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Control of the development of limb musculature

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SUMMARY

Limb muscle development is an embryonic multistep process including paraxial mesoderm segmentation, somite compartmentalisation, muscle precursor cell delamination (epithelial-mesenchymal transition), proliferation-migration and, finally, terminal differentiation. Classical as well as the latest research articles in this developmental biology field are reviewed and discussed. A general overview of molecular mechanisms controlling muscle development in the limb of the developing embryo is presented.

Key words: Embryology – Segmentation – Somitogenesis – Somite compartmentalisation – Hypaxial muscles – Myogenesis

INTRODUCTION

Limb muscle development is an embryonic multistep process that initially includes paraxial mesoderm segmentation, somite compartmentalisation and, later, muscle precursor cell delamination (epithelial-mesenchymal transition), proliferation-migration and finally terminal differentiation. Each of these steps is tightly controlled by molecular mechanisms that in recent years have been studied in depth . The temporal overlap of some of these processes, as well as the role of certain molecular mechanisms in more than one process, makes it difficult to clearly delineate the parts of this review without being –in some cases– redundant, but I have tried to keep this to a minimum in the hope of presenting a clear overview of what is and what is not known about this developmental process. I have also decided not to analyze and review all the data available about the molecular control of myogenesis, as this is a major task that would require several individual reviews, and I have preferred to focus on describing the process of limb muscle development as a embryological/morphological process and introducing some of the better known molecular mechanisms that control some of the steps involved. I therefore have to apologise to all authors whose works are not included here.

The current review will focus mainly on the molecular mechanisms involved, starting with muscle precursor delamination, and passing all the way through to terminal differentiation in the limb, even though a general introduction will be included for the initial steps.

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Paraxial mesoderm segmentation

Paraxial mesoderm segmentation has been shown to be regulated by the so-called segmentation clock, which can be summarised as cyclic waves of gene activation and repression in the cranio-caudal axis of the embryonic unsegmented paraxial mesoderm or presomitic mesoderm. These cycles determine the time necessary for the epithelialisation of each somite pair, and each species has a specific time: for example, 90min in the chicken embryo incubated at 37ºC. Both genetic/transcriptional as well as post-transcriptional/translational mechanisms have been reported to control this segmentation clock and have been satisfactorily reviewed elsewhere and hence will not be reviewed here (Aulehla and Pourquie, 2008; 2010; Dequeant and Pourquie, 2008; Gomez et al., 2008; Lewis et al., 2009; Cibois et al., 2010; Gibb et al., 2010).

Somite compartmentalisation

Once the paraxial mesoderm has become organized into epithelial somites, each of these can be compartmentalised in different spatial axes (dorso-ventral and medio-lateral).The formation of the different compartments has been shown to be controlled or influenced by signals from surrounding tissues such as the ectoderm, the neural tube, the floor plate and notochord, the lateral plate and the intermediate mesoderm; removal of any of these tissues during embryonic development interferes or alters the compartmentalisation of the somite, resulting in the expansion or retraction of specific compartments. Each of these tissues is a source of different growth factors or secreted factors that can act in a paracrine manner (Yusuf and Brand-Saberi, 2006; Alexander et al., 2009).

Based on morphological criteria, the epithelial somite can be divided into a ventral and dorsal half; the ventral somitic half, after an epithelial to mesenchymal transition, will form the sclerotome, which will give rise to the axial skeleton, including the vertebral column but also the ribs (Christ et al., 1998, 2000; Huang et al., 2000a; Alexander et al., 2009); the dorsal somitic half will form the dermomyotome, an epithelial structure that will give rise not only to dermis and muscle, as its name implies and has been known traditionally, but also to part of the scapula and angioblasts (both vascular and lymphatic) (Wilting et al., 1995, 2000; Zhi et al.,1996; Christ et al., 1998, 2000; Huang et al., 2000b; He et al., 2003). The ventral compartment is molecularly characterised by the expression of Pax-1 and Pax-9, while the dorsal compartment is characterised by Pax-3 and Pax-7 expression, although many other molecular markers have also been discovered (Stockdale et al., 2000; Yusuf and Brand-Saberi, 2006).

However, when muscle development is taken into consideration, lateral and medial compartments have been defined. These compartments do not designate a morphological structure but rather are differentiated by specific gene expression patterns and also by the prospective muscle groups that they will form. Thus, the medial compartment will form the epaxial musculature, which includes the muscles of the back, while the lateral compartment will form the hypaxial musculature, which will give rise to the muscles of the limbs and body wall (Scaal and Christ, 2004). While the epaxial domain is homogenous along the cranio-caudal body axis, the growth of the hypaxial domain is different at limb or interlimb level. At interlimb level, the lateral dermomyotomal lip will grow as a continuous sheet, while at limb level the hypaxial domain will undergo an epithelial to mesenchymal transition, resulting in the delamination of myogenic precursors from the lateral dermomytomal lip and migration into the limb mesenchyme (Scaal and Christ, 2004).

