

Homeogenes and Vertebrate Skeletal Myogenesis: A Perspective

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Abstract

Homeogenes, identified initially in *Drosophila* and subsequently in vertebrates, are good candidates as regulators of muscle patterning and differentiation. Gain-of-function and loss-of-function mutations for these genes in mice give rise to morphogenetic changes [1, 2]. Nonetheless, little is known regarding the cellular consequences of expression and their role during skeletal myogenesis. Myogenic precursors express patterning genes during development, yet cell lines show either low or absent levels suggesting that clues normally present *in vivo* are absent under culturing conditions or that cell lines are no longer capable of receiving patterning signals. Nonetheless, cell lines have proven highly valuable in dissecting the cellular and molecular pathways that govern myogenesis *in vivo*. We and others, have taken advantage of using myogenic cell lines by introducing forced-expression of homeotic genes to elicit changes in cell behavior. At least two homeogenes, *Msx1* and *Pax3* can function as myogenic repressors *in vitro*. This *in vitro* effect correlates well with their respective patterns of expression in the embryo. Other homeogenes do not exert this effect and are potential candidates as positional or muscle-specific regulators during differentiation. One example of this latter case is *Engrailed 2* which is expressed in the jaw. As the study of skeletal myogenesis becomes more focused upon development *in vivo*, a role for homeogenes, and patterning genes in general during skeletal muscle development promises to emerge.

Key words: homeogenes, skeletal muscle, embryogenesis, engrailed, *Msx1*, *Pax3*.

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Experiments involving forced expression of the MRFs in cell culture provide evidence that this gene family can phenotypically convert 'fibroblastic' or committed cells (adipocytes, chondrocytes, etc.) into myogenic cells [3, 4]. *Myf5* (in the mouse embryo) is expressed very early during development in the medial portion of the dermamyotome in response to interactions with surrounding axial structures that promote the skeletal muscle lineage (see Nakovitz and Stockdale; this issue). From these observations, it was proposed that the MRFs may be lineage determination genes [3]. However, several observations persist that challenge this assumption. First, although *myf5* is expressed in the right place at the right time to account for the early axial muscle program, their progeny account for a small subset of skeletal muscle in the final vertebrate body plan. Chick-quail transplantation experiments demonstrate that the lateral portion of the somite dermamyotome (that portion furthest from the neural tube) delaminates and migrates out to distal structures including the limbs [5]. Their progeny will give rise to a significant muscle mass however, myogenic regulatory factor (MRF;

MyoD, myogenin, *myf5* and *MRF4*) expression is detected only shortly before the first signs of differentiation are evident [6-8]. If the forelimb buds are removed at the 25-28 somite stage, prior to MRF expression and placed into culture, cells with myogenic potential can be seen after several days [9]. Thus, the migratory population of myogenic precursors does not actively express the MRFs. The potential role of cell-cell interactions in repressing the myogenic program can be demonstrated by dissociating these limbs into single cell preparations which results in a precocious and rapid initiation of MRF expression [9]. These results support the notion that myogenic cells are already established prior to overt MRF expression. It has recently been reported that myogenic cells can be obtained at high frequency upon culturing chick epiblast cells [10]. These observations indicate that the myogenic capacity may be a default and widespread early cell fate in the embryo prior to gastrulation [10]. In these experiments, only dissociated cells give rise to muscle, demonstrating that cell-cell interactions repress subsequent differentiation analogous to the situation in the limb bud. Taken

together, it can be proposed that the myogenic lineage is established early in vertebrate embryogenesis, prior to gastrulation, however, specific cell-cell interactions repress the muscle program and restrict its distribution to the somite (see George-Weinstein et al., this issue).

Two mechanisms must therefore take place during early development. The first mechanism is that genes are required and expressed prior to the MRFs to establish myogenic potential. The second is that cell-cell interactions (signaling) results in a 'repressed' state which allows the muscle precursors to proliferate and migrate prior to MRF expression and subsequent terminal differentiation. In this review, we will consider the evidence for the later proposal, and examine how homeogenes may mediate the regulation of differentiation of 'early' myoblasts. I suggest that the homeogene family exerts crucial effects at the cellular level which underlie this early myogenic repression. I also consider the example of homeogenes that are expressed in specific muscles during differentiation and speculate that they may play a role in positional specification of muscle groups.

