REPRODUCTION
REVIEW

Many routes to the same destination: lessons from skeletal muscle development

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Abstract

The development and differentiation of vertebrate skeletal muscle provide an important paradigm to understand the inductive signals and molecular events controlling differentiation of specific cell types. Recent findings show that a core transcriptional network, initiated by the myogenic regulatory factors (MRFs; MYF5, MYOD, myogenin and MRF4), is activated by separate populations of cells in embryos in response to various signalling pathways. This review will highlight how cells from multiple distinct starting points can converge on a common set of regulators to generate skeletal muscle.

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Introduction

Understanding the process of differentiation is a central concern of developmental biology, and the study of vertebrate skeletal muscle provides an excellent paradigm to understand this problem. Investigations over many years have shed light on the inductive processes within embryos that lead to myogenesis and the molecular events that underpin this. As well as providing basic insights into developmental biology, this work has the potential to inform regenerative medicine as, at least in some respects, the myogenic programme active in adult muscle stem cells recapitulates that of the embryo.

One of the striking features apparent from recent work is the variety of ways in which skeletal muscle cells can be generated. From diverse developmental starting points, precursor cells converge on a small number of core transcriptional regulators that govern the myogenic programme. In amniote embryos (mammals, birds and reptiles), muscle development starts at late neurula/pharyngula stages (described in Box 1). Although there are significant differences between species in early development, the myogenic programme is highly conserved, and both mammalian and avian embryos have been widely used in its analysis. This review will describe the core transcriptional network of the myogenic programme, how it is induced in different cells and what implications this has for our understanding of differentiation.

The origins of muscle

There are a number of embryonic tissues capable of producing skeletal muscle, all of which are mesodermal in origin. The locations of these areas in the embryo are shown schematically in Fig. 1A. Most muscles are derived from paraxial trunk mesoderm (i.e. the mesoderm lying immediately adjacent to the midline, see Fig. 1A and B), which forms somites, and head mesoderm. Additionally, some lateral neck mesoderm has also been shown to have myogenic potential. Trunk, head, neck and limb muscles are all produced by distinct developmental pathways, which are briefly described below.

Myogenesis in somites

Somites are segmentally repeated embryonic structures that bud off from unsegmented paraxial mesoderm alongside the neural tube (shown in Fig. 1). Somites are found throughout the chordates and are the source of all the skeletal muscle of the trunk and limbs as well as some head muscles, such as the tongue muscles (Noden *et al.* 1999, Brent & Tabin 2002, Christ *et al.* 2007). Somites begin forming at the anterior of the embryo and are added posteriorly as the embryo extends. This means that anterior somites are older and more developed than posterior somites, and there is a range of somite stages within a single embryo. This can be seen in Fig. 2A, which shows an early pharyngula chicken embryo (see Box 1) at Hamburger–Hamilton stage 16 (Hamburger & Hamilton 1951) where MYF5, an early marker of muscle differentiation, is expressed in anterior somites, while the most recently formed somites (I–VI) do not yet express myogenic markers. Below the newly formed somites is the presegmented paraxial mesoderm, which continues to segment to produce more posterior somites.

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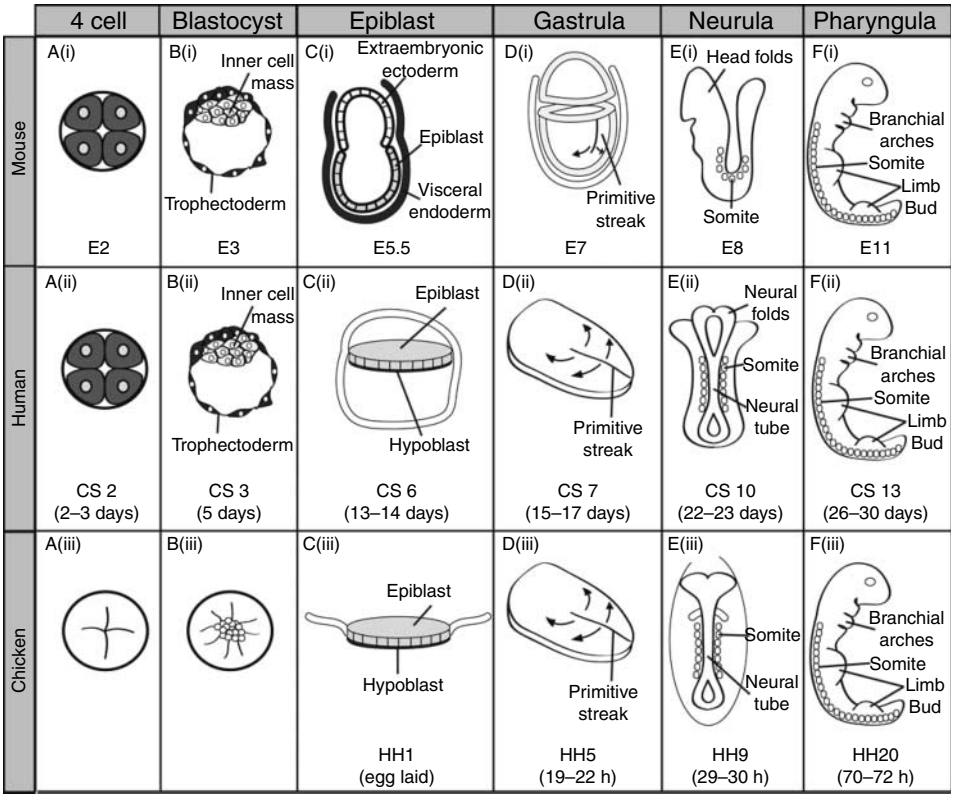
Box 1 Comparative development of amniote embryos