It is noteworthy that the hypaxial domain has also been described as a source of angioblasts migrating into the limb (and is clearly delineated by the expression of the vascular endothelial growth factor receptor 2, VEGFR2; Nimmagadda et al., 2007). Interest in knowing whether muscle precursors and angioblasts might have a common precursor is high, and the two working hypothesis are that myogenic precursors and angioblasts are specified either in the somite or in the limb. A highly complex retroviral library has been used to address this question and the conclusion reached is that both endothelial and myogenic precursor cells in the limbs have a common somitic precursor (Golden et al., 1995; Kardon et al., 2002). Further indications that this may be the case have been obtained more recently by showing that dif-

ferent populations of migratory cells exist and that one or another fate could be influenced by modulating CXCR4 (Vasyutina et al., 2005; Yusuf et al., 2006). The confirmation or refutation of these facts is only a matter of time, as interest in cellular pluripotency has increased vastly in recent years. Interestingly, it has also been shown that the endothelial and muscle precursors of somitic origin migrate and become positioned differently at their fate in the limb, opening a large number of questions that so far have remain unaddressed (Huang et al., 2003).

Molecularly, the epaxial and hypaxial domains can be clearly differentiated by a series of well characterised markers (Stockdale et al., 2000; Yusuf and Brand-Saberi, 2006).

Once the somite compartments have formed, the actual process of limb muscle process can start, and it is from here onwards that I would like to present a more detailed review of the state of the art.

The molecular mechanisms that control limb muscle development have been elucidated using different models (such as mice, chick, zebrafish, xenopus, etc…), each of them having different limitations. Many different knock-out mice displaying muscle defects have been generated over the years, including those involving myogenic regulatory factors (Myf5, Mrf4, MyoD and Myogenin) or other transcription factors such as Pax3, Pax7, Meox2, Six1 and Six4, etc… (Mankoo et al., 1999; Grifone et al., 2005; 2007; Giordani et al., 2007; Buckingham and Vincent, 2009; Bismuth and Relaix, 2010; Carvajal and Rigby, 2010). All such knock-out mice have been shown to have defects in limb musculature, but in many cases the exact molecular mechanisms that justify the observed phenotype have remained unsolved and may involve different processes, such as proliferation-differentiation-apoptosis, etc… In many instances, the chick embryo has served as an alternative tool to overcome the limitations of the mouse model. However, many open questions remain to be elucidated.

Delamination of muscle precursor cells from the lateral dermomyotomal lip (epithelial-mesenchymal transition)

As stated above, many different knock-out mice display limb muscle defects, but how these defects originated remains unclear. Nevertheless, one mechanism that has been characterized, if not completely at least in greater detail, is the mechanism that drives the epithelial-mesenchymal transition that takes place in the lateral dermomyotomal lip and that allows muscle precursor cells to abandon the somite and migrate into the outgrowing limb mesenchyme. The dermomyotomal lips that contain limb muscle precursor cells are clearly delineated by Lbx1 gene expression (Dietrich et al., 1998) and also express c-Met, the receptor for scatter factor/hepatocyte growth factor, which is produced in the limb mesenchyme and which upon binding to its cognate receptor induces an epithelial to mesenchymal transition of the lateral dermomyotome, leading to a delamination of muscle precursors (Brand-Saberi et al., 1996; Dietrich et al., 1999; Scaal et al., 1999). The expression of c-Met is regulated by Pax3, a transcription factor whose expression is also restricted to the dermomyotome; SF/HGF, c-Met or Pax3 knock-out mice have a defect in limb musculature due to the lack of delamination of muscle precursors (Daston et al., 1996; Epstein et al., 1996; Yang et al., 1996).

Migration and positioning of muscle precursor cells

The former interaction (c-Met/SF/HGF) has also been shown to be responsible for muscle migration as limb outgrowth progresses. The myogenic precursors follow the distal migration of the SF/HGF source (Dietrich et al., 1999; Scaal et al., 1999). However, taking into consideration that limb muscles are not only positioned in the areas where SF/HGF is being produced it is not unreasonable to surmise that other factors that counteract or modulate its action must exist (fig. 1). This hypothesis was confirmed by showing that if either the distal, posterior or anterior mesenchyme was excised, the muscle precursors migrated differently; this effect could be also mimicked implanting a Bmp antagonist (Noggin) in the anterior or posterior mesenchyme. Therefore, it was concluded that Bmps may be the factors counteracting SF/HGF attractive potential (Bonafede et al., 2006). Furthermore, it has been shown that TCF4, a transcription factor in the Wnt signaling pathway, delineates the mesenchymal fields within the limb where myogenic precursors will reside once migration has

