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Invited Review

Skeletal muscle development in the mouse embryo

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Summary. In this review we discuss the recent findings concerning the mechanisms that restrict somitic cells to the skeletal muscle fate, the myogenic regulatory factors controlling skeletal muscle differentiation and specification of myogenic cell lineages, the nature of inductive signals and the role of secreted proteins in embryonic patterning of the myotome. More specifically, we review data which strongly support the hypothesis that Myf-5 plays a unique role in development of epaxial muscle, that *MyoD* plays a unique role in development of hypaxial muscles derived from migratory myogenic precursor cells, and that both genes are responsible for development of intercostal and abdominal muscles (hypaxial muscles that develop from the dermatomal epithelia). In addition, while discussing upstream and post-translational regulation of myogenic regulatory factors (MRFs), we suggest that correct formation of the myotome requires a complex cooperation of DNA binding proteins and cofactors, as well as inhibitory function of non-muscle cells of the forming somite, whose proteins would sequester and suppress the transcription of MRFs. Moreover, in the third part of our review, we discuss embryonic structures, secreted proteins and myogenic induction. However, although different signaling molecules with activity in the process of somite patterning have been identified, not many of them are found to be necessary during in vivo embryonic development. To understand their functions, generation of multiple mutants or conditional/tissue-specific mutants will be necessary.

Key words: Myogenesis, Patterning, Induction, Cell lineage, Mouse embryo

Introduction

In all vertebrates, the development of skeletal muscle occurs in a nearly related pattern (reviewed in Ordahl and LeDouarin, 1992; Wachtler and Christ, 1992; Hauschka, 1994; Christ and Ordahl, 1995; Currie and Ingham, 1998). Epithelia1 spheres named somites arise from the paraxial mesoderm in a stereotypical cranial to caudal progression on either side of the neural tube. Somites represent a source of all skeletal muscle for the embryonic body (trunk and limb muscles) and some head muscles. The remaining head muscles arise from more anterior nonsomitic paraxial and prechordal head mesoderm. Subsequently, somites become compartmentalized into a dorsal epithelia1 dermamyotome (source of dorsal dermis and myotomes) and a ventral mesenchymal sclerotome (source of axial skeleton). It is believed that medially located cells of the derma-myotome, adjacent to the neural tube, migrate laterally to form the myotome, the compartment of the somite that gives rise to the skeletal muscle (Kaehn et al., 1988; Denetclaw et al., 1997; Kahane et al., 1998).

The myogenic regulatory factors (MRFs), a group of basic helix-loop-helix (bHLH) transcription factors consisting of MyoD, myogenin, Myf-5, and MRF4, play essential regulatory functions in the skeletal-muscle developmental program. The introduction of null mutations in Myf-5, MyoD, myogenin, and MRF4 into the germline of mice has revealed the hierarchical relationships existing among the MRFs, and established that functional redundancy is a feature of the MRF regulatory network (reviewed in Megeney and Rudnicki, 1995; Rudnicki and Jaenisch, 1995). Importantly, the entire embryonic lineage that gives rise to skeletal muscle never forms in compound-mutant animals lacking both Myf-5 and MyoD, as evidenced by the absence of myoblasts and myofibers throughout development (Rudnicki et al., 1993; Kablar and M.A. Rudnicki, unpublished).

Lineage tracing experiments in avian embryos indicate that epaxial (originating in the dorsal-medial half of the somite, e.g. back muscles) and hypaxial (originating in the ventral-lateral half of the somite, e.g. limb and body wall muscles) musculature at the limb level have distinct origin (reviewed in Chevallier et al., 1977; Christ et al., 1983; Ordahl and Le Douarin 1992; Christ and Ordahl 1995; Denetclaw et al., 1997). Recent reports provide the first definitive evidence for unique roles of MyoD and Myf-5 in the emergence of myogenic lineages within the developing somite (Kablar et al.,

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1997, 1998; reviewed in Ordahl and Williams, 1998).

In this review we summarize recent findings that provide a link between MRF regulatory network and signals secreted from embryonic structures that regulate skeletal muscle fate and differentiation. For example, a number of studies have concerned the products of the Wnt, Hedgehog (Hh) and Bone morphogenetic protein (Bmp) gene families the key myogenic regulators, controlling initiation of myogenesis and fate of myoblasts. We primarily focus on the cell autonomous factors controlling skeletal muscle differentiation, on the identification of structures and molecules that induce compartmentalization of the somite into myotome and on the molecular biology of the distinct cell lineage formation within the somite.

