to myosin X
PDE3A	similar to cyclic nucleotide phosphodiesterase PDE3A
PDGFD	platelet derived growth factor D
PTGFR	
RGS9BP	RGS9-1 anchoring protein R9AP
RHOJ	ras homolog gene family, member J
WNT9A	wingless-type MMTV integration site family , member 9A
ZAK	similar to mixed lineage kinase-related kinase MRK-beta
Structural	
CDC42EP3	similar to CDC42 effector protein 3
DMD	dystrophin
ECM2	extracellular matrix protein 2
MID1	midline 1
MYOM3	
SHROOM3	similar to shroom-related protein
Transcription	
ANKRD1	ankyrin repeat domain 1
EBF1	early B-cell factor 1
EMX2	empty spiracles homolog 2
EYA2	eyes absent homolog 2
EYA4	eyes absent homolog 4
FOXO1A	forkhead box 01A
HOXD8	homeobox D8
LHX9	LIM homeobox 9
MEOX2	mesenchme homeobox 2
MYCBP2	myc binding protein 2
NFIB	nuclear factor I/B
PPARA	peroxisome proliferative activated receptor, alpha
PRRX1	paired related homeobox 1
RARB	retinoic acid receptor, beta
TSHZ2	zinc finger protein 218
TSHZ3	zinc finger protein 537
YAF2	YY1 associated factor 2

Gene Symbol	Gene Title/Comments
Transport	
BIN1	bridging integrator 1
COLEC12	collectin 1 precursor CL-3
CYB5A	cytochrome B-5
FTD	ferritoid
RBP7	retinol binding protein 7, cellular
SCP2	sterol carrier potein-2
SYTL2	similar to synaptotagmin-like 2 isoform B