Much of the data discussed in this review have come from experiments on mouse or chicken embryos, because non-amniote vertebrates, such as zebrafish and *Xenopus*, have some significant differences in their myogenic programmes. Here, we show a schematic comparison of mouse, chicken and human development, demonstrating some of the similarities seen between these species' embryos. Also shown is the time at which these stages are reached along with the Carnegie stages (CS) for human embryos (Hill 2007) and the Hamburger–Hamilton stages (HH) for chicken embryos (Hamburger & Hamilton 1951). Mouse embryos are described with the embryonic day (E) since fertilisation (Kaufman 1992).

Following fertilisation and initial cell division, the embryo segregates into embryonic and extra-embryonic lineages. At blastocyst stages in mammals (B, i–iii), the outer cell layer forms the trophoblast, which will form the placenta, while the embryo itself is derived from the inner embryonic stem cells. These inner cells then form the epiblast (C, i–iii). In most amniotes, including humans and chickens, the epiblast is an epithelial disc with a thin layer of underlying cells, the hypoblast. In rodents, the epiblast forms a cup shape contained within the visceral endoderm (VE). The tissues of the embryo are almost exclusively derived from the epiblast, and the main role of the hypoblast and VE is to provide spatial cues for the epiblast as it forms its primary axes.

At gastrula stages (D, i–iii), the embryo produces the three germ layers of ectoderm, mesoderm and endoderm. Epiblast cells migrate into the primitive streak where they undergo epithelial to mesenchymal transition. Cells from the streak migrate out between the epiblast and hypoblast (or VE in rodents). These cells will generate endoderm and mesoderm and, in humans and chickens, displace the hypoblast to produce the trilaminar embryo.

Ectoderm cells then give rise to the neural plate and neural tube (neurula stages, E, i–iii). Mesoderm alongside the neural tube begins to segment to form somites, while at the posterior of the embryo, the tail bud continues to extend and generate more mesoderm. At this stage, rodent embryos begin to turn as they return to a more typically amniote embryonic programme. It is at this point that the most anterior somites are beginning the myogenic programme.

Pharyngula embryos are characterised by the appearance of the branchial (or pharyngeal) arches (F, i–iii). The mesenchymal core of the arches will generate the facial and jaw muscles. In anterior somites, myogenesis is well advanced, while posterior somites are still undergoing the early stages of differentiation. At these stages, myoblasts are beginning to migrate into the limb buds where they will produce the limb muscles.

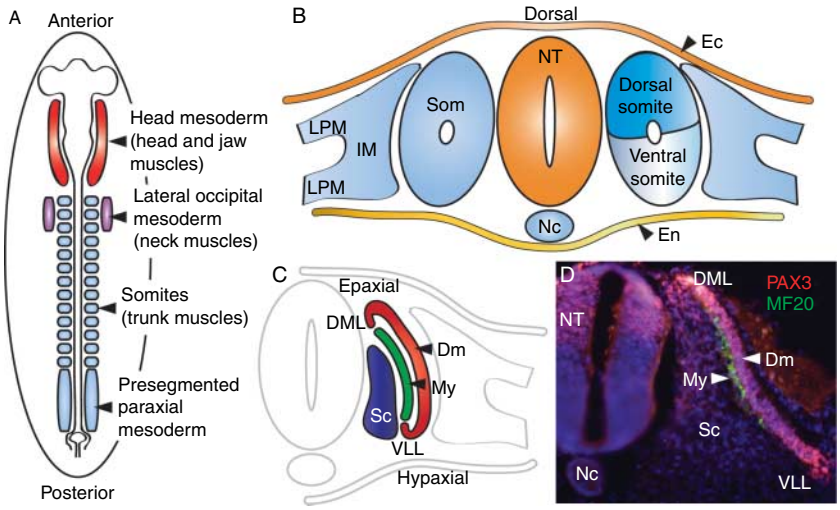


Figure 1 (A) Schematic of 13 somite amniote embryo corresponding to Carnegie stage 11 in human (~24 days), E8.5 in mouse, HH11 in chicken (40–45 h) showing location of myogenic regions. (B) Transverse section of embryo showing spatial arrangement of mesodermal derivatives in blue, ectodermal derivatives in orange and endoderm in yellow. The dorsal somite will produce the dermomyotome, and the ventral somite will produce the sclerotome. (C) Spatial organisation of somite compartments in the embryo as somites undergo differentiation; the dermomyotome is shown in red, myotome in green and sclerotome in blue. (D) Immunostaining of transverse section of HH stage 18 chicken embryo; PAX3 staining in red, MF20 (antibody to sarcomeric myosin, marker of differentiated muscle) in green. LPM, lateral plate mesoderm; IM, intermediate mesoderm; Som, somite; NT, neural tube; Nc, notochord; Ec, ectoderm; En, endoderm; Dm, dermomyotome; My, myotome; Sc, sclerotome; DML, dorsomedial lip of dermomyotome; VLL, ventrolateral lip of dermomyotome.