MRFs and myogenic cell lineages

Since the discovery of MyoD in 1987, and thereafter other MRFs, such as: myogenin, *Myf-5* and MRF4, there has been remarkable progress toward resolving the molecular mechanisms controlling skeletal muscle development. Through clarification of the functions of the myogenic bHLH transcription factors, skeletal muscle development has become a paradigm for reasoning about the mechanisms of genetic redundancy, cell differentiation and cell fate specification.

The role of the four MRFs during myogenesis has been elucidated by gene targeting in mice. Null mutations in *myogenin* cause a substantial reduction in skeletal muscle tissues (Hasty et al., 1993; Nabeshima et al., 1993), probably because of a failure in differentiation of already specified cells (Ordahl and Williams, 1998). Mutation in the other three genes results in essentially normal patterning and amount of skeletal muscle tissue (Braun et al., 1992; Rudnicki et al., 1992; Zhang et al., 1995). However, mice carrying null mutations in both *MyoD* and *Myf-5* genes completely lack differentiated muscle and myoblasts (Rudnicki et al., 1993; Kablar and Rudnicki, unpublished). Taken together, these data led to the proposal that *Myf-5* and *MyoD* (primary MRFs) are required for the determination of skeletal myoblasts, while myogenin and MRF4 (secondary MRFs) act later as differentiation factors (reviewed by Megeney and Rudnicki, 1995; Rudnicki and Jaenisch, 1995). In addition, when the *Myf-5* coding region is replaced by myogenin, transgenic mice appear normal (Wang et al., 1996), but in the *MyoD* null background Myf-5$^{myg\text{-}ki/myg\text{-}ki}$ mice fail to fully rescue the muscle deficit observed in *Myf-5*$^{-/-}$:*MyoD*$^{-/-}$ embryos, suggesting that myogenin has a reduced ability to substitute for *Myf-5* (Wang and Jaenish, 1997). However, what is less clear is the role of individual genes in control of the formation of distinct cell fates or lineages within the myotome.

The examination of the expression patterns of two *MyoD-lacZ* (258/-2.5*lacZ* and MD6.0-*lacZ*) transgenes in wild-type, *Myf-5* and *MyoD* mutant embryos, followed by an immunohistochemical analysis (Kablar et al., 1997, 1998, 1999), furthered our understanding of how *Myf-5* and *MyoD* genes cooperate during skeletal muscle specification. The MD6.0-*lacZ* transgene is expressed in differentiated myocytes (Asakura et al., 1995; Kablar et al., 1997, 1998), while the 258/-2.5*lacZ* transgene (Goldhamer et al., 1995), is expressed in determined mpc following translocation (Kablar et al., 1998, 1999). A reduced ability of myogenic precursor cells (mpc) to progress through their normal developmental program, and not a defect in migration of mpc, is suggested to be the reason for the delayed onset of muscle differentiation in the branchial arches, tongue, limbs and diaphragm of *MyoD*$^{-/-}$ embryos. The mpc for intercostal and abdominal wall musculature in *MyoD*$^{-/-}$ embryos arrive on time to their normal location in the embryo, but only some of these cells undergo timely differentiation. By contrast, both the inability of mpc to timely arrive and differentiate in the absence of *Myf-5*, is suggested to be the reason for the delayed onset of back,

