Differential activation of Myf5 and MyoD by different Wnts in explants of mouse paraxial mesoderm and the later activation of myogenesis in the absence of Myf5

S. Tajbakhsh 1, U. Borello 2, E. Vivarelli 2, R. Kelly 1, J. Papkoff 3, D. Duprez 4, M. Buckingham 1 and G. Cossu 2,*

1 Département de Biologie Moléculaire CNRS URA 1947, Institut Pasteur, 25 Rue du Dr Roux, 75724 Paris Cedex 15, France
2 Istituto Pasteur-Cenci Bolognetti, Dipartimento di Istologia ed Embriologia Medica. Università di Roma “La Sapienza”, Via A. Scarpa 14, 00161 Rome, Italy
3 ARIAD Pharmaceuticals Inc., 26 Landsdowne St, Cambridge, MA 02139, USA
4 Institute d'Embryologie Cellulaire et Moléculaire du CNRS et du College de France, 49bis Avenue de la Belle Gabrielle, 94736 Nogent Sur Marne Cedex, France
*Author for correspondence (e-mail: cossu@axrma.uniroma1.it)

Accepted 28 July; published on WWW 30 September 1998

SUMMARY

Activation of myogenesis in newly formed somites is dependent upon signals derived from neighboring tissues, namely axial structures (neural tube and notochord) and dorsal ectoderm. In explants of paraxial mesoderm from mouse embryos, axial structures preferentially activate myogenesis through a Myf5-dependent pathway and dorsal ectoderm preferentially through a MyoD-dependent pathway.

Here we report that cells expressing Wnt1 will preferentially activate Myf5 while cells expressing Wnt7a will preferentially activate MyoD. Wnt1 is expressed in the dorsal neural tube and Wnt7a in dorsal ectoderm in the early embryo, therefore both can potentially act in vivo to activate Myf5 and MyoD, respectively. Wnt4, Wnt5a and Wnt6 exert an intermediate effect activating both Myf5 and MyoD equivalently in paraxial mesoderm. Sonic Hedgehog synergises with both Wnt1 and Wnt7a in explants from E8.5 paraxial mesoderm but not in explants from E9.5 embryos.

Signaling through different myogenic pathways may explain the rescue of muscle formation in Myf5 null embryos, which do not form an early myotome but later develop both epaxial and hypaxial musculature. Explants of unsegmented paraxial mesoderm contain myogenic precursors capable of expressing MyoD in response to signaling from a neural tube isolated from E10.5 embryos, the developmental stage when MyoD is present throughout the embryo. Myogenic cells cannot activate MyoD in response to signaling from a less mature neural tube.

Together these data suggest that different Wnt molecules can activate myogenesis through different pathways such that commitment of myogenic precursors is precisely regulated in space and time to achieve the correct pattern of skeletal muscle development.

Key words: Myogenic induction, MyoD, Myf5, Wnt, Sonic Hedgehog

INTRODUCTION

Skeletal muscles of the body are derived from segmental mesodermal structures called somites. Shortly after their formation, somites give rise dorsally to the epithelial dermomyotome and ventrally to the mesenchymal sclerotome. Myotome formation is initiated as cells from the epaxial dermomyotome lip, adjacent to the neural tube, become positioned underneath the epithelium (Denetclaw et al., 1997; see also Cossu et al., 1996a). These early myotomal cells are postmitotic and constitute the first differentiated skeletal muscle in the embryo. The epaxial myotome later contributes to deep back muscles. Cells from the lateral domain of the dermomyotome will form the hypaxial component of the myotome as well as other muscles such as those of the body wall and limb (see Christ and Ordahl, 1995; Tajbakhsh and Cossu, 1997). It has been shown that cells in the medial and lateral halves of the dermomyotome constitute separate myogenic domains, although their fate remains plastic as shown by medial/lateral switch-grafts of newly formed somites (Ordahl and Le Douarin, 1992).