As somites develop, they form a number of distinct compartments that will contribute to distinct tissues of the animal. These include the sclerotome, which will give rise to vertebrae and ribs, the dermatome, which produces the dorsal dermis, the syndetome, from where axial tendons are derived, and the myotome, which is where muscle differentiation first occurs (Brent *et al.* 2003, Christ *et al.* 2007). Myogenic precursors can first be detected in the dermomyotome, a characteristic 'C'-shaped epithelial structure containing a mixture of myogenic and dermal progenitors (see Fig. 1C and D) which subsequently segregate to give rise to the dermatome and myotome. The first molecular markers that label myogenic precursors in this structure are the paired box transcription factors PAX3 and PAX7, the expression of which is induced by signals from surrounding tissues (Otto *et al.* 2006). PAX3 and 7 label proliferating myoblasts, the precursors of skeletal muscle, in the dermomyotome where they form a regulatory network with other factors, such as the DACH2, SIX1 and EYA2 proteins, to initiate the myogenic programme (Heanue *et al.* 1999, Kardon *et al.* 2002, Grifone *et al.* 2007). The structure of the developing somites is shown in Fig. 1D, which shows PAX3 expression labelled in red in the dermomyotome and expression of sarcomeric myosin, a marker of differentiating muscle cells, labelled in green in the underlying myotome.

PAX3 and PAX7 are important regulators of muscle development and are upstream of myogenic genes in somites, limb muscles and satellite cells. *Pax3* is expressed in somite muscle precursor cells, and mice carrying mutations in this gene lack limb muscles (Goulding *et al.* 1994), although myogenesis in trunk muscle precursors still takes place. However, in mice lacking both *Pax3* and *Pax7*, major defects in myogenesis occur, suggesting that together these genes are required

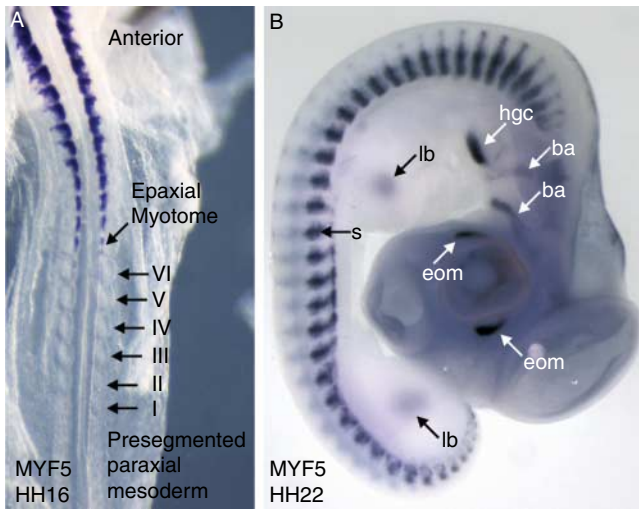


Figure 2 (A) *In situ* hybridisation of MYF5 in HH stage 16 embryos. Anterior is towards the top, posterior is towards the bottom. Expression is seen in anterior somites starting in the epaxial myotome and then spreading more widely through the somite. Posterior somites (I–VI) do not express muscle-specific genes. (B) *In situ* hybridisation of MYF5 in HH stage 22 embryos. Expression is seen in all muscle types including myogenic cells in somites (s), limb buds (lb) and head. Tongue muscle precursors are seen in the hypoglossal cord (hgc), jaw and facial muscles in the branchial arches (ba) and extraocular muscles (eom) around the eye.

for normal muscle development. While PAX3/7 are not unique to muscle cells, as they also have roles in neural development, they have an important role in the determination of trunk myoblasts, and in their absence, myogenesis is not sustained, although early muscle cells in the somite are still formed via a PAX-independent mechanism (Relaix *et al*. 2005). Analysis of mouse mutants and overexpression in chick embryos has shown that PAX3/7 control the expression of myogenic regulators such as MYF5 and MYOD (Maroto *et al*. 1997, Bajard *et al*. 2006, Sato *et al*. 2010). They also support the proliferation and survival of myoblasts before differentiation (Buckingham & Relaix 2007, Collins *et al*. 2009).

Following induction, myoblasts then migrate around the edges of the dermomyotome to form the myotome, the region of the somite where myogenic differentiation is first observed. This process has been extensively studied using cell labelling and confocal microscopy in chicken embryos (Kahane *et al*. 1998, Cinnamon *et al*. 1999, Denetclaw *et al*. 2001, Ordahl *et al*. 2001, Venters & Ordahl 2002, Gros *et al*. 2004). This process begins in the dorsomedial lip of the dermomyotome where myoblasts migrate ventrolaterally to form a layer of cells lying directly under the dermomyotome, the epaxial myotome. Figure 1C and D show the structure of the differentiating somite and its separation into three compartments. Subsequently, myoblasts also migrate from the ventrolateral lip and from lateral edges of the dermomyotome into this layer to produce the primary myotome. The ventrolateral lip myoblasts contribute to the hypaxial myotome, while cells from the lateral edges of the dermomyotome contribute to both epaxial and hypaxial myotomes (Gros *et al*. 2004). It is here that myogenic differentiation starts, as cells exit the cell cycle and begin to express muscle-specific markers while PAX3 is down-regulated. It is this switch from proliferative myoblast to post mitotic myocyte, which marks the start of differentiation. MYF5 is the earliest marker of determined muscle cells, closely followed by MYOD. MYF5 expression in the epaxial myotome is shown in Fig. 2A. At later stages, cells from the central dermomyotome also move directly into the underlying myotome, rather than by migrating around the edges of the dermomyotome, and lineage tracing in both chicken and mouse embryos has shown that it is from these cells that satellite cells, adult muscle stem cells, are derived (Gros *et al*. 2005, Relaix *et al*. 2005, Lepper & Fan 2010).