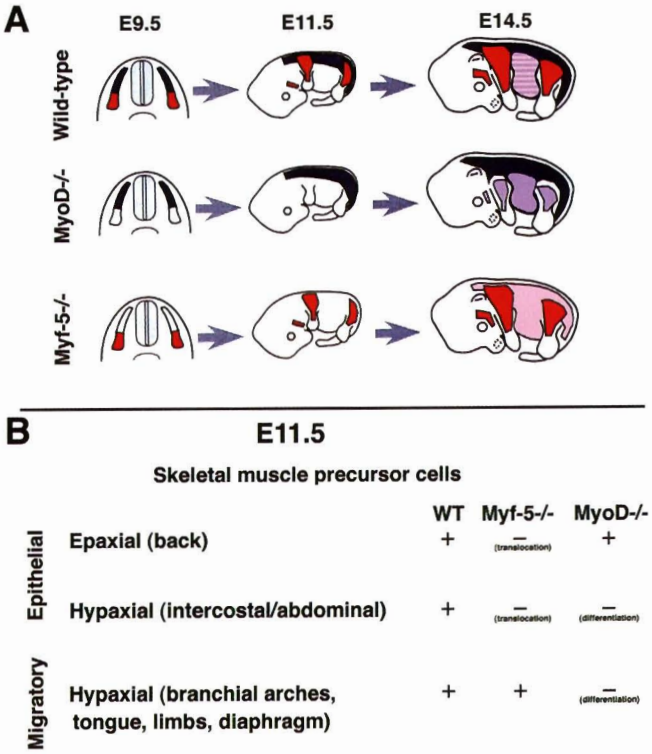

Fig. 1. Myogenic cell lineages. **A.** Examination of the expression pattern of two *MyoD-lacZ* transgenes and immunohistochemistry against MRFs and skeletal muscle proteins, reveals that the MD6.0-lacZ transgene (-5 kb enhancer of *MyoD* promoter) is expressed in differentiated myocytes, while the 258/-2.5lacZ transgene (-20 kb enhancer of *MyoD* promoter) is expressed in determined mpc following translocation. *MyoD* null embryos have 2 day delay in differentiation of all hypaxial musculature (violet in E14.5), regardless of the origin of their mpc (e.g. epithelial: intercostal and abdominal; migratory: branchial arches, tongue, limbs, diaphragm), and normal epaxial musculature (blue). *Myf-5* null embryos have 2 day delay in translocation of all mpc for epithelia-derived musculature (e.g. back, intercostal and abdominal; pink in E14.5) and normal development of musculature deriving from migratory mpc (red). **B.** Summarized data on myogenic cell lineage dependence on *Myf-5* and *MyoD*.

intercostal and abdominal wall musculature development in *Myf-5*$^{-/-}$ embryos. Tajbakhsh et al. (1996) and Kablar et al. (1999) have also shown that mpc migrate abnormally in *Myf-5*n*lac*Z knock-in mice and in 258/-2.5*lac*Z transgenic mice, respectively. The mpc expressing *lac*Z are found to coexpress cartilage and dermal markers in the absence of *Myf-5* or *Myf-5* and *MyoD*, suggesting that mpc remain multipotent. Therefore, together with the data on the targeted inactivation and the protein expression patterns, these recent observations strongly support the hypothesis that *Myf-5* plays a unique role in development of epaxial muscle, while *MyoD* plays a unique role in the development of hypaxial muscles derived from migratory mpc (Fig. 1). In addition, the development of intercostal and abdominal muscles (hypaxial muscles that develop from the dermatomal epithelia; Ordahl and Williams, 1998) appears to be dependent on both genes and, therefore, these muscles may originate from two myogenic lineages.

Upstream and post-translational regulation of MRFs in somites and limb buds

Several members of the *Pax* family of homeobox genes are expressed in distinct regions of the developing somite. Pax-3 and Pax-7 are expressed in the paraxial mesoderm and, then, in the dorsal half of somites, prior to the formation of medial and lateral domains of the dermamyotome (Goulding et al., 1991; Jostes et al., 1991). Neither of them is expressed in the myotome, but Pax-3 is expressed in the population of migratory mpc. The first indication of a role for Pax-3 in skeletal muscle development came from *sploch* mice, that lack *Pax-3* and limb muscles (Bober et al., 1994).

The induction of myogenesis is thought to be an exclusive property of MRFs. However, new evidences have emphasized the role of Pax-3 as an upstream regulator of *MyoD* in the mouse developing somite (Maroto et al., 1997; Tajbakhsh et al., 1997). By the analysis of *sploch*:*Myf-5*n*lacZ*$^{-/-}$ mice (Tajbakhsh et al., 1997), it has been shown that Pax-3 is necessary and sufficient for the induction of myogenesis. The body proper of *sploch*/*Myf-5*$^{-/-}$ embryos entirely lacks skeletal muscles and *MyoD* is not activated in the myotome. In addition, Pax-3 transfected non-muscle cells activate *MyoD* and differentiate into myoblasts (Maroto et al., 1997). Taken together, these findings suggest that either *Myf-5* or Pax-3 activity is required for the initiation of *MyoD* transcription and a consequent onset of myogenesis (Fig. 2). Indeed, the myotomal expression of *MyoD* in *sploch* mice indicates the existence of Pax-3-independent pathway of *MyoD* activation and necessity for *Myf-5*-dependent regulatory pathway of *MyoD* expression. To better understand how direct is the relationship between Pax-3 and MyoD, examination of *sploch*:*MyoD*$^{-/-}$ embryos would elucidate whether Myf-5-dependent myogenesis is completely independent of Pax3 and MyoD.