In higher vertebrates, myogenic differentiation of somites is known to be dependent upon signals provided by the adjacent structures. Experiments performed with explants of chick embryos suggest that signals from the notochord (ventral) and the dorsal neural tube co-operate to activate myogenesis (reviewed in Cossu et al., 1996a). Surgical extirpation of the axial structures (neural tube and notochord), however, compromises the formation of epaxial but not limb and body wall muscles (Rong et al., 1992; see Christ and Ordahl, 1995).
In keeping with this observation, we and others observed that myogenic differentiation of cells derived from the hypaxial domain of the dermomyotome depends upon the presence of adjacent dorsal ectoderm (Kenny-Mobbs and Thorogood, 1987; Fan and Tessier-Lavigne, 1994, Cossu et al., 1996b). In explants of unsegmented paraxial mesoderm, the neural tube induces myogenesis by activating Myf5 first, while the dorsal ectoderm leads to the preferential activation of MyoD (Cossu et al., 1996b).

In explants from avian embryos, the induction of myogenesis by axial structures can be replaced by the combinatorial activity of Sonic Hedgehog (SHH) and certain members of the Wnt family of signaling molecules (Münsterberg et al., 1995; Stern et al., 1995). It was suggested that SHH may induce competence for subsequent activation of myogenic genes in response to Wnt1, Wnt3 and Wnt4 but not Wnt7a or Wnt7b. SHH, however, appears to be only transiently required and at later developmental stages is no longer necessary (Münsterberg et al., 1995). In ovo experiments have underlined the central role of SHH in initiating the myogenic program (Borycki et al., 1998).

In the mouse embryo, Myf5 is the first to be activated in the epaxial domain of newly formed somites at E8 following a rostrocaudal gradient. MyoD expression, however, appears about 2 days later in the epaxial myotome (see Buckingham, 1992), while in the hypaxial myotome, it is expressed from about E9.75 in the interlimb somites (Tajbakhsh et al., 1997). In contrast, in avians, both Myf5 and MyoD are activated in the epaxial domain shortly after somites form. Since the onset of MyoD and Myf5 expression is spatially and temporally regulated in mouse embryos, we investigated whether the differential activation of Myf5 and MyoD may be promoted by different members of the Wnt family, differentially expressed in neural tube and dorsal ectoderm (Parr et al., 1993; Parr and McMahon, 1995). Here we show that the action of the neural tube in activating Myf5 can be replaced by cells expressing Wnt1 and, to a lesser extent, Wnt4 while MyoD activation by dorsal ectoderm can be replaced by Wnt7a-expressing cells. Consistent with this differential expression pattern, Myf5 null embryos have predominantly epaxial muscle defects whereas MyoD null embryos have predominantly hypaxial muscle defects (Kablar et al., 1997; S. T. and M. B., unpublished results).

We also investigated whether a differential response of Myf5 and MyoD to different signals (Wnts and/or other unidentified molecules) may explain the later rescue of epaxial muscles in Myf5 null embryos. These embryos do not form epaxial myotome initially, since this depends on the medial activation of Myf5. If epaxial myotome formation were exclusively Myf5-dependent, the phenotype of Myf5 null embryos would be similar to that of embryos whose neural tube has been extirpated. However, these latter embryos lack epaxial muscles (Rong et al., 1992) whereas in Myf5 null embryos epaxial muscles do form at later stages (Braun et al., 1992; Tajbakhsh et al., 1996a). We show that the older neural tube will induce MyoD in epaxial muscle precursors, thus explaining this discrepancy.

**MATERIALS AND METHODS**

**Mouse lines**

The Myf5a2 allele was created by inserting nlacZ in frame into the first exon of the Myf5 gene such that the expression of this reporter gene is under the transcriptional and translational control of the endogenous Myf5 locus (Tajbakhsh et al., 1996a). Heterozygous males were crossed with CD1 outbred female mice. To provide homozygous embryos, heterozygous mice were mated and the pregnant female was killed at embryonic day (E) 9.5.

The MLC3F-nlacZ construct contains 2 kb of the mouse fast myosin light chain 3 (MLC3F) promoter together with a 3' muscle-specific enhancer, regulating expression of an nlacZ-SV40 poly(A) sequence. In two independent transgenic lines, the nlacZ reporter gene is strongly expressed in skeletal muscle from E9 (Kelly et al., 1995). Hetero- or homozygous transgenic mice were crossed with CD1 outbred female mice.

**Cell and embryonic explant cultures**

QT6 fibroblasts expressing chick Sonic Hedgehog (SHH) and AtT20 cells expressing various Wnts have been previously described (Duprez et al., 1998; Smolich et al., 1993).

