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Skeletal myogenesis and *Myf5* activation

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Myogenic regulatory factors (MRFs) are the master regulators of skeletal myogenesis. Among the MRFs, Myf5 is the earliest to be expressed and is regulated by a complex set of enhancers. The expression of Myf5 defines different muscle populations in the somite. Furthermore, Myf5 expression is also found in non-muscle tissues, such as preadipocytes and neurons. Here, we present a current view on the regulation of skeletal myogenesis by transcription factors and cellular signals, with an emphasis on the complexity of transcriptional activation of Myf5. We also discuss Myf5 expression in different populations of myoblasts, preadipocytes and neuronal tissue.

Introduction

The process of skeletal myogenesis during embryonic development has been the focus of studies for some time. Identification of myogenic regulatory factors (MRF) was instrumental to the understanding of this process. MRFs are the master regulators for the commitment of myogenic precursor cells (MPCs) and the terminal differentiation of myoblasts. Traditionally, Myf5 and MyoD were considered to be the early or commitment MRFs, while myogenin and MRF4 were considered to be the late or differentiation MRFs.[1-5] However, later studies showed additional function of MyoD in terminal differentiation, making it also a late MRF,[6] and of MRF4 in the commitment of MPCs, suggesting it is also an early MRF.[7] Among the four MRFs, Myf5 is the earliest to be expressed and is regulated by a complex set of enhancers spanning 140 kb of *Myf5* regulatory region.[8,9] Recent studies have identified additional levels of regulations in the equilibrium of *Myf5* enhancers with transcriptional balancing sequences.[10] The expression of Myf5 defines different muscle populations in the somite,[11,12] whereas Myf5 expression is also found in non-muscle tissues, such as preadipocytes and neurons.[13-16]

Somitogenesis

In the mammalian embryo, the skeletal muscles of trunk, limbs, diaphragm and tongue develop from somites, while the craniofacial muscles develop from prechordal, presomitic, as well as somitic paraxial mesoderm.[17,18] The somites are generated in rostro-caudal direction by segmentation of paraxial mesoderm on both sides of the neural tube. The segmentation unfolds by a "clock and wavefront" model. The cells of paraxial mesoderm express genes in a cyclical pattern governed by a negative feedback loop in a time delayed fashion. The cyclical expression represents the clock, and in mice it involves genes of the Wnt, Notch and FGF pathways. The wave is generated by a gradient of FGF-Wnt-retinoic acid signaling. When cells are in the permissive stage of cycle and the gradient of wave reaches a threshold, the formation of segment occurs (reviewed in ref. 19). Segmentation starts from embryonic day 8 (E8.0). After segmentation, somites give rise to epithelial dermomyotome on the dorsal side and mesenchymal sclerotome on the ventral side. The sclerotome later forms the cartilage and the bone of spine and ribs, while dermomyotome gives rise to dermatome which forms dermis of the back and myotome which forms the skeletal musculature.[20]

The myotome is formed by involution of cells from dermomyotome. This occurs in two waves. First, the "pioneer" cells from the dorsomedial lip delaminate and position themselves underneath the dermomyotome. These cells orient themselves rostro-caudally as they differentiate into myofibers and cover the underside of the dermomyotome. The second wave involves cells delaminating from all four lips of dermomyotome. Cells from the dorsomedial and ventrolateral lip migrate to the rostral and caudal lip where they enter the myotome, differentiate, and take a direction parallel to existing cells from first wave.[21] The back muscles are derived from the epaxial myotome, while the body wall and limb muscles are derived from the hypaxial myotome.[20] Hypaxial somite contains migratory MPCs, which delaminate to the limb bud where they form the muscles of the limb. In the limb bud, myogenic regulatory genes are expressed to initiate the differentiation of these cells (reviewed in ref. 22). The migratory cell precursors are also present in occipital and cervical somites. These progenitor cells, however, give rise to hypoglossal cord and eventually tongue and pharyngeal muscles.[23]