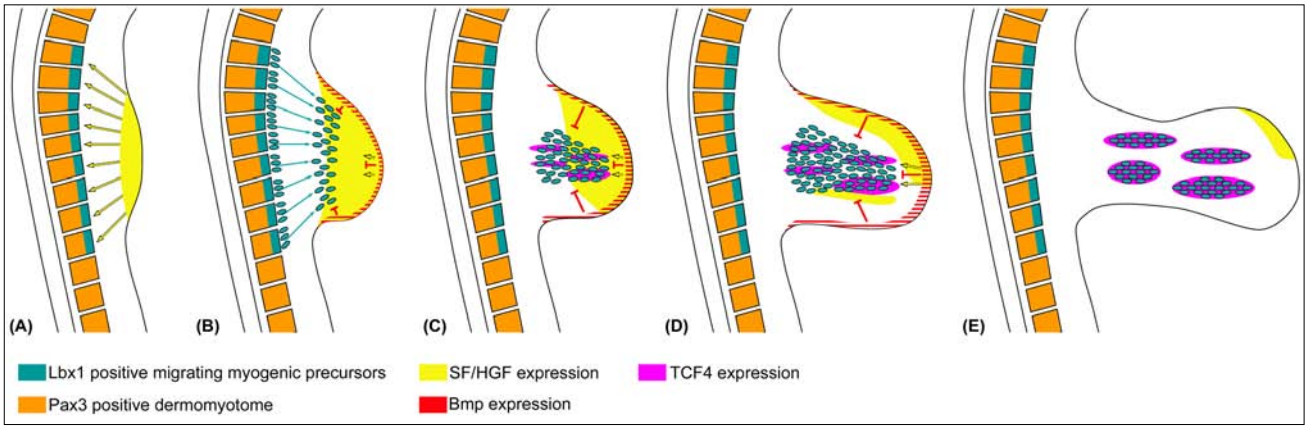

Figure 1. Scheme showing the progression of limb muscle development. (**A**) Limb outgrowth begins and with it the production of SF/HGF. (**B**) SF/HGF will bind to the c-Met receptor present in the membrane of the hypaxial Lbx1-positive muscle precursor cells and will delaminate from the epithelial dermomyotome and migrate into the limb.(**C, D**) During their migration, the Lbx1 positive muscle precursor cells will be under the influence of positive (SF/HGF) and negative (Bmps) signals that will drive them forward into the distal limb tip but without reaching the tip and neither the limb anterior nor posterior borders. The expression of TCF4 begins and will mark the future fields were muscle cells will reside. (**E**) Muscle cells adopt their final position within the limb, overlapping or coinciding with the position of TCF4 expression.

occurred; these fields are not dependent on muscle cell migration since limbs devoid of muscle cells still express this TCF4 and misexpression of TCF4 in different limb territories results in the mispositioning of myogenic precursor cells (Kardon et al., 2003). Bmps have been shown to influence these TCF4-positive mesenchymal fields (Bonafede et al., 2006)

Lbx1 expression in myogenic cells is normal in c-Met mutants, indicating that Lbx1 and c-Met are working is a spatially, but not genetically, coordinated fashion (Dietrich et al., 1998). Lbx1 knock-out mice are able to undergo the epithelial mesenchymal transition of the lateral dermomyotomal lip, but myogenic precursors are unable to migrate into the limb; the exact mechanisms of how this happens remains unsolved (Brohmann et al., 2000).

Control of the proliferation-differentiation balance

The myogenic precursor cells that have migrated into the limbs do not express any of the four myogenic regulatory factors (Myf5, Mrf4, MyoD and Myogenin) known to control myogenesis. Other transcription factors, such as Six1 and Six4, have been shown to control the expression of MRF in the limb, and genetic hierarchies have been established. The activation of all these genes occurs in the limbs, and their inactivation results in limb muscle defects, although the exact molecular mechanisms remain unclear (Mankoo et al., 1999; Grifone et al., 2005; 2007; Giordani et al., 2007; Buckingham and Vincent, 2009; Bismuth and Relaix, 2010; Carvajal and Rigby, 2010).