Embryos were dated taking day E0.5 as the morning of the vaginal plug. For most experiments, embryos at E9.5 (20 to 24 somites) were isolated in PBS. The cranial myotomes of the Myf5-nlacZ embryos, were stained for β-galactosidase activity (β-gal) and homozygous, heterozygous and wild-type embryos were pooled separately. The typical appearance of cranial somites of Myf5 null embryos made it possible to easily distinguish them from heterozygous embryos (Tajbakhsh et al., 1996a).

The tissues were then digested with 0.25% pancreatin-0.1% trypsin for 5 minutes at 4°C. After the enzymatic digestion, the various tissues were mechanically separated according to the experimental scheme and then cultured in complete medium (see below). In experiments where reaggregation of different tissues was required, the tissue fragments were seeded together (under the dissecting microscope) on a layer of 10T1/2 cells, to which they adhered within 10 minutes, and the dishes were then carefully transferred to the incubator. Preliminary experiments had shown that 10T1/2 cells did not alter the extent of myogenic differentiation under these conditions (Cossu et al., 1995). Also 10T1/2 cells do not undergo myogenesis or MyoD activation upon co-culture with neural tube or Wnt-expressing cells (Cossu et al., 1996b and unpublished observations). Unless otherwise indicated, unsegmented paraxial mesoderm refers to paraxial mesoderm alone with no associated structures.

In one set of experiments, the UPM from Myf5a2+/- and Myf5a2-/- embryos was cut in medial and lateral halves. The medial half was cultured with its adjacent half neural tube, without dorsal ectoderm and notochord; the lateral half with its own dorsal ectoderm and without lateral mesoderm. The intact contralateral UPM served as a control and was cultured with adjacent structures (half neural tube, notochord and dorsal ectoderm). The explants were grown in culture for 5 days and assayed for the expression of MyHC.

All cultures were grown in RPMI medium (GIBCO) supplemented with 10% fetal calf serum (Flow), 300 μM β-mercaptoethanol and 50 μg/ml gentamycin. At the times indicated, cultures were fixed and stained for β-galactosidase activity and/or incubated with different antibodies (Tajbakhsh et al., 1994).

**Dil injection**

A 0.5% solution of DiI (1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate, Molecular Probe) in absolute ethanol was diluted just before use in 0.3 M sucrose to a final concentration of 0.05% and injected with an electrically pulled glass capillary into a single site underneath the ectoderm at the level of the UPM of E9.5 embryos. After the injection, the embryos were cultured as described (Serbedzija et al., 1990) at 37°C for 24 hours in DMEM supplemented with 50% rat serum in a slowly rotating 15 ml culture tube, gassed with 10% oxygen and 5% carbon dioxide in nitrogen. After the culture, the embryos were fixed in 4% paraformaldehyde, cryostat-sectioned and observed using fluorescence microscopy.
Differential activation of Myf5 and MyoD by different Wnts

Immunocytochemistry

Immunocytochemistry on cultured cells was carried out as described (Cusella-De Angelis et al., 1994; Tajbakhsh et al., 1994) using the following antibodies:

1. anti-β-galactosidase monoclonal antibody (from Sigma);
2. MF20 anti-sarcomeric myosin monoclonal antibody (Bader et al., 1982);
3. a rabbit polyclonal anti-MyoD antibody (Koishi et al., 1995);
4. a rabbit polyclonal anti-β-galactosidase antibody (Tajbakhsh et al., 1994);
5. a rabbit polyclonal anti-sarcomeric myosin antibody (Cusella De Angelis et al., 1994).

All polyclonal antibodies and the anti-β-galactosidase monoclonal antibody were diluted 1:100 before use; other monoclonal antibodies were used as undiluted supernatant.

Whole-mount in situ hybridization

Embryos were prepared for whole-mount in situ hybridization as previously described (Tajbakhsh et al., 1997) with some modifications to detect dorsal ectoderm expression of Wnt7a. Proteinase K (5 μg/ml) treatment was carried out for 2, 4, or 8 minutes and post-antibody washes in MABT were for 3-24 hours. The Wnt1 and Wnt7a antisense probes used in this study have been described (Smolich et al., 1993). Photographs of whole-mount stained embryos were taken with an Olympus SZH10 stereomicroscope.