Patterning, Proliferation and Plasticity

Adult skeletal muscle tissue consists of terminally differentiated myofibers which are not capable of further mitosis [11]. When muscle is damaged, a stem cell population of myogenic precursors referred to as satellite cells is stimulated to divide and ultimately differentiate into mature myotubes [12, 13]. Recent evidence involving long term analysis of mice carrying a null allele for MyoD suggest that MyoD may serve an important role in either satellite cell generation or survival as MyoD^{-/-} mice show severely perturbed regeneration capacity [14]. It has been proposed that the proper patterning of regenerated muscle consists of interactions of the satellite cells with the original basal lamina of the damaged myotubes [12]. It should be noted that when myogenic cell lines are injected into the muscle mass of an adult mouse, the cells migrate through basal lamina, and ultimately fuse with fibers, indicating that cell lines are capable of responding to morphogenetic clues [15]. Studies by Stockdale and colleagues reveal that avian myoblasts are pre-programmed to differentiate into specific fiber types and that several distinct populations may be generated quite early in development (see Nakovits and Stockdale, this issue). Furthermore, patterning genes such as the homeobox gene family have been identified which are expressed in several discrete early myogenic populations such as Pax3 and Engrailed 2. It is well established that the regulated expression of these genes leads to proper formation of the embryo although a specific role in skeletal muscle development has yet to be resolved. Thus, a molecular basis for any intrinsic factor that specifies one myogenic cell from another remains to be identified.

Patterning events require that cells remain in an undifferentiated and proliferative state. Since the myogenic program results in terminal differentiation, it is critical that a sufficiently large population of myogenic founder cells are

generated and maintained to form the large muscle mass typical of the final vertebrate body. The newly formed limb bud is highly proliferative and contains cells which are positionally plastic [16]. While the cell fate of the limb mesenchyme and myoblasts is presumably fixed, a large variety of experimental manipulations can alter their positional fate. Presumably, myogenic cells respond to changes in the limb context by undergoing additional proliferative steps to generate new muscle masses. A highly dramatic illustration of this concept is seen when ectopic limbs are induced by FGF [17]. In this context, limbs can be induced along the flank of the embryo which will induce the neighboring somites to send out a population of myogenic cells which participate in the new limb. Whereas these cells would have normally given rise to axial muscle masses which are small by comparison to limb muscles, they are recruited to undergo pronounced increases in proliferation. It is noteworthy that once the positional fate of muscle is fixed in the early limb bud, MRF expression begins to be detected in the proximal pre muscle masses [8, 16]. At the same time, the expression of patterning genes declines in these regions. Thus, at the molecular and cellular level, proliferation and patterning are coincident, whereas differentiation and MRF expression are closely linked temporally and spatially.

Homeobox Genes as Myogenic Repressors-Msx1 and Pax3 During Limb Muscle Development

We have previously demonstrated that the Embryonic Day (ED) 9.25-10 mouse limb bud contains cells capable of myogenic differentiation [7-9, 16]. Whereas the first transcripts for the MRFs becomes detectable by ED10.5, dissociation of cell-cell contacts in ED9.5 limbs leads to a rapid induction of the MRFs suggesting that the limb environment exerts either a repression of differentiation and/or induction of high rates of cell proliferation [9]. At least two well characterized homeogenes have been identified which show strong expression during this early myogenic cell migration. These are Msx1, which is expressed in the limb mesenchyme and lateral flank mesoderm, and Pax3, which is similarly expressed but appears to be restricted to the myogenic cells in the lateral mesoderm and limb. Expression of both genes decreases prior to MRF expression and subsequent differentiation.

Msx1 is one candidate for regulating the proliferative/undifferentiated state of cells in the limb mesenchyme. The evidence that Msx1 plays a role in cell differentiation is: 1) Msx1 is expressed in the proliferative and undifferentiated region of the limb (and face) prior to MRF expression. 2) As Msx1 expression declines, overt signs of differentiation can be observed and *in situ* analysis demonstrates that the MRFs begin to accumulate. 3) Disruptions of cell-cell contacts in the limb leads to an acceleration of muscle and cartilage differentiation and a loss of Msx1 expression in limb bud mesenchyme [9]. 4) Msx1 forced-expression in myoblasts can block terminal differentiation, induce tumorigenic behavior, and results in a down-regulation of

steady-state levels of MyoD [18]. This oncogenic effect is not seen with *Msx2*, a closely related homeogene indicating that the effects of *Msx1* are specific [18]. Weintraub and colleagues have reported that activation of the mouse *MyoD1* locus and myogenic conversion by the human *myoD* locus, is repressed by human chromosome 4 whereas other chromosomes do not exert this effect in somatic cell hybrids [19]. In collaboration with these investigators, we have determined that this repression correlates to the presence of the active *MSX1* locus on this chromosome [20]. Furthermore, the repression conferred by chromosome 4 can be relieved by antisense *Msx1* [20]. The coincidence of these results strongly support the hypothesis that *Msx1* acts as a repressor of *MyoD* and subsequent muscle differentiation. Gruss and colleagues have observed that several *Hox* genes and *Pax* genes can also lead to tumorigenesis following forced-expression in non-transformed cells [21, 22]. Several homeotic gene loci have been implicated as break-points in chromosomal translocations associated with leukemia [23]. Thus, an emerging view that patterning genes control cell proliferation and cell differentiation provides a new framework for understanding the cellular basis of morphogenesis and identification of homeotic gene targets. Consistent with this notion, we observe that the Inhibitor of Differentiation gene (*Id*) is expressed in a temporal and spatial pattern largely overlapping with *Msx1* [24]. It is likely that the apparent decrease in steady-state levels of *MyoD1* expression in *Msx1* expressing cells contributes to the inhibition of terminal differentiation.