The myotome can be divided into two regions, the epaxial and hypaxial myotomes. The epaxial myotome gives rise to the deep muscles of the back, while the hypaxial myotome produces the muscles of the body wall and limbs. Spatially, the epaxial myotome is located in the dorsal somite, while the hypaxial myotome is ventral. There is no obvious morphological division between these domains in somites, but they can be distinguished at the molecular level by the expression of epaxial markers such as EN1 and hypaxial markers such as SIM1 (Cheng *et al*. 2004). The epaxial and hypaxial myotomes are largely derived from dorsomedial and ventrolateral regions of the dermomyotome respectively (Huang & Christ 2000), although careful examination of labelled cells has shown a more complex distribution of cells in the myotome including myoblasts migrating from the lateral edges of the dermomyotome contributing to both epaxial and hypaxial muscles (Gros *et al*. 2004).

Epaxial and hypaxial muscles are induced by distinct signalling events from neighbouring tissues. The induction of myogenesis in somites has been examined using somite explant culture where dissected somites from either chicken or mouse embryos are cultured *in vitro* and by surgical manipulation of chicken embryos. These approaches allow co-culture with other tissues to examine inductive events or the addition of defined growth factors either in culture or directly to the embryo. This has shown that myogenesis is driven by signals from the neural tube, notochord, overlying ectoderm and lateral mesoderm (Munsterberg & Lassar 1995). A combination of WNT1 and WNT3A from the dorsal neural tube and SHH from the floor plate of the neural tube and notochord induces myogenesis in the epaxial myotome (Munsterberg *et al*. 1995, Tajbakhsh *et al*. 1998, Borycki *et al*. 1999). The bone morphogenetic protein (BMP) inhibitor noggin is also produced in dorsal somites and plays an important role, as otherwise BMP signals from the dorsal neural tube inhibit myogenesis (Hirsinger *et al*. 1997, Marcelle *et al*. 1997). However, in hypaxial muscles, a combination of BMP from lateral mesoderm and WNTs from overlying ectoderm is responsible for myogenic induction (Dietrich *et al*. 1998, Otto *et al*. 2006), demonstrating that even within a single somite, different myogenic programmes are used to generate skeletal muscle.

Development of limb muscles

Although the musculature of the limb is derived from somites, just as trunk muscles are, the developmental pathways leading to their production have several distinct features, which are worth noting.

Using quail–chick grafts to produce chimeric embryos has determined that limb muscles are derived from the hypaxial myotome. The muscles of the chicken forelimb are derived from somites 16–21 (Beresford 1983) and those of the hindlimb from somites 26–33 (Lance-Jones 1988). Detailed analysis of PAX3-expressing cells migrating into limb buds has shown that in mouse embryos, forelimb muscles come from somites 9–14 and hindlimb muscles from somites 26–32 (Houzelstein *et al*. 1999). Delamination of PAX3-positive myoblasts from the ventrolateral dermomyotome is induced by hepatocyte growth factor (HGF)/Scatter factor, which causes cells to migrate into the developing limb buds as shown by the overexpression of HGF in chicken embryos and loss of function of MET (c-met), the HGF receptor, in

mice (Bladt *et al*. 1995, Brand-Saberi *et al*. 1996, Scaal *et al*. 1999). The migrating muscle cells form two blocks of tissue, the dorsal and ventral muscle masses, and only then do they begin to differentiate and express muscle-specific genes, a process requiring the transcription factors MEOX2 and PITX2 (Mankoo *et al*. 1999, L'Honoré *et al*. 2010). This process is shown schematically in Fig. 3 along with sections through chicken embryo limbs, which have been immunolabelled for PAX3 to detect myoblasts. The molecular signals responsible for limb muscle differentiation have not been fully elucidated, partly because many of these same signals also regulate limb patterning, and disruptions to patterning can also affect myogenesis (Robson *et al*. 1994). Grafting of growth factor-soaked beads in chicken embryos has shown that BMP signalling can maintain PAX3-positive myoblasts in an undifferentiated state in the limb bud (Amthor *et al*. 1998) as does expression of HGF (Scaal *et al*. 1999); however, the factors required for induction of myogenic differentiation in limbs are not known, and it has been suggested that these myoblasts simply differentiate when inhibitory signals of BMPs are down-regulated (Amthor *et al*. 1998). Some signals are known to regulate limb myogenesis, and inhibition of FGFR4 can prevent myogenic differentiation (Marics *et al*. 2002). In contrast to myotome induction in somites, β-catenin-dependent WNT signalling is not required for differentiation of limb myoblasts (Hutcheson *et al*. 2009) and may in fact inhibit myogenesis by inducing expression of the anti-myogenic factor MSX1 (Miller *et al*. 2007), although WNT signalling has been shown to affect fast/slow fibre type differentiation in limb muscles (Anakwe *et al*. 2003). WNT6 from limb ectoderm can also induce expression of PAX3, MYF5 and myogenin, while it appears to negatively regulate MYOD suggesting distinct regulatory functions in different parts of the myogenic network (Geetha-Loganathan *et al*. 2005). It has also been shown that in somites, PITX2 is regulated by WNT signalling which is not the case in limb muscle, confirming that distinct molecular pathways are operating in these muscle groups (Abu-Elmagd *et al*. 2010). NOTCH signalling can induce MYOD in limbs, but appears to be downstream of PAX3 and MYF5 (Delfini *et al*. 2000), while SHH, another factor known to positively induce myogenesis in somites, seems to regulate slow muscle formation rather than induction of differentiation (Bren-Mattison & Olwin 2002, Li *et al*. 2004). What is clear is that the myogenic programme in the limb does not simply recapitulate that seen in somites and that the differentiation of committed PAX3-positive myoblasts requires a distinct set of signals.