RESULTS

Different Wnt molecules preferentially activate medial or lateral myogenic pathways

As a preliminary experiment, we co-cultured unsegmented paraxial mesoderm (UPM) from E9.5 MLC3F-nlacZ embryos with or without notochord from wild-type siblings and with either control AtT20 pituitary cells or AtT20 cells expressing various Wnts. The UPM was isolated from mice with a MLC3F-nlacZ transgene, the expression of which reveals differentiated muscle cells (Kelly et al., 1995). Control AtT20 cells or notochord did not activate myogenesis while control AtT20 cells together with notochord induced modest differentiation; AtT20 cells expressing Wnt1, Wnt4, Wnt5a, Wnt6 and Wnt7a all induced robust differentiation independent of the presence of notochord (data not shown).

Since different Wnts are expressed at different developmental stages in the neural tube and in the dorsal ectoderm (Parr et al., 1993), we asked whether the specific activation of MyoD or Myf5 may be induced by specific Wnts. To examine this, we co-cultured the UPM from Myf5a2+/- embryos with AtT20 cells expressing various Wnts. In these mice, the nlacZ gene is targeted into the Myf5 locus and thus allows easy monitoring of cells that have activated the Myf5 gene. The cultures were double stained for the expression of MyoD or Myf5 may be induced by specific Wnts. To examine this, we co-cultured the UPM from Myf5a2+/- embryos with AtT20 cells expressing various Wnts. In these mice, the nlacZ gene is targeted into the Myf5 locus and thus allows easy monitoring of cells that have activated the Myf5 gene. The cultures were double stained for the expression of MyoD and β-galactosidase (which identifies cells expressing Myf5) after 1, 2 and 3 days in vitro. Fig. 1 shows representative fields of cells double stained for MyoD and β-galactosidase after 2 days of co-culture with AtT20 expressing Wnt1 (Fig. 1A,B), Wnt4 (Fig. 1C,D), Wnt6 (Fig. 1E,F) and Wnt7a (Fig. 1G,H). The presence of Wnt1 results predominantly in Myf5 expression, Wnt4 and Wnt6 lead to the expression of both genes, and Wnt7a results predominantly in MyoD expression.

As observed previously (Cossu et al., 1996b), after 3 days of culture most myogenic cells express both genes with variable intensity (data not shown). Quantitative analysis of these data is reported in Fig. 2. Furthermore, since explants were completely surrounded by Wnt-expressing cells, we could observe whether or not activation of Myf5 or MyoD would occur on both sides of the UPM. Fig. 3 shows that explants that were surrounded by cells expressing Wnt7a, did express MyoD peripheral to the center of the explant. A similar result was obtained with Myf5 in explants surrounded by Wnt1-expressing cells (data not shown).

To test whether Wnt7a would co-operate with SHH in activating myogenesis in UPM from younger embryos (as previously shown for Wnt1 and Wnt3 in avian explants; Münsterberg et al., 1995), we co-cultured UPM from E8.5 (6-10 somites) MLC3F-nlacZ embryos with AtT20 cells expressing Wnt7a (or Wnt1 as a positive control), with QT6
cells expressing SHH, or with both cell types together. Fig. 4 shows that the UPM cultured with SHH-expressing cells alone did not contain any β-galactosidase-positive cells, UPM cultured with either Wnt7a or Wnt1-expressing cells contained a few β-galactosidase-positive cells. However, the UPM cultured with cells expressing Wnt7a or Wnt1 together with SHH-expressing cells contained many more β-galactosidase-positive cells. Thus, at early developmental stages, Wnt7a as well as Wnt1 appear to co-operate with SHH to induce myogenesis.

Expression of Wnt1 and Wnt7a in vivo
To verify that the various Wnts are expressed at the right time and place to play an inductive role in vivo, we performed whole-mount in situ hybridization with probes specific for various Wnt transcripts. In agreement with previous reports (Parr et al., 1993), we observe strong expression of Wnt1 in the neural tube (Fig. 5A), adjacent to the dorsomedial domain of newly formed somites where Myf5 is first activated, starting from E8. (Ott et al., 1991). We show expression of Wnt7a in the dorsal ectoderm (Fig. 5B) overlying the unsegmented paraxial mesoderm and newly formed somites. Interestingly, Wnt7a is expressed in dorsal ectoderm overlying the lateral mesoderm and lateral edge of somites (Fig. 5C; E9.5, 22 somites); it is indeed in this lateral somitic domain that MyoD is first activated in vivo, but only at a slightly later stage (E9.75; about 27 somites). This is probably due to an inhibition mediated by the lateral mesoderm (Pourquié et al., 1996, Cossu et al., 1996b).