Knockout Models of MRFs

Skeletal myogenesis is regulated by four MRFs: Myf5, MyoD, myogenin and MRF4. In the mouse embryo, Myf5 is the earliest MRF to be expressed. It is first expressed in the dorsomedial lip of dermomyotome at E8.0, which soon forms the epaxial myotome.[8] It is also expressed in the hypaxial myotome.[24] Myogenin is expressed after Myf5 at E8.5 and MRF4 at E9.0. MyoD is the last to be expressed in the somite at E10.5.[25,26] Cells migrating toward the limb do not express MRFs until they have reached the limb bud.[27]

The mouse models of MRF null mutations are important in delineating the function of MRFs in vivo. Introduction of a *MyoD*

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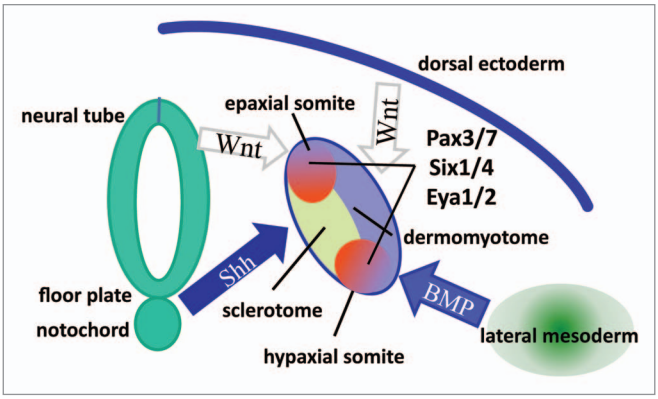

Figure 1. Myogenesis in the somite is regulated by signaling molecules from neighboring tissues and factors expressed by MPCs. Epaxial myogenesis is positively regulated by Wnt from neural tube and Shh from floor plate and notochord. Wnt signals from dorsal ectoderm induce myogenesis in hypaxial myotome, whereas BMP from lateral mesoderm inhibits. Pax 3/7, Six 1/4, Eya 1/2 expressed by MPCs positively regulate myogenesis.

null mutation in mice does not have a negative effect on skeletal muscle development. *MyoD*$^{-/-}$ mice are viable with normal physiology and morphology of skeletal muscles, although the expression of Myf5 is increased and prolonged.[28] The skeletal muscles of *Myf5*$^{-/-}$ mice also appear morphologically normal and the levels of MyoD, myogenin or MRF4 do not change compared to the wild type. The *Myf5* knockout mice, however, die perinataly due to the loss of distal parts of the ribs and inability to breathe. The only abnormality of skeletal muscle development in *Myf5*$^{-/-}$ mice is a delayed appearance of myotomal cells until MyoD is expressed.[29] The absence of muscle deficiency in the *MyoD*$^{-/-}$ or *Myf5*$^{-/-}$ mice was unexpected and indicated a functional redundancy. A *MyoD*/*Myf5* double knockout model shows a clear phenotype of redundancy between the two genes. Mice deficient in both *MyoD* and *Myf5* lack skeletal muscle completely.[1] As myoblasts do not form in the *MyoD*/*Myf5* double knockout, these two genes are considered as the early or commitment MRFs.