The *sploch* phenotype in the limb buds is similar to that of mice lacking tyrosine kinase receptor c-met, which binds scatter factor, the migratory peptide growth factor (Bladt et al., 1995). The *sploch* mice lack the expression of c-met, suggesting for *c-met* to be a target of Pax-3 and a reason for inability of *sploch* migratory mpc to arrive into the limbs (Yang et al., 1996). To further our understanding about the role of Pax-3 in specification and migration of mpc a *Myf-5*$^{-/-}$:*c-met*$^{-/-}$ phenotype should be compared to the *sploch*:*Myf-5*$^{-/-}$ phenotype.

Limb bud mpc migrate during early embryogenesis from somites to limb buds where migration stops and differentiation occurs (Fig. 2). In addition to *Pax-3* and *c-met*, that mark these migratory mesenchymal mpc, there is a third population of mpc, intercalated between the epaxial and hypaxial somitic bud, that can be specifically marked by *Engrailed-1 (En-1)* and *Drosophila single minded (Sim-1)* homologue (Loomis et al., 1996; Tajbakhsh and Spörle, 1998). Analysis of *sploch* embryos have demonstrated that Pax-3 is not necessary for specification of these cells (Tajbakhsh and Spörle, 1998), but the proliferation of mpc in the limb is linked to Pax-3 expression (Amthor et al., 1998). *Drosophila ladybird (lbx-1)* homologue (Mennerich et al., 1998) and a transcription factor Sp-1-related gene 26M15 (Tajbakhsh and Sprole, 1998) are two new

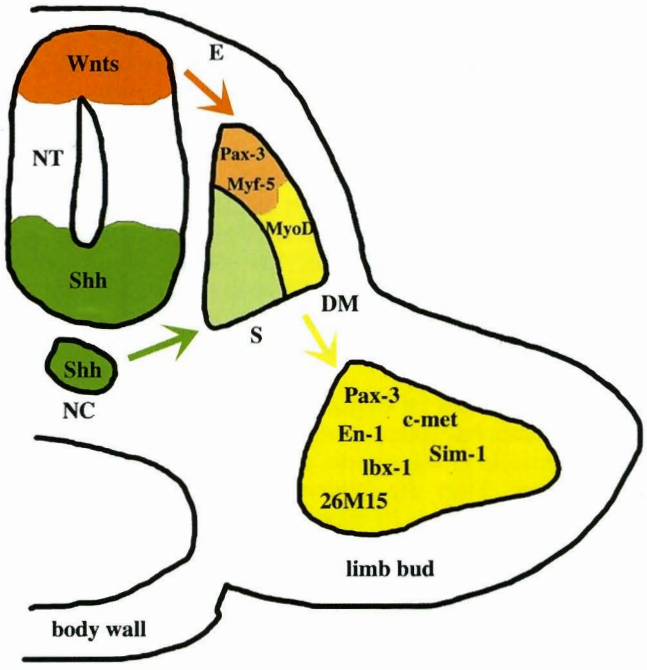

Fig. 2. A model for molecular interactions during myogenesis. Shh and Wnts, produced by the neural tube (NT) and notochord (NC), induce Pax-3 and *Myf-5* in the somites (DM: dermamyotome; S: scelrotome). Either of them can activate the initiation of *MyoD* transcription and myogenesis. Surface ectoderm (E) is also capable of inducing *Myf-5* and *MyoD*. In addition, Pax-3 regulates the expression of c-met, necessary for migratory ability of myogenic precursor cells, that also express: En-1, Sim-1, lbx-1 and 26M15.

markers for limb bud mpc. lbx-1 is present in the trunk of *c-met* null embryos, but absent in *sploch* mice (Tajbakhsh and Sprole, 1998). Limb buds of *sploch* embryos are also devoid of *lbx-1* transcripts, while a low level of c-met is still detectable (Mennerich et al., 1998). The presence of c-met-expressing cells in *sploch* limb buds suggests that Pax-3 is not the only molecule controlling migration of mpc into the limb. It is postulated that Pax-3 is necessary for *lbx-1* expression to occur in somites, but in limb buds, some additional and unknown signals would be needed to initiate lbx-1 expression in mpc (Mennercih et al., 1998).