The appearance of epaxial muscles in Myf5 null embryos and signaling from the older neural tube
As shown above, different Wnts can provide differential signaling potentially leading to the activation of epaxial or hypaxial myogenic pathways. We further investigated this phenomenon in the context of epaxial muscle formation in Myf5 null embryos. Myotome is missing in these embryos until the period that corresponds to MyoD activation in the somite. Subsequently epaxial as well hypaxial muscles are present. We therefore examined MyoD activation in Myf5 null embryos.

Cultures of cells from somites and axial structures of Myf5a2–/– embryos revealed that myogenic precursors capable of activating the Myf5-nlacZ alleles, are already present at the onset of somitogenesis. In these mutant embryos, however, these muscle progenitors fail to differentiate initially in vitro (data not shown), as observed in vivo (Tajbakhsh et al., 1996a). Rescue of epaxial musculature in Myf5a2–/– embryos may depend either upon later MyoD activation in epaxial myogenic precursors or migration of MyoD-dependent, myogenic precursors from the lateral domain of the somite to invade and colonize the medial domain. To investigate the latter possibility, we labeled with DiI the lateral domain of UPM in embryos at 22-24 somites and then cultured these embryos for 24 hours in vitro. These conditions are similar to those used to observe migration of neural crest cells (Serbedzija et al., 1990). However, as shown in Fig. 6, no labeled cells from the lateral domain of paraxial mesoderm could be found in the medial domain, adjacent to the neural tube. We then dissected the
medial and lateral halves of UPM from Myf5a2+/− and Myf5a2−/− embryos, with their adjacent inducing structures (i.e. neural tube/notochord and dorsal ectoderm) respectively. The explants were grown in culture for 5 days and assayed for the expression of MyHC. As a control, we cultured the contralateral UPM of each experimental embryo as an intact explant with its own half neural tube and dorsal ectoderm. The results of this series of experiments, described in Fig. 7, indicated that myogenesis occurred in explants from the medial half of UPM from Myf5a2−/− embryos. This is reduced to about 30% compared to cultures from Myf5a2+/− embryos. We suggest that, during the 5 day culture period, the neural tube has matured to a point where MyoD-dependent myogenesis is now activated (see below). As expected, myogenesis also occurred in the lateral halves of cultures from both Myf5a2+/− and Myf5a2−/− embryos, although this was also reduced in the latter (see Discussion). Finally the results indicated that, in all samples, the two separate halves contained a number of differentiated cells roughly comparable to the culture from the contralateral intact UPM. In a separate experiment, the medial third of the UPM was dissected (with the neural tube) from the lateral two thirds (with dorsal ectoderm), to minimize the possibility of contamination by cells located more laterally in the dermomyotome. In this case also, myogenesis occurred in medial explants from Myf5a2−/− embryos (data not shown).

We then tested whether MyoD-dependent muscle progenitors that can respond to the neural tube from embryos at a later developmental stage, are present in the UPM. To do this, we separated the UPM of Myf5a2+/− embryos from all adjacent structures and then recombined it in vitro with SHH. UPM from E9.5 or E10.5 wild-type embryos. After 1, 2 or 3 days of culture, the samples were double stained for the presence of MyoD and β-galactosidase. Fig. 8 shows that, as previously reported (Cossu et al., 1996b), UPM incubated with the neural tube from E9.5 embryos activated Myf5 first; in contrast UPM with the neural tube from E10.5 embryos activated predominantly MyoD. After 3 days of culture, the majority of myogenic cells expressed both proteins, although to a variable extent. These findings show that myogenic precursors already present in the UPM at E9.5 can be activated by signals from a E10.5 neural tube through a Myf5-independent, MyoD-dependent pathway.

**DISCUSSION**

**Different Wnt signals and different myogenic pathways**

The data presented here provides the first evidence that two different members of the Wnt family can activate expression of two different determination genes in mammalian embryonic cells. When unsegmented paraxial mesoderm was co-cultured with AtT20 cells expressing Wnt1, preferential induction of Myf5 was observed. In contrast AtT20 cells expressing Wnt7a preferentially activated MyoD in such explants. We confirm that Wnt1 is expressed in the dorsal neural tube (Parr et al., 1993) and show that Wnt7a transcripts are detected in the dorsal ectoderm overlaying the UPM and the lateral edge of newly formed somites.