Myogenin null mice also display a severe deficiency in skeletal muscle. However, they are able to form myoblasts but fail to fuse into myotubes. The *myogenin* null mice die perinatally and only a few myofibers are observed at birth.[30-32] The lack of myofibers places myogenin in the later stages of differentiation and hierarchically downstream of MyoD and Myf5.[2] Three *MRF4* knockout mice have a range of phenotype from viable with no muscle defects, to lethal phenotype with some muscle defects.[3-5,33] An increase in myogenin expression and some deficiencies in myotomal myogenesis and deep back muscle or intercostal muscle formation in *MRF4* knockout mice[3,4] lead to the conclusion that MRF4 may have a function in terminal differentiation similar to myogenin. Since *Myf5* and *MRF4* are adjacent to each other on the same chromosome, if one is knocked out there is, in most cases, a cis effect by which the expression of the other is also decreased or lost.[3,7,33] In newer *MyoD*/*Myf5* double knockout models, mutations are made in such manner that MRF4 expression is still present. The expression of MRF4 in these knockout mice is enough to support both epaxial and hypaxial differentiation, establishing MRF4 as a commitment MRF.[7] The view on MyoD function has also changed. A double null mutant of *MyoD* and *MRF4* has almost identical phenotype to that of *myogenin* null mutants, suggesting that MyoD, along with myogenin and MRF4, plays a role in terminal differentiation of myoblasts.[6]

Regulation of MRFs Expression

During somitogenesis, tissues surrounding the somites produce signals directing myogenesis. Factors from the notochord, floor plate, neural tube, dorsal ectoderm and lateral mesoderm affect the expression of MRFs (**Fig. 1**). Sonic hedgehog (Shh) from the notochord and floor plate induces both Myf5 and MyoD in epaxial but not in hypaxial myotome. The effect of Shh on Myf5 is direct while on MyoD indirect. Loss of *Shh* leads to the loss of Myf5 expression but not MyoD.[34] Furthermore, Shh activates *Myf5* through a Gli binding site in the epaxial enhancer.[35] Also, Shh cannot activate *MyoD* in absence of Myf5.[36,37] The Wnt factors take part in the specification of the somite as well. Wnt1 expressed by neural tube preferentially activates *Myf5*, while Wnt7a expressed by dorsal ectoderm activates *MyoD*. Wnt4, Wnt5a and Wnt6 activate both *MyoD* and *Myf5,* but less effectively than Wnt1 and Wnt7a.[38] Wnt signaling affects the epaxial expression of Myf5 directly through a β-catenin binding site in the extended epaxial enhancer.[39]

Bone morphogenic proteins (BMPs) from lateral plate mesoderm have a negative effect on the expression of MyoD and Myf5. BMP4 inhibits MyoD expression in lateral somite.[40] The level of BMP expression is also important. Low levels of BMP2, BMP4 and BMP7 maintain Pax3 expression in proliferating populations of the limb, whereas high levels inhibit myogenic differentiation.[41] Noggin, a BMP antagonist, is produced in the lateral somite and inactivates BMP4 signals. Noggin expression in lateral somite is induced by Wnt1 from the neural tube and possibly by Shh from notochord.[40]

The expression of MRFs is also regulated by factors expressed by the somitic cells. Pax3 and Pax7 (paired box proteins 3 and 7), together with Six family of proteins and their cofactors Eya, regulate MRFs expression and myogenesis. Pax3 is involved in the development of both epaxial and hypaxial muscle, as defects in both can be observed in *Pax3* deficient mice.[42-44] However, more severe defects are observed in the hypaxial muscle.[45] Pax3 and Myf5 are hierarchically above MyoD, since MyoD is not expressed in trunk and limbs of *Pax3*/*Myf5* double null mutant mice.[24] Pax3 also has a role in the survival of muscle precursors in hypaxial dermomyotome.[46] Severe loss of limb muscle in *Pax3* null mice may be a consequence of loss of c-Met and Lbx1, which regulate migration of MPCs to the limb.[47] Pax3 affects differentiation of limb muscle not only through migration but also by MRF regulation. The expression of Myf5 is directly regulated by Pax3 through the limb bud enhancer of *Myf5*.[48] Pax3 regulates Myf5 expression indirectly through the epaxial enhancer, in which Pax3 directly regulates the expression of Dmrt2, which then regulates the expression of Myf5.[49] Pax3 also directly regulates expression of MyoD in C2C12 myoblast cells.[50]