Recent biochemical and genetic analysis have demonstrated that members of the myocyte enhancer factor-2 (MEF-2) family of MADS (MCM1, agamous, deficiens, serum response factor)-box transcription factors play multiple roles in skeletal, cardiac and smooth myogenesis and morphogenesis (reviewed by Olson et al., 1995; Black and Olson, 1998). MEF-2 proteins act in a combinatorial pattern through protein-protein interactions with other transcription factors to control specific sets of target genes. They are also found to act in conjunction with the bHLH transcription factors to direct muscle-specific gene expression (Kaushal et al., 1994; Molkentin et al., 1995, 1996), although the precise character of the action of these genes in provoking myogenesis remains controversial (reviewed by Ludolph and Konieczny, 1995). Transfection experiments have indicated that MEF-2 proteins bind cooperatively *MyoD* to synergistically activate E-box and MEF-2-site containing promoters. During somitogenesis, *MEF-2* gene expression follows *myogenin* expression. Moreover, MEF-2D is expressed in C2 myoblasts, while other three MEF-2 proteins (MEF-2A, B and C) are not expressed until after differentiation. Taken together, it appears that MEF-2 proteins act as differentiation factors during skeletal myogenesis. In addition, the ubiquitous E proteins, that also contain a bHLH domain, are found to interact with MRFs, as well. They are thought to be the cofactors of the myogenic transcription factors, probably in order to correctly initiate transcription of muscle-specific genes.

Another class of bHLH proteins, not expressed in the myotome, but found to regulate the correct activation of myogenesis in the somite, consists of: Id, Twist and I-mf proteins. They are expressed at a high level in the cells of sclerotome. In cultured muscle cells, they are found to inhibit myogenesis. Id protein has been shown to inhibit *MyoD* function by competing with *MyoD* for dimerisation with its bHLH cofactors, the E proteins, preventing creation of the active bHLH-E protein heterodimers (Jen et al., 1992). Twist has been shown to *in vitro* inhibit myogenesis by both its ability to sequester E proteins and by its ability to directly prevent transactivation via MEF-2 (Hebrok et al., 1994; Spicer et al., 1996). Twist sclerotomal localization in the embryo and its in vitro functions suggest that Twist inhibits inappropriate myogeneis in the sclerotomal compartment of the developing somite. I-mf has been shown to operate by binding the MRFs and anchoring them in the cytoplasm, therefore, masking their nuclear signalling. I-mf can also directly interfere with the process of binding the nuclear targets of the MRFs (Chen et al., 1996). Taken together, it appears that correct formation of the myotome requires a complex cooperation of DNA binding proteins and cofactors, as well as inhibitory function of non-muscle cells of the forming somite, whose proteins would sequester and suppress the transcription of MRFs.

Embryonic structures, secreted proteins and myogenic induction

As a consequence of morphogenetic movements during gastrulation, the anterior-most portion of the unsegmented paraxial mesoderm is formed. The environment for somitogenesis to take place is now established and, over several days, paraxial mesoderm segments to transient epithelial spheres or somites. There is very little information on the molecular mechanism controlling segmentation and boundary formation in vertebrates. For instance, a zebrafish homologue of the *Drosophila* pair-rule gene hairy (her-1) is suggested to play a role in the segmentation of paraxial mesoderm, because of its appropriate expression pattern (Muller et al., 1996). Moreover, gene targeting has established a role in segmentation and somite epithelialization for a mouse homologue of a *Drosophila* gene *Delta (Delta-like-1* or *Dll-1*) (Hrabe de Angelis et al., 1997), whereas *Notch-1* (*Delta-1* is a ligand of *Notch*) null embryos have a less severe phenotype (Conlon et al., 1995). Both, the analysis of *Dll-1* embryos (Hrabe de Angelis et al., 1997) and the analysis of embryos mutant in the bHLH transcription factor *paraxis* (Burgess et al., 1996), suggest that the epithelialization of somites is not required for specification of the dermamyotome and sclerotome (reviewed in Yamaguchi, 1997).