Formation of head and neck muscle

The muscles of the vertebrate head form a complex and heterogeneous group including extraocular muscles, jaw and tongue muscles, and the muscles required to control head movements (Noden & Francis-West 2006). They have various origins with some, such as the tongue, being derived from somites, while others (jaw muscles and extraocular muscles) are derived from the cephalic and prechordal mesoderm that lies anterior to the segmented trunk mesoderm and which does not form somites.

Interestingly, these non-somite-derived muscles also have some distinct molecular mechanisms driving their differentiation and do not express PAX3/7 but instead are specified by other transcription factors such as PITX2 (Dong *et al*. 2006, Shih *et al*. 2007) and TBX1 (Dastjerdi *et al*. 2007); mice carrying mutations in these genes show defects in head muscle development, while overexpression studies in cultured chicken embryo

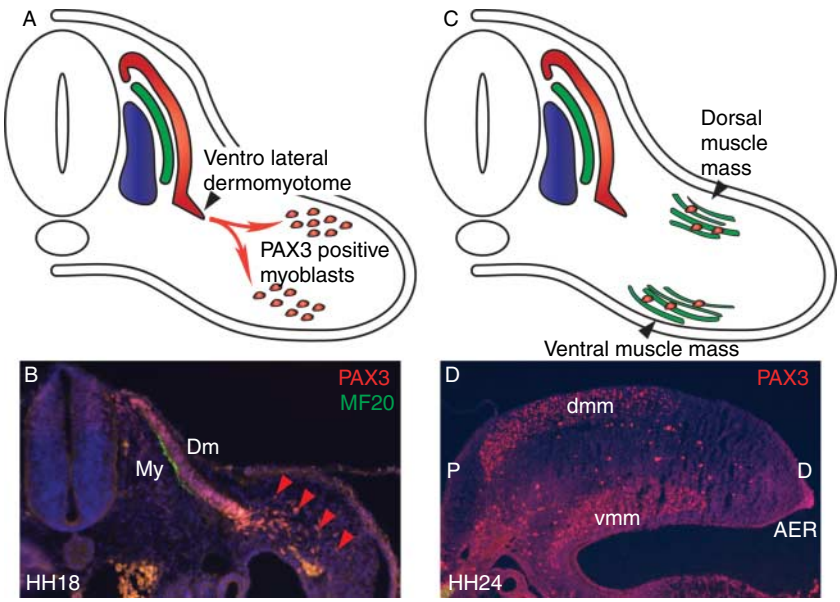


Figure 3 (A) Schematic of myoblast migration from ventrolateral dermomyotome into limb buds. (B) Immunostaining of transverse section of HH stage 20 chicken embryo. PAX3 (red) -positive cells are shown delaminating and entering limb bud (red arrowheads). (C) Schematic of dorsal and ventral muscle masses formed by limb bud myoblasts. Undifferentiated precursors (some of which will give rise to satellite cells in adult muscles) are shown in red with differentiated myotubes shown in green. (D) Immunostaining of transverse section of HH stage 24 chicken embryo. PAX3 (red) cells are restricted to the dorsal and ventral regions of the limb bud. My, myotome; Dm, dermomyotome; P, proximal; D, distal; dmm, dorsal muscle mass; vmm, ventral muscle mass; AER, apical ectodermal ridge of limb bud.

myogenic cells lead to an increase in muscle cell number. They also share some early markers, such as ISL1 (islet1), with cardiac muscle (Nathan *et al*. 2008) and, as the jawed vertebrates evolved, may have used these cardiac precursors to develop at least some of their head muscles (Tzahor 2009). Confirmation of this has recently come from studies in the simple chordate, Ciona intestinalis, and clonal analysis of mouse embryos where some cardiac precursors have also been found to contribute to jaw muscles and their evolutionary precursors (Lescroart *et al*. 2010, Stolfi *et al*. 2010).

Although the early specification of head and neck muscles is distinct from those of the trunk, they still express the core myogenic regulators and require these genes for their differentiation. This is shown in Fig. 2B, which shows the expression of MYF5 in the precursors of the tongue, jaw, facial and extraocular muscles. The signals responsible for induction of head muscle from mesoderm have not been fully determined, but in chicken embryos, head mesoderm does not undergo myogenesis when grafted into trunk regions (Mootoosamy & Dietrich 2002), and therefore a different signalling environment is necessary. Using explant culture of head mesoderm and retroviral expression in chicken embryos has shown that inhibitors of both WNT and BMP signalling can promote head myogenesis (Tzahor *et al*. 2003) but, as in the limb, positive inductive events have yet to be identified.