Pax7$^{-/-}$ mice exhibit no overt muscle phenotype.[51] However, *Pax3* and *Pax7* double mutant mice have a more severe muscle phenotype than *Pax3* null mice, indicating a redundant role of Pax3 and Pax7 in myogenesis.[52] Pax3 and Pax7 also have a function in marking a population of MPCs in adult mice to become satellite cells.[52] Furthermore, Pax7 may have a function in the renewal and propagation of satellite cells.[51]

Six1 and *Six4* are paralogues of *Drosophila sine oculis* genes. *Six1* null mice have deficiencies in hypaxial and some epaxial muscles.[53] *Six1/Six4* double null mutants have an even more severe phenotype with compromised expression of MyoD, myogenin, MRF4 and Pax3.[42,54] The expression of Pax3 in hypaxial muscle is directly regulated by Six1 via the hypaxial enhancer.[42] Six1 and Six4 also directly regulate expression of Myf5 in the limb bud by binding to the limb bud enhancer,[55] which is regulated by Pax3 as well.[48] Six1 and Six4 also directly regulate *myogenin* promoter.[56] The phenotype of *Eya1/Eya2* double knockout mice is almost identical to that of *Six1/Six4* double knockout, with a similar loss of Pax3 expression.[42]

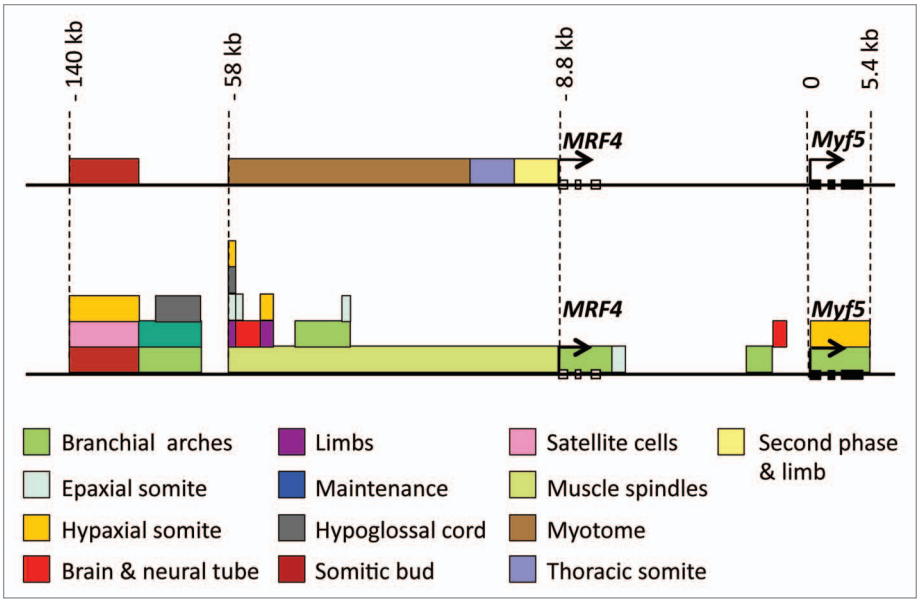

Figure 2. *Myf5* and *MRF4* genes are both located on chromosome 10, about 8.8 kb apart. Elements regulating *MRF4* located upstream of *MRF4* start site are shown on the top part. *Myf5* enhancers spanning 140 kb upstream of *Myf5* start site and the intragenic region of *Myf5* are shown on the bottom part.