Pax3 is a member of the paired-like vertebrate homeobox genes and has also been implicated in early myogenic regulation in the embryo [25]. Expression of *Pax3* is observed in the lateral portion of the dermamyotome of the somite and in cells that appear to migrate from the somite into the limb [25-27]. The mouse genetic mutant, *Splotch* was identified to carry an altered *Pax3* locus and fails to develop limb musculature when bred to homozygosity [28, 29]. A similar phenotype is obtained with both the *c-met* knockout and with the scatter factor (*HGF/SF*) knockout [30]. *C-met* encodes a receptor that specifically binds to *SF* [30]. The expression of *c-met* in the somite and limb is coincident with that of *Pax3* and the *c-met* transcript is not detected in *Splotch* mutant homozygous embryos [31] suggesting that *Pax3* may be 'upstream' of *c-met*. *SF*, which was originally identified as a factor that increase cell motility in culture is expressed in the early limb bud and thus may serve to guide or at least stimulate dermamyotomal cells to migrate into the limb from the somite.

The potential role of *Pax3* in the early myoblasts may be quite similar to that of *Msx1* and may account for the lack of a limb phenotype in the *Msx1* null mutation mice [32]. It was recently demonstrated that *Pax3* can inhibit myogenic differentiation of C2C12 myoblasts normally induced by exposure to low serum [33]. When 10T1/2 cells are converted to myogenic cells by *MyoD*, *Pax3* can inhibit this conversion in a manner analogous to *Msx1*. If the same

phenotypic conversion is performed with myogenin, *Pax3* has no overt effect [33]. Thus, *Pax3* likely serves to keep the migratory myogenic precursors in a proliferative state. This anti-myogenic capacity is shared by a *PAX3*-forkhead fusion protein resulting from a t(2;13) chromosomal translocation found in pediatric alveolar rhabdomyosarcomas [34].

Based on these specific observations with *Pax3* and *Msx1*, it can be proposed that a general effect of homeobox gene expression on myoblasts is to inhibit differentiation. This can be accomplished by direct repression of *MRF* expression, or more likely, is a result of several cellular changes elicited by homeobox gene expression.

Homeobox Genes as Myogenic Positional Markers-Engrailed 2 During Jaw Muscle Development

Muscle cells in the vertebrate embryo originate from the somites except in the craniofacial region, where muscle cells derive from the somitomeres [35]. In the mouse, seven somitomeres form as pairs on both sides of the neural epithelium from the cranial paraxial mesoderm [36]. Heterotopic transplant studies performed on mouse embryos have shown that the somitomeres contribute to specific branchial arches based on their relative rostro-caudal position [37]. These results are consistent with those in the chick which show that muscle patterning information is dictated by connective tissue precursors (in this case cephalic neural crest cells) rather than by muscle precursors [38, 39]. Patterning in this region is perturbed by the disruption of the *Hoxa-2* gene which is expressed in all cells in the arches [40, 41]. Mice homozygous for this mutation have a 'homeotic' transformation of the second arch derivatives to first arch elements [40, 41]. Although this highlights the importance of the 'Hox-code' in craniofacial development, the patterning and specification of distinct muscles is poorly understood. As outlined below, the notion that connective tissue entirely dictates the morphogenesis and fiber type of cranial facial muscles does not apply in all instances, and in particular with regard to jaw musculature.

Muscular derivatives of the first branchial arch include the *masseter*, *temporalis*, *tensor tympani*, and *tensor veli palatini*, the *pterygoids*, the *mylohyoid*, and the anterior belly of the *digastric* [42]. The *masseter* and *temporalis* are the major jaw-closing muscles [43]. These muscles have physiological demands which differ from other commonly studied skeletal muscles [43]. One measure of skeletal muscle diversity is the variation of fiber types (e.g. fast, slow, tonic, etc.) [44]. The pattern of fiber composition is dependent upon a number of factors [45]. Extrinsic factors such as innervation and extra cellular matrix have been shown to effect fiber type, but these do not satisfy many observations [46]. For instance, transplantation studies of jaw muscle cells in the cat to non-jaw muscle beds gives rise to muscles that show a maintenance of jaw specific myosin expression [46], implying that intrinsic factors are present in the myoblasts of the jaw muscles. These differ-