Therefore, the patterning of the somite anterior-posterior axis differs from the patterning of its dorsal-ventral and medial-lateral axes, where the later two appear to be also dependent on the environmental signals from the adjacent embryonic tissues (reviewed by Tajbakhsh and Cossu, 1997; Yamaguchi, 1997; Currie and Ingham, 1998; Tajbakhsh and Spörle, 1998). It is now accepted that presomitic and somitic cells are multipotent and that their fates are determined by association of signals from axial (e.g. neural tube and notochord) and lateral (e.g. surface ectoderm and lateral mesoderm) structures that act along dorsal-ventral and medial-lateral axes.

The nature and source of different environmental influences is the subject of intensive investigations (Tajbakhsh and Spörle, 1998). It is proposed that axial structures stimulate the process of epaxial (back) skeletal muscle differentiation and not the differentiation of hypaxial (e.g. limb) muscles (Teillet and Le Douarin, 1983; Rong et al., 1992). Lateral somitic lineage specification results from signals emanating from lateral

plate mesoderm (Pourquie et al., 1995, 1996; Cossu et al., 1996) and dorsal ectoderm (Kenny-Mobbs and Thorogod, 1987; Fan and Tesier-Lavigne, 1994; Cossu et al., 1996). However, a number of recent *in vitro* studies have generated contrary results concerning the precise source of the signal(s) (Buffinger and Stockdale, 1994, 1995; Munsterberg and Lassar, 1995; Stern and Hauschka, 1995; Pownall et al., 1996).

The current view (Fig. 3) suggests that the dorsal neural tube and the overlying non-neural ectoderm are sources of signaling molecules belonging to the family of Wnt secreted proteins, whereas the notochord and the ventral neural tube are sources of the family of Hedgehog secreted proteins (Johnson et al., 1994; reviewed by Bumcrot and McMahon, 1995; Munsterberg et al., 1995; reviewed by Currie and Ingham, 1998). They apparently positively regulate the onset of myogenesis and the induction of the myotome. For instance, when the dorsal neural tube is infected with a retrovirus containing Sonic Hedgehog (Shh), somitic tissues express some myogenic molecular markers (Johnson et al., 1994). *Shh* null mouse embryos (Chiang et al., 1996) have reduced expression of *Myf-5* (medial myotome) and unaffected expression of *MyoD* (lateral myotome), suggesting that there is no absolute requirement for Shh in the induction of myogenesis. These results also reinforces the notion that axial signals (Shh) specify medial, but not lateral myotomal fates. It has also been shown that several members of the Wnt family of secreted proteins associate with Shh to induce myogenesis in somitic explants (Munsterberg et al., 1995). Lassar and Munsterberg (1996) explain that presegmental plate mesoderm requires both Shh and Wnt signals to activate *MyoD* expression, while more mature somites require only Wnt signaling, suggesting that both the dorsal neural tube and the notochord are required for high level MRF activation (Pownall et al., 1996). Moreover, the action of the neural tube in activating *Myf-5* can be replaced by cells expressing Wnt-1, while *MyoD* activation by dorsal ectoderm can be replaced by cells expressing Wnt-7a (Tajbakhsh et al., 1998). Taken together, these results suggest that activation of myogenesis by different Wnt molecules is executed through different pathways that regulate spatio-temporal commitment of mpc, as supported by recent findings that *Myf-5* and *MyoD* null embryos have epaxial and hypaxial muscle deficits, respectively (Kablar et al., 1997).