The Regulation of *Myf5/MRF4* Locus

Myf5 and *MRF4* are in mouse chromosome 10, approximately 8.8 kb apart.[57] The link between *Myf5* and *MRF4* is conserved among birds,[58] mice[59] and humans.[60] The transcription regulatory elements of *Myf5* and *MRF4* span a 140 kb region upstream of the *Myf5* start site (**Fig. 2**) and are well characterized.[9,57,59,61-66] The large number of enhancer elements in this locus allows for complex regulation of gene expression. The equilibrium between the enhancers, minimal promoters and transcription balancing sequences (TRABS) further fine tunes the spatiotemporal expression.[10]

Expression of Myf5 in epaxial dermomyotome of the somite is regulated by the early epaxial enhancer. This enhancer regulates Myf5 expression at the earliest known time point and locates immediately downstream of the *MRF4*.[63,64] The epaxial enhancer by itself is activated by Shh through a Gli binding site.[35] When a 195 kb upstream regulatory region is connected with *Myf5* minimal promoter, only the maintenance of Myf5 in the epaxial dermomyotome appears to be dependent on the Gli site in the early epaxial enhancer.[67] The extended epaxial enhancer expanding 5' from the enhancer is positively regulated by Wnt signaling via Lef/Tcf sites found immediately upstream of the early epaxial enhancer.[39] Furthermore, Pax3/Dmrt2 cascade also regulates the epaxial enhancer. Pax3 directly regulates expression of Dmrt2, which then regulates the early expression of Myf5 through the epaxial enhancer.[49] Once Myf5 is expressed, the cells of epaxial dermomyotome delaminate to form epaxial myotome.[68,69] The expression of Myf5 here is regulated by an element located -57/-56.5 kb from the *Myf5* start site.[62] A region within -23 kb also regulates expression in a sub domain of epaxial myotome.[57] The early hypaxial expression of Myf5 is regulated by an intragenic enhancer overlapping with *Myf5* coding region.[63] Hypaxial expression is further regulated by a region located between -53.3 and -48 kb[62] and a distant element located at -140/-88.2 kb.[9] As the somite matures, Myf5 expression in both hypaxial and epaxial somite is regulated by an element located at -57.5/-57 kb[62] and a region between -88.2 and -63 kb, which ensures the maintenance of Myf5 in axial muscles after E11.5.[9]

Myf5 expression in limbs is directed by elements located between -58 and -48 kb.[57,62,63] The element located at -57.5/-57 kb region regulates expression in both fore and hind limbs[62] and it is under direct control of Pax3, Six1 and Six4.[48,55] A second element located between -53.3 and -48 kb regulates Myf5 expression preferentially in the hind limbs.[62] Expression in the branchial arches is initiated by two elements: an intragenic element overlapping with the coding region of *Myf5*, and the proximal arch element immediately upstream of *Myf5* transcription start site.[63] Negative arch element in the intragenic region of *MRF4* downregulates the early expression in branchial arches.[63] The effect of the negative branchial arch element is overcome by more distal hyoid (-45/-23 kb) and mandibular arch (-88.2/-63 kb) elements.[70] The expression of Myf5 in the hypoglossal cord is directed by two elements located at -57.5/-57 kb[62] and -81/-63 kb.[9]

Expression of Myf5 in central nervous system is regulated by two elements. The proximal element, located 294 bp upstream of *Myf5* transcriptional start site, regulates expression in neural tube.[63] The distal element, located at -56.6/-53.7 kb, directs

expression in brain and in neural tube.[62] Myf5 expression in the adult mice is regulated by two regulatory regions. Expression in the satellite cells is regulated by an element located in the -140/-88 kb region, whereas expression in muscle spindles is regulated by region from -59 to -8.8 kb.[65]

Expression of MRF4 is regulated by a set of enhancers that overlap with those of *Myf5*. In both dorsal and ventral region of caudal and rostral somites, MRF4 expression is regulated by an element located between -58.6 and -17.3 kb.[70] The region between -17.3 and -15.3 kb regulates early MRF4 expression in central myotome of thoracic somites.[71] The expression in the ventral myotome of thoracic somites is regulated by an element located at -140/-88 kb. The expression of MRF4 in the limb and the second phase are regulated by an element between -15.3 and -8.8 kb.[59]