Fig. 4. Co-operative effect of SHH with Wnt7a and Wnt1. UPM from MLC3f-nlacZ E8.5 (6-10 somites) embryos was co-cultured for 2 days with AtT20 cells expressing Wnt1 or Wnt7a and/or with QT6 cells expressing SHH. The cultures were then stained for β-galactosidase activity with X-gal. Different experiments (n=2) were performed in triplicate (total, n=6) with individual UPM and averaged. Error bars, standard error of the mean.

Fig. 5. Wnt7a is expressed in the dorsal ectoderm. Whole-mount in situ hybridizations of Wnt1 (A; E9.5) and Wnt7a (B,C; E9/E9.5) showing Wnt1 transcripts in the dorsal neural tube along the anterior-posterior axis while Wnt7a transcripts are detected in the dorsal ectoderm overlaying the UPM and the lateral edge of newly formed somites.

Fig. 6. Co-operative effect of SHH with Wnt7a and Wnt1. UPM from MLC3f-nlacZ E8.5 (6-10 somites) embryos was co-cultured for 2 days with AtT20 cells expressing Wnt1 or Wnt7a and/or with QT6 cells expressing SHH. The cultures were then stained for β-galactosidase activity with X-gal. Different experiments (n=2) were performed in triplicate (total, n=6) with individual UPM and averaged. Error bars, standard error of the mean.
respectively. Wnt7a is a potent dorsalizing agent as revealed by recent knock-out experiments (Parr and McMahon, 1995). Although muscle is present in these mutant mice, it is not clear whether epaxial or hypaxial myogenesis is perturbed.

β-catenin/TCFs have been implicated as intracellular mediators of Wnt signaling to activate nuclear gene transcription (Cadigan and Nusse, 1997). Recent studies, however, have suggested that, while this model may be true for certain Wnts such as Wnt1, Wnt7a appears to function via a β-catenin-independent pathway (Kengaku et al., 1998). These observations may have important consequences in the subsequent activation of Myf5 or MyoD genes by these functionally different Wnts.

Other members of the family such as Wnt4, Wnt5a and Wnt6 appear to activate both Myf5 and MyoD. Multiple Wnt receptors, as well as soluble receptor-like molecules have been described (Banhot et al., 1996; Leyns et al., 1997; Wang et al., 1997), but it is not known at present how the affinities of different Wnts for these molecules may vary. It remains to be seen how this differential activation is achieved in the embryo. It is also clear that other molecules, including SHH, cooperate with the various Wnts thus adding to this complexity. Interestingly, we observe the effect of SHH with both Wnt1 and Wnt7a in explants from early embryos (6-10 somites), suggesting that activation of myogenesis both by the neural tube and the dorsal ectoderm may depend upon Sonic Hedgehog signaling from the notochord. This requirement for the notochord is not observed at later developmental stages (20-24 somites) perhaps because the UPM from these stages has been exposed to SHH for a longer period and may thus be more developmentally advanced.

A previous report had shown that among the various Wnt family members, Wnt1, Wnt3 and Wnt4 were capable of activating myogenesis in chick segmental plates, but Wnt7a or Wnt7b were not (Münsterberg et al., 1995). At present, we have no explanation for this discrepancy except for possible species differences; the differential expression of MyoD and Myf5 genes in medial and lateral somite domains has not been examined in detail in the chick. Furthermore, the 2-day delay in the onset of MyoD activation with respect to Myf5 is observed in the mouse but not in the chick. Recently, Wnt6 was reported to be a candidate molecule for the induction of Pax3, and hence potentially myogenesis, by murine dorsal ectoderm (Fan and Tessier Lavigne, 1997). We have not examined in detail the expression of Wnt6 but show here that it will activate myogenesis in chick segmental plates, but Wnt7a or Wnt7b were not (Münsterberg et al., 1995). At present, we have no explanation for this discrepancy except for possible species differences; the differential expression of MyoD and Myf5 genes in medial and lateral somite domains has not been examined in detail in the chick. Furthermore, the 2-day delay in the onset of MyoD activation with respect to Myf5 is observed in the mouse but not in the chick. Recently, Wnt6 was reported to be a candidate molecule for the induction of Pax3, and hence potentially myogenesis, by murine dorsal ectoderm (Fan and Tessier Lavigne, 1997). We have not examined in detail the expression of Wnt6 but show here that it will activate myogenesis, thus suggesting some possible redundancy among certain members of the Wnt family.