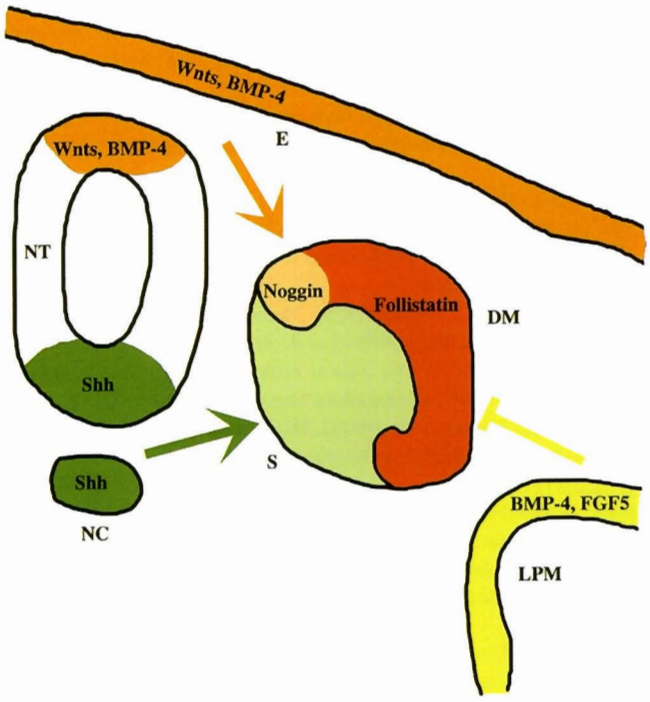

Fig. 3. Embryonic structures and myogenesis. The current view suggests that the dorsal neural tube (NT) and the overlying non-neural ectoderm (E) are sources of signaling molecules belonging to the family of Wnt secreted proteins and BMP-4, whereas the notochord (NC) and the ventral neural tube (green) are sources of the Shh. They positively regulate the onset of myogenesis and the induction of the myotome. By contrast, the lateral plate mesoderm (LPM) produces BMP-4 and FGF5, negatively regulating muscle terminal differentiation in the lateral part of the myotome lineage. Response to the BMP-4 signal may be mediated by its binding proteins noggin and follistatin (DM: dermamyotome; S: sclerotome).

By contrast, the lateral plate mesoderm produces a diffusible signal, most likely BMP-4 (a member of the transforming growth factor family, TGF), that negatively regulates muscle terminal differentiation in the lateral part of the myotome (Pourquie et al., 1996; Tonegawa et al., 1997) and possibly controls the specification of hypaxial somitic lineage (reviewed by Currie and Ingham, 1998; Dietrich et al., 1998). In addition, low concentrations of BMP-2, BMP-4 and BMP-7 maintain proliferative capacity of *Pax-3*-expressing population of mpc in the limb bud, while high BMP concentrations induce cell death (Amthor et al., 1998). Moreover, Shh upregulates some BMPs and delay muscle differentiation, suggesting that skeletal muscle development requires skeletal muscle differentiation to be delayed (Amthor et al., 1998). The maintenance of committed (and *Pax-3*-expressing) mpc in an undifferentiated state allows migration to the limb or body wall, having as a consequence a delayed muscle differentiation in the limbs compared to the trunk (Buckingham, 1992).

The existence of gradients of secreted factors across the dermamyotome in order to specify cell fates is proposed, but the fact that BMP-4 is also expressed in the dorsal neural tube, compromise the model. Alternativelly, response to the BMP-4 signal may be mediated by its binding proteins noggin and follistatin. Noggin, a BMP antagonist, is expressed within the paraxial mesoderm and neural tube, followed by a restriction of its expression only to dorsomedial lip of the dermamyotome (Connolly et al., 1997; Hirsinger et al., 1997; Reshef et al., 1998). Noggin is found to upregulate molecular markers of medial and

downregulate markers of lateral somite differentiation, possibly counteracting with BMP-4 and Wnt-1 (lateralizing signals) in the dorsal neural tube. BMPs and noggin control the timing and pattern of MRF expression, since it is found that BMP inhibits the expression of primary MRFs in *Pax-3*-expressing cells, Wnt-1 induces noggin expression in the medial somite and the ectopic noggin expression induces formation of a lateral myotome (Reshef et al., 1998).

Another molecule that is suggested to have a role in mediating BMP activity is follistatin. *follistatin* null embryos do not have early patterning defects, but later in development their skeletal muscle mass is reduced, suggesting that follistatin have a role in morphogenesis of the myotome (Matzuk et al., 1995). The expression patterns of *follistatin* and *follistatin related* genes have led to a proposal that *follistatin* antagonizes BMP-4-dependent muscle fate repression (Amthor et al., 1996). It is tempting to speculate that *follistatin* regulate BMP-4 activity, providing a balance between proliferative and differentiating states of mpc.

Taken together, various signaling molecules with activity in the process of somite patterning have been identified, but not many of them are found to be necessary during *in vivo* embryonic development. To understand their functions, generation of multiple mutants in case of redundancy, or conditional/tissue-specific mutants in case of early lethality, will be necessary.

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