ences are reflected in the distinct fiber composition of these muscles [44]. Such fiber type variance, in large part, results from the expression of specific isoforms of myofibril proteins [44]. The initial distinctions between various muscle types (I, IIA, IIB) were based on myosin ATPase histochemistry and speed of relaxation [43]. The MyLC2 gene is a myosin light chain-2 family member which has been shown to have jaw muscle specific expression in the cat which is limited to the jaw-closing muscles [47]. Taken together, jaw muscles share many common features with other more commonly studied skeletal muscles (i.e. leg, diaphragm, etc.) including myogenic factor expression, etc.; however, jaw muscles have several unique characteristics suggesting that unique 'jaw' regulatory mechanisms are present. These features include the expression of unique myosin isoforms, sex-steroid responsivity [48, 49], and *Engrailed-2* expression (see below).

Engrailed expression in the jaws of vertebrates was initially reported in zebrafish using an anti-EN polyclonal antibody [50]. With this antisera, it was not possible to distinguish between specific En loci gene products (*EN-1* versus *EN-2*). Reactivity was correlated to sites of initial jaw muscles formation and remained detectable during innervation and then decreased. Concurrent with these analyses, an enhancer region containing 9.5 kb of genomic DNA was isolated that could drive lacZ reporter constructs in both the mid-hind brain region and first arch [51]. This region could be further subdivided into two fragments of 1.5 and 1 kb which could drive reporter gene expression in the brain and jaw respectively [51]. From previous studies on the expression of the myogenic factors and sarcomeric actins and myosins [7, 8, 52], it is clear that expression of *En-2* precedes the overt differentiation of skeletal muscle in this region and closely coincides with the initial accumulation of myogenic factor transcripts (myf5; [6, 51]). This data therefore supports the notion that *En-2*, and not *En-1*, plays a role during 'early' jaw muscle development. During this stage of development, myoblasts which originate from head mesoderm migrate with cranial neural crest to the first visceral arch [35, 39]. It is unclear whether the labeled myoblasts will give rise to all jaw muscles or are already specified to give rise to the *masseter* and *temporalis*.

En-2 is expressed in specific muscles of the jaw:

By day 15 p.c. in the mouse embryo, the facial muscles have formed fibers and individual muscles are discernible. Using the transgenic reporter mice described above, a histological analysis of whole mount lacZ stained embryos was undertaken. Although this staining is difficult to detect with transmitted light in sectioned material, staining appears as red grains under dark field polarized optics. These studies reveal that both the *masseter* and the *temporalis* express the transgene. The anterior belly of the *digastric* and the *mylohyoid*, other mandibular arch derivatives, showed no detectable staining. The *tensor tympani*, and *tensor veli palatini* were not distinguishable from other structures in sections examined, although no staining was

seen in the pharyngeal region, nor in the inner ear region. The pterygoids were also not observed to be stained. Thus, the muscles which clearly show *En-2* expression are those that are functionally related in lifting the jaw (Degenhardt and Sassoon; unpublished observations). This is comparable to the situation in the zebrafish where *engrailed* expression is in the *levator arcus palatini* and *dilator operculi* [50]. These two muscles are also functionally related, but they open and expand the oral cavity rather than close the jaw [50]. These data indicate that *En-2* also plays a role in the differentiated muscles of the jaw. Detailed analysis reveals that staining is confined to the myotubes and is not detectable in the surrounding connective tissue. Although all skeletal muscles share many biochemical and molecular properties, they also have differences with respect to morphologies and biochemical composition that likely reflect specific functional requirements. Preliminary studies using forced expression of *En-2* in myogenic cells reveal that this homeogene does not block differentiation consistent with its maintained expression in myotubes in vivo (Degenhardt and Sassoon; unpublished observations).

Conclusions

The cell and molecular biology of skeletal myogenesis has seen rapid advances in recent years. The isolation of the MRFs and the study of their role and regulation during embryogenesis has allowed the field to establish spatial and temporal landmarks for the understanding of how the myogenic program is established and regulated. As reviewed here, homeogenes such as *Msx1* and *Pax3* are good candidates as myogenic repressors whereas *En-2* may serve a role in positional specification of a distinct muscle group. The paucity of homeogene expression in muscle may reflect the fact that either currently identified genes have not been carefully examined in muscle, or that other novel homeogenes remain to be identified. It is unlikely a coincidence that jaw musculature has many unique cellular and molecular characteristics as well as specific and restricted *En-2* expression. In the case of *Pax3*, *Msx1* and *En-2*, the future challenge will be to understand what homeogene expression does to a myoblast and how this information is integrated in a multicellular environment to result in proper muscle patterning.

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