Another level of regulation of *Myf5/MRF4* locus is the equilibrium formed between enhancer sequences, minimal promoters of *Myf5* or *MRF4* and TRABS. A recent study shows that in absence of *Myf5* minimal promoter, the enhancer elements of *Myf5* can drive transcription from *MRF4* promoter and also from alternative transcription start sites or cryptic promoters; and in some cases from both *MRF4* and cryptic promoter at the same time. Furthermore, some enhancers can also interact nonproductively with the cryptic promoters, thus demonstrating that enhancer elements in the *MRF4/Myf5* locus are not simply interacting with their respective promoters or cryptic promoters with an on- or off-mode, but rather in equilibrium. These cryptic promoters are thus termed transcription balancing sequences.[10]

The same study shed additional light on previous *Myf5* and *MRF4* knockout mice.[10] The three *MRF4* knockout mice had a range of phenotype from viable with no muscle defects, to lethal phenotype with some muscle defects.[3-5,33] The strength of the promoter used to drive a selection gene, the direction of transcription and the amount of deletion of original *MRF4* promoter all caused a greater or lesser interaction of *Myf5* enhancer elements to promoter of the selection marker, and thus a greater or lesser loss of Myf5 expression.[7,33] A similar effect was observed in a *Myf5* knockout study that used three different alleles which inhibited MRF4 expression to a different extent.[7]

Myf5 and Adipogenesis

Study of different expression profiles between white and brown preadipocytes discovered a significant enrichment of muscle transcripts in brown preadipocytes. These include Myf5, MyoD and myogenin, which were previously considered specific for skeletal myogenesis.[13] This finding agrees with an earlier lineage tracing study which showed that cells of central dermomyotome give rise to cells of dermis, skeletal muscle and brown fat.[72] A second study shows more specifically that the progeny of Myf5-expressing cells forms brown fat and skeletal muscle.[14] Collectively, these findings indicate that brown fat cells and skeletal muscle cells share a common precursor.

The expression of Myf5 in brown preadipocytes is driven by a region of 6 kb immediately upstream from start site.[14] This region includes previously characterized neural tube enhancer, early and late branchial arch element, and a part of early epaxial enhancer and negative branchial arch element.[63] It is likely that the early epaxial enhancer is driving Myf5 expression in the lineage tracing study since it is active in epaxial dermomyotome from which brown fat and skeletal muscle arise. MRFs are downregulated as preadipocytes continue to differentiate.[13] Furthermore, the switch between the lineages and the downregulation of MRFs are governed by the expression of PRDM16.[14] It is not known if Myf5 or other MRFs have a function required for adipogenesis, but for now, it appears that they simply drive myogenesis once a switch is turned between the two lineages.

Myf5 and Neuronal Expression

Myf5 is expressed in central nervous system, in the ventral neural tube from cervical to sacral level at E10.5[15] and in the brain in mesencephalon, more specifically in prosomer p1 at E8 and in secondary prosencephalon in prosomer p4 at E10.[16] Axons of these *Myf5*-expressing neurons form medial longitudinal fasciculus and mammillotegmental tract. Some olfactory tracts also express Myf5. In the adult mice brain, Myf5 is also expressed in ventral regions possibly arising from embryonic Myf5 expressing structures.[73]

Myf5 positive cells from neural tube but not from the brain continue to differentiate into skeletal muscle in vitro and express myosin heavy chain.[15,16] This indicates that myogenesis is suppressed by factors present in the neural tube which are lost in culture. Expression of Myf5 in the neural tube is regulated by a proximal element at -0.7/-0.3 kb from the start site and a distal element at -56.6/-53.3 kb, which also regulates Myf5 expression in the brain.[62,63] The distal element can be further narrowed down to 700 bp at -55/-54.3 kb, which recapitulates the expression pattern of the larger element.[74] The expression of Myf5 in the explants of brain cell is upregulated by Wnt signaling. The function of distal element is dependent on four Tcf sites indicating that the regulation of distal element by Wnt is direct. Furthermore, the activity of this element also depends on an Oct6 site.[73,74]