A recent study on the origins of neck muscles has provided more evidence of the diversity of muscle origins (Theis *et al*. 2010). A combination of grafting in avian embryos and genetic analysis of mouse embryos demonstrates that these muscles show head muscle-like characteristics in that they are derived from cells expressing ISL1 and TBX1 and do not require PAX3/7. However, these muscles are not derived from the head mesoderm but are derived from lateral plate mesoderm adjacent to the most anterior somites (lateral occipital mesoderm, see Fig. 1A). The expansion of a myogenic programme into lateral, as opposed to paraxial, mesoderm provides another example of evolutionary plasticity in which distinct signalling events converge on core myogenic regulators to generate diverse muscle patterns.

Muscle stem cells in adult animals

Adult muscle satellite cells are found underlying the basal lamina and are able to both self-renew and to contribute muscle growth and regeneration upon activation. Quail–chick chimeras, confocal imaging of labelled cells in chicken embryos and lineage tracing in mice have shown that these stem cells are derived from the same compartment as embryonic myogenic cells (Gros *et al*. 2005, Kanisicak *et al*. 2009), and their function depends on PAX3/7 and the myogenic regulators required in embryonic muscle (Buckingham 2007). Intriguingly, recent evidence using an inducible knockout approach has questioned the absolute requirement for *Pax* gene function during muscle regeneration in adult mice and suggested that it is only essential in younger animals (Lepper *et al*. 2009). These experiments used mice carrying an oestrogen receptor/Cre recombinase fusion protein, which was able to delete a floxed *Pax7* allele after tamoxifen injection to bypass earlier defects seen in *Pax7* knockout mice. Strikingly, even in the absence of PAX7 and PAX3, muscles from adult mice were able to regenerate following damage, while juvenile mice treated in this way showed a severely compromised regenerative ability. This may imply that alternative myogenic pathways, such as those utilised in head muscle specification, can be used in older animals but, as yet, no candidates have been identified. There is also recent evidence that a PAX7-dependent non-satellite cell compartment can contribute to muscle regeneration in adults (Mitchell *et al*. 2010).

The core transcriptional programme in skeletal muscle

When cells differentiate into skeletal muscle, they begin by activating a unique set of transcriptional regulators, the myogenic regulatory factors (MRFs). These are the key inducers of myogenesis and are absolutely required for differentiation of skeletal muscle.

The MRFs and muscle determination

Myogenesis is initiated by the expression of the MRFs, a group of four related basic helix-loop-helix transcription factors, which share the ability to induce myogenic differentiation when expressed in non-muscle cells (Pownall *et al*. 2002). They are MYF5, MYOD, MRF4 (sometimes referred to as MYF6) and myogenin. However, it is also clear that they have distinct functions and may act to specify different subsets of muscles. A further complication is that there is a degree of functional redundancy between these genes, and, in some cases, loss of one member can be compensated for by up-regulation of another (Rudnicki *et al*. 1992).

MRF expression is seen in all muscles of the embryo, although there are some differences in timing, with MYF5 normally being expressed first and others being activated in various sequences depending on the muscle programme, and patterns such that not all muscles express all MRFs; for example, MRF4 is not expressed in facial muscles. The expression pattern of MYF5 in all muscle groups of the embryo is seen in Fig. 2, which shows expression in myogenic cells of somites, limb buds and the head.

Activation of one of the MRFs will often result in the expression of the others; this self-reinforcing mechanism presumably serves to lock down the myogenic programme and inactivate other differentiation programmes (Delfini & Duprez 2004, de la Serna *et al*. 2005,

Sweetman *et al*. 2008). The exception to this is MYF5, which is not activated by the expression of other MRFs and seems to act at the top of the myogenic cascade. The regulation of MYF5 is complex and controlled by multiple enhancers covering over 150 kb of DNA, each of which drives expression in a particular subset of muscle cells which can be seen by fusing a LacZ reporter to specific enhancer elements in transgenic mice (Carvajal *et al*. 2001, 2008). An example of this is the early epaxial enhancer that drives expression of MYF5 in the epaxial myotome (see Fig. 3A). This enhancer contains TCF/LEF transcription factor binding sites, which are governed by β-catenin-dependent WNT signalling, and Gli-binding sites that respond to SHH signalling. Mutation of the TCF sites results in a loss of MYF5 expression in the epaxial myotome (Borello *et al*. 2006), while when the Gli-binding sites are lost, initial epaxial expression is still seen but not maintained (Teboul *et al*. 2003). This analysis has complemented the embryo manipulations that showed the requirement for WNT and SHH signalling in the epaxial myotome (Munsterberg *et al*. 1995, Tajbakhsh *et al*. 1998, Borycki *et al*. 1999) and provided a link to the underlying genetics of this process.