In order to obtain a correct muscle mass, a tight control between the proliferation and differentiation phases must be brought into play during the migratory process. It has been stated that an early cell cycle withdrawal, with the consequent loss of proliferation and start of differentiation, would result in a lower muscle mass (Amthor et al., 1999). In contrast, if differentiation is inhibited, then on a long-term basis more muscle would be obtained since the cell would continue proliferating (Amthor et al., 1999). The point where muscle cells stop proliferating is not exactly known. However, several factors influencing the proliferation-differentiation balance have been discovered (Myostatin, Follistatin, Wnt6, Shh, Bmps, SF-HGF, FGFs, Sfrp2, etc…) (Amthor et al., 1998, 1999, 2002a; 2002b; Scaal et al., 1999; Marics et al., 2002; Anakwe et al., 2003; Francis-West et al., 2003; Geetha-Loganathan et al., 2005).

Thus, there would presumably be factors that would inhibit proliferation by inducing an increase in differentiation and factors that would increase proliferation, resulting in a decrease in differentiation (Amthor et al., 1999). However, this simple model is challenged by the fact that FGFR-4 is expressed in

myogenic precursors and is essential for myogenic differentiation because its inhibition results in a down-regulation of myogenic differentiation markers such as MyoD and Myf-5 without affecting Pax3 and without affecting proliferation (Marics et al., 2002). Accordingly, the inhibition of differentiation does not automatically result in an increase in proliferation and there may be proteins involved in regulating proliferation; some of them involved in controlling differentiation and some that affect both processes.

In the limb, it has been reported that at least the dorsal ectoderm is a source of signals that maintain muscle cells undifferentiated since its removal induces the expression of MyoD, which characterises differentiating muscle cells (Amthor et al., 1998). It has been shown that the dorsal ectoderm produces Wnt-6, among others (Schubert et al., 2002, Rodríguez-Niedenführ et al., 2003). Wnt-6 overexpression not only leads to a decrease in *MyoD* expression but also rescues the effect of ectoderm removal. Interestingly, it does not affect Myf-5 expression (Geetha-Loganathan et al., 2005). It has recently been shown that Wnt-6 also controls early chondrogenesis in the chicken limb, increasing the complexity of Wnt-6 signalling during limb development and further reducing our understanding of the process (Geetha-Loganathan et al., 2010).

Other factors within the Wnt signalling cascade have been shown to change/modify cell number. These factors reside in the mesenchyme rather than in the ectoderm, indicating that, similarly to what happens in the somite, myogenesis in the limb is also controlled by all surrounding tissues (Anakwe et al., 2003).

Terminal differentiation

After migration, positioning, and the initial phases of differentiation, myogenic precursor cells must fuse and start forming the characteristic multinucleated muscle fibres. However, when and how this step takes place remains largely unknown.

The determination of fibre contractile characteristics, such as differentiation into slow or fast fibers, has received little attention *in vivo* (Bren-Mattison and Olwin, 2002; Anakwe et al., 2003; Sacks et al., 2003; Tee et al., 2009). The formation of fast and slow fibers has been shown to be influenced by both the Shh and Wnt signaling pathways (Bren-Mattison and Olwin, 2002; Anakwe et al., 2003; Francis-West et al., 2003; Sacks et al., 2003; Tee et al., 2009). Specific information regarding the control of this process in the limb musculature is scarce (Bren-Mattison and Olwin, 2002; Anakwe et al., 2003; Francis-West et al., 2003).

Myogenic precursor cells in the limb express Sfrp2 (a Wnt antagonist), which is inactivated when cells start terminal differentiation, allowing active Wnt signalling. *In vivo*, Wnt11 decreases the number of slow and increases the number of fast fibres, while overexpression of Wnt5a, similarly to Shh, has the opposite effect (Bren-Mattison and Olwin, 2002; Anakwe et al., 2003). This apparently simple balance is complicated by the fact that there are other factors that simultaneously affect fibre numbers and types (Anakwe et al., 2003), and therefore it remains to be clarified whether the observed effects are a switch from one fibre type to another or whether the factors affecting fibre type are effectively controlling the proliferation/apoptosis of a subset of muscle cells, as hinted at in some studies (Bren-Mattison and Olwin, 2002).

Future Directions

Knowledge of the control of limb muscle development has increased impressively since Bodo Christ described the somitic origin of limb muscles in 1974 (Christ et al, 1974, 1977). The inherent limitations of different developmental biology models (chick, mouse, xenopus, zebrafish, drosophila, etc…) have led to many open questions that are difficult, if not impossible, to address in an *in vivo* setting and may need the help of cell biology to shed some light on certain aspects of limb muscle development. On the other hand, many researchers have addressed issues concerning muscle development *in vitro* but its relevance in most cases in *in vivo* models remain to be elucidated. It is for these reasons that many years of hard work are still needed to gain further insight into these intriguing matters of developmental biology.

In these review I have not mentioned aspects such as functional units –i.e. innervation of muscles– etc… an issue that further hinders our knowledge of the area, and the

combination of both muscle and nerve may contribute certain scientific aspects largely unknown or overlooked to date.

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