The expression of Myf5 in the nervous system raises the question about the function of Myf5 in developing brain. The homozygous *Myf5-nlacZ* mutants show no abnormalities in brain structures, indicating that Myf5 does not have a significant function in brain development. In fact Myf5 protein does not accumulate in brain cells as the translation of Myf5 mRNA is inhibited by microRNAs on the 3'UTR.[74] Interestingly, the 700 bp distal element regulating Myf5 expression in both brain and neural tube shares no significant sequences homology among human, chicken or dog. The exception is a short 100 bp region with homology to a rat sequence, but this segment does not contain any of the sites that regulate Myf5 expression in the brain. Since there is no conservation of the distal element and Myf5 performs no function in the brain, the expression of Myf5 in the brain appears to be accidentally brought about by a genome rearrangement after the mouse and rat split.[74] The Myf5 expressing cells from neural tube do differentiate to skeletal muscle in explant culture,[15] indicating that Myf5 protein is possibly produced in these cells. The function of Myf5 in the neural tube, however, remains to be determined.

Perspectives

The existence of Myf5 and MyoD specific populations in the somite has been debated for some time. Myf5 is expressed earlier than MyoD and turned off when MyoD expressed.[8,25,26] Therefore, MyoD is possibly expressed in cells expressing Myf5 or in an independent cell population. Ablation of Myf5-expressing ES cells during differentiation reveals the presence of MyoD-expressing cells independent of Myf5,[75] which is corroborated in a mouse model. When a Myf5-expressing cell population is ablated by Cre induced diphtheria toxin, skeletal muscle forms normally, suggesting the existence of a Myf5-independent muscle population. In contrast, conditional ablation of myogenin expressing cells leads to almost complete loss of skeletal muscle.[11,12] Furthermore, when Myf5 lineage is traced with Cre-induced GFP, both epaxial and hypaxial myotome populations of cells are found to express Myf5 only, MyoD only, or Myf5 and MyoD both.[11]

The finding of populations expressing Myf5 or MyoD only, as well as populations expressing both Myf5 and MyoD, underscores the dynamics between cell populations and MRF regulation. When Myf5 expressing population is ablated, there is no impact on muscle phenotype,[11,12] due to the ability of MyoD expressing population to compensate for the loss of Myf5. It is however unclear what signals maintain the balance between different populations of MPCs and guide the MyoD population to increase in numbers and takeover the function of ablated Myf5 population. In addition, MRF4 supports myogenesis in the absence of Myf5 and MyoD.[7] The expression profiles of MRF4 in different MyoD and Myf5 populations remain to be determined, as does the existence of a MRF4 only population.

The regulation of MRFs expression also needs to be revisited in light of distinct Myf5 and MyoD populations. Factors such as Shh and Wnt are known to directly regulate expression of Myf5 via epaxial enhancer.[35,39,67] Furthermore, Pax3, Six1 and Six4 all play a part in skeletal myogenesis.[42-44,53,76] It remains to be determined which enhancer elements regulate the expression of Myf5 in populations only expressing Myf5 or in those expressing both Myf5 and MyoD. The expression of Myf5 in both hypaxial and epaxial somite is regulated by several different enhancers,[9,57,62-64] suggesting that Myf5 expression in different populations may be regulated by different enhancers or different combination of transcription factors.

Spatio-temporal expression of Myf5 is regulated by a large number of enhancers and specific transcription factors have been identified only for the epaxial, limb and distal neural tube enhancers.[35,39,48,49,55,67,73,74] Furthermore, molecular basis for the equilibrium between enhancers, *Myf5* or *MRF4* promoters, and TRABS is still unclear. More integrated studies will provide invaluable molecular insights into the complexity of myogenesis and gene regulation in general.

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