Although the interactions between MRFs are complex, mouse mutants have given some indication of specific functions of individual MRFs. The clearest phenotype is seen in mice lacking myogenin that have severe defects in skeletal muscle formation (Hasty *et al*. 1993). In contrast, mice carrying mutations in either MYF5, MYOD or MRF4 are viable and produce morphologically normal muscle; severe muscle defects in the embryo are only observed when all three genes are removed (Rudnicki *et al*. 1993, Kassar-Duchossoy *et al*. 2004). Defects in regeneration of adult muscle have also been reported in the absence of MYF5 (Gayraud-Morel *et al*. 2007, Ustanina *et al*. 2007) and MYOD (Macharia *et al*. 2010). Recently, it has also been shown that ablation of MYF5-expressing cells using a Myf5-Cre to drive expression of diphtheria toxin in all MYF5-expressing cells in the embryo also produces apparently normal muscle development (Gensch *et al*. 2008, Haldar *et al*. 2008). This surprising result suggests that a MYF5-independent population of cells can expand to fill the niche left when the MYF5 cells are removed and undergo myogenesis without expressing MYF5. Taken together, these findings indicate a high degree of plasticity and the presence of compensatory mechanisms that are able to induce myogenesis even when normal mechanisms are severely compromised.

Biochemical studies of MYOD and other MRFs have revealed how these genes are able to activate muscle-specific gene expression by recruitment of co-activators and co-repressors to target gene loci (Berkes & Tapscott 2005); however, the issue of how MRFs regulate only muscle-specific gene expression remains unresolved. E boxes, the DNA motif recognised by MRFs, are widespread throughout the genome, and, using ChIP-seq analysis, MYOD has been shown to bind to a huge number of these, including a great many which are presumably non-functional in terms of inducing muscle gene expression; it has been suggested that these interactions may help define the epigenetic state of the cells, priming them for myogenic differentiation (Cao *et al*. 2010). However, this does not address the issue of how MRF binding can specifically activate transcription at some sites in the genome but not in others. A possible answer to this has come from the analysis of other muscle specification genes such as the *Six* genes (Heanue *et al*. 1999, Grifone *et al*. 2005). These proteins form a complex with the EYA and DACH proteins, which can bind DNA and activate transcription. Muscle-specific gene promoters often contain both MRF and SIX complex-binding motifs, and co-operation between these modules may be required for transcriptional activation of muscle-specific genes (Liu *et al*. 2010).

MicroRNAs in muscle development

The discovery that microRNAs, small, non-coding RNAs, can play major roles in the development and differentiation of cells has been the subject of a great deal of fascinating recent research. Several microRNAs have been shown to be directly regulated by MRFs, and others have been identified in the introns of muscle-specific genes. MicroRNAs are negative regulators of gene function and act by interacting with complementary sequences in the 3′ UTRs of target mRNAs, which are then either degraded or translationally repressed (Fabian *et al*. 2010).

Muscle-specific microRNAs have been isolated and shown to be expressed during muscle cell differentiation in cell culture (Anderson *et al*. 2006), in embryonic muscles in normal development (Sweetman *et al*. 2006) and in regenerating adult muscle (Yuasa *et al*. 2008). They are also up-regulated in muscle diseases such as Duchenne muscular dystrophy (Greco *et al*. 2009) and myotonic dystrophy (Gambardella *et al*. 2010), presumably as part of the regenerative response seen in these diseases. Of particular interest have been the so-called myomiRs, a group of microRNAs consisting of the miR-1/206 family and the miR-133 family. These microRNAs are derived from three separate loci; each of these produces a single transcript, which is then processed to produce one microRNA of the 1/206 family and another of the 133 family. Interestingly, these appear to have opposing effects with miR-1/206 enhancing differentiation, while miR-133 seems to drive myoblast proliferation (Chen *et al*. 2006). The mechanisms of these effects include the ability of miR-1/206 to negatively regulate follistatin and utrophin, both of which retain myoblasts in an undifferentiated state (Rosenberg *et al*. 2006). Other confirmed targets include DNA polymerase α (Kim *et al*. 2006), HDAC4 (Chen *et al*. 2006), gap junction protein α1 (GJA1, also known as connexin43; Anderson *et al*. 2006), calmodulin and

MEF2A (Ikeda *et al.* 2009), while miR-133 can target serum response factor (SRF) to maintain cells as proliferative myoblasts (Chen *et al.* 2006). This is far from an exhaustive list of targets of these microRNAs, and the true number of mRNAs regulated in this way remains unknown. There is good evidence that these microRNAs are directly regulated by MRFs in muscle development (Rao *et al.* 2006, Rosenberg *et al.* 2006, Sweetman *et al.* 2008), and they are good candidates for core components of the myogenic programme. Satellite cells have also been shown to express miR-1 and miR-206, which regulate the switch from proliferation to differentiation by controlling PAX7 (Chen *et al.* 2010) and PAX3 (Hirai *et al.* 2010) following microRNA induction by MYOD. This feedback loop may form an important part of the myogenic programme. However, the myomiRs are not restricted to skeletal muscle, and both miR-1 and miR-133 are also expressed in cardiac muscle where they also regulate differentiation (Townley-Tilson *et al.* 2010).

As well as the muscle-specific myomiRs, other microRNAs have also been implicated in muscle development. Examples include miR-24, which can regulate transforming growth factor β in skeletal muscle (Sun *et al.* 2008), miR-181, which regulates HOXA11 (Naguibneva *et al.* 2006), miR-221 and -222, which control cell cycle regulators in skeletal muscle differentiation (Cardinali *et al.* 2009), and miR-214, which regulates the epigenetic state of muscle cells (Juan *et al.* 2009). MicroRNAs have also been identified encoded in the introns of muscle-specific genes such as myosins. These can regulate muscle fibre type and may provide a mechanism, whereby cell fate decisions can be locked down by co-expression of mRNAs and miRs which act together (van Rooij *et al.* 2009).

The study of microRNAs in development remains at an early stage, and it is not clear to what extent they are likely to be major drivers of differentiation or have more subtle roles in the fine tuning of cell behaviour and identity; for example, it seems likely that myomiRs act to regulate the balance between proliferation and differentiation rather than as direct inducers of cell fate. Understanding the roles of small RNAs in differentiation and development is an important challenge, especially given their ability to regulate numerous target genes, and will undoubtedly have a major impact on our understanding of the molecular control of biological processes.

Conclusions: conservation of the core myogenic programme in different cellular contexts

During development, myogenic cells are produced in numerous distinct regions of the body and have to differentiate in very different cellular contexts. The distinct programmes leading to MRF activation in these tissues are summarised in Fig. 4. This is also true during adult life, as regenerating muscles also need to activate the myogenic programme. It is this requirement for flexibility that has required muscle cells to evolve the ability to activate the core modules of the myogenic transcriptional network (centred on the MRFs) in different ways in response to different stimuli. This flexibility explains the plasticity of early muscle compartments and the ability of muscles to regenerate throughout adult life. It has even been reported recently that MRFs can be bypassed altogether in some muscle stem cells and that PAX3 alone can drive differentiation in these cells (Young & Wagers 2010). This unexpected result may well cause a re-evaluation of the absolute requirement for MRFs in myogenesis; nevertheless, it is clear that most myogenic pathways converge on these regulators and the extent to which non-MRF-dependent mechanisms exist is not yet known.

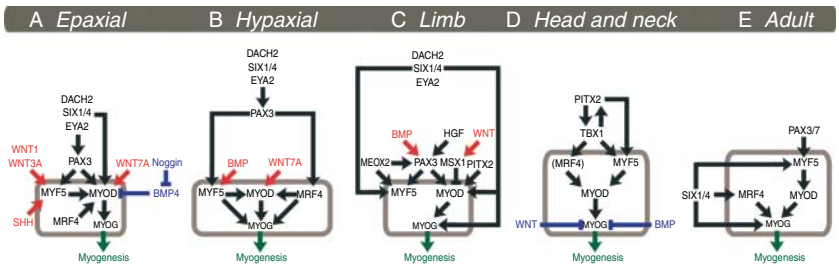


Figure 4 Comparison of the signalling pathways leading to MRF expression and myogenesis in epaxial and hypaxial somite, limb, head and neck, and adult. In grey boxes are the core network of MRFs in activating myogenesis from different tissues. Positive regulatory signals are shown in red, and inhibitory signals are shown in blue. Black arrows show genetic interactions. (A) Epaxial somite: DACH2, SIX1/4, EYA2, PAX3, MYF5 and MRF4 induce MYOD, thus leading to myogenin (MYOG) expression and myogenesis. WNT1, WNT3A and SHH up-regulate MYF5 expression, and WNT7A up-regulates MYOD. BMP4 negatively regulates MYOD but is itself inhibited by noggin. (B) Hypaxial somite: DACH2, SIX1/4 and EYA2 are capable of inducing PAX3. Subsequently, PAX3 induces MYF5 and MRF4, which in turn activate MYOD expression, followed by MYOG expression and myogenesis. BMP and WNT7A positively regulate MYF5 and MYOD expression. (C) Limb: DACH2, SIX1/4, EYA2, PAX3, MEOX2 and PITX2 are capable of inducing MYF5 and MYOD; subsequently, MYOD induces MYOG expression and myogenesis. MSX1 can inhibit MYOD expression. BMP and HGF up-regulate PAX3 expression, and WNT regulates MSX1. (D) Head and neck: PITX2 and TBX1 induce each other and MYF5. TBX1 is capable of inducing MYF5 expression (and MRF4 in those head muscles which express it) before activating MYOD expression. Subsequently, MYOD induces MYOG expression followed by myogenesis, but inhibitory signals from WNT and BMP can also regulate MYOG expression. (E) Adult: PAX3/7 directly induce MYF5 expression, whereas SIX1/4 can induce MRF4, MYF5 and MYOG. MRF4 and MYOD activate MYOG expression followed by myogenesis.

One of the striking lessons from the study of myogenesis is that activation of a specific differentiation programme can be mediated by a simple core programme containing only a few genes. This has also been shown in iPS cells where a pluripotent state can be induced by the expression of just four transcription factors (Yamanaka & Blau 2010) and, more recently, in heart tissue where three transcription factors can induce conversion of fibroblasts into cardiomyocytes (Ieda *et al*. 2010). In both these cases, a small suite of master regulators appear to be able to overwrite existing cellular programmes and redefine a cell's identity. One fascinating aspect of this is the implication that these proteins are able to change the epigenetic state of the cell and restructure chromatin to allow wholesale changes in gene expression as cells change fate. It is intriguing to speculate that the MRFs required for myogenesis may have similar activities as part of their well-documented ability to induce changes in cell fate.

Understanding myogenesis has important implications for regenerative biology, as activation and differentiation of adult stem cells are often governed by these core transcriptional programmes. In addition, our understanding of the basic cellular and developmental mechanisms controlling cell fate and differentiation has been greatly informed by these studies. Future work to elucidate the developmental basis and epigenetic mechanisms controlling these numerous myogenic programmes will continue to shed light on some of the most fascinating aspects of biology.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review reported.

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