**How do epaxial muscles form in Myf5 null embryos?**

Results accumulated over the last few years have led to a simple scheme where two signaling tissues (neural tube/notochord and dorsal ectoderm) activate two responding genes (Myf5 and MyoD) in two populations of precursor cells (medial and lateral) thus leading to the origin of epaxial and hypaxial muscles, respectively (Cossu et al., 1996a). While this scheme may be correct in principle, it is also oversimplified and cannot explain by itself the spatiotemporal complexity of further development of skeletal muscle tissue. Notably, in vivo, MyoD activation is delayed in the hypaxial myotome of Myf5 null embryos (Tajbakhsh et al., 1997) in spite of the fact that the β-galactosidase+ progenitor cell population is present, suggesting that initial activation of hypaxial myogenesis depends also on Myf5. This difference between these in vivo observations and those with explant cultures is probably related

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**Fig. 6.** Laterally marked muscle precursors are not subsequently detected in the medial-half somite. Rhodamine fluorescence (A) and phase contrast (B) of a transverse cryostat section (somite VIII) from an E9.5 mouse embryo which was injected with Dil in the lateral UPM, and then cultured for 1 day. Note that the ventrolateral domain of paraxial mesoderm is uniformly labeled, but no labeled cells are seen in the medial domain of the somite, adjacent to the neural tube (nt); dm: dermomyotome. Bar: 15 μm.

**Fig. 7.** In vitro differentiation of medial and lateral halves of UPM, isolated from E9.5 (20-24 somite) Myf5a+/− (open bars) and Myf5a2−/− (hatched bars) embryos. The right UPM of each embryo was dissected into a medial half (which was left attached to its own side of the neural tube) and into a lateral half (with its dorsal ectoderm but without lateral mesoderm). The contralateral left UPM was cultured intact with its own side of the neural tube, the notochord and dorsal ectoderm. After 5 days, the cultures were stained with MF20 antibody and the number of myosin-positive cells was counted in each culture. Data are the average of at least ten different cultures from individual embryos. Error bars: standard error of the mean.
and Ordahl, 1995). Thus axial structures are required to later have epaxial muscles in the back, while the latter show a structures: the former do not form the myotome initially, but Myf5 gene and embryos that lack the axial requires further investigation.

It has been shown to retard the latter (Cossu et al., 1996b). This to the disruption of embryo architecture in explants. Absence of tissues, such as lateral mesoderm, may have differential effects on the activation of Myf5 and MyoD. Indeed in explants it has been shown to retard the latter (Cossu et al., 1996b). This requires further investigation.

Here, we have investigated the difference between embryos that lack the Myf5 gene and embryos that lack the axial structures: the former do not form the myotome initially, but later have epaxial muscles in the back, while the latter show a total absence of these muscles (Rong et al., 1992; see Christ and Ordahl, 1995). Thus axial structures are required to produce more signals than those that simply activate Myf5. To address this problem, we first examined the possibility that colonization of the medial domain of paraxial mesoderm by precursors located in the lateral domain may rescue epaxial myogenesis in Myf5a2−/− embryos. Dissection of the unsegmented paraxial mesoderm showed that the medial half of Myf5a2−/− embryos contains myogenic precursors that are capable of in vitro terminal differentiation, thus ruling out this possibility. This differentiation was observed after a 5 day culture period, during which time the neural tube presumably ages, acquiring the potential to activate MyoD (see below).

In this context, it is necessary to discuss the apparent discrepancy between the data reported here and those of Gamel et al. (1996), who showed that myogenesis occurred in chick somites and segmental plates, which had been divided into medial and lateral halves, in the absence of inducing tissues. They concluded from their studies that reciprocal inhibition of myogenesis must be exerted by different parts of a developmental unit such as a newly formed somite. We do not observe this phenomenon in the mouse. While there is at present no explanation for this species difference, it should be remembered that default myogenesis has been reported in the early chick embryo (George-Weinstein et al., 1996) and this may be related to some basic difference to mammalian cells, which depend upon a community effect to undergo myogenic commitment (Cossu et al., 1995).

Fig. 8. Time course of in vitro appearance of Myf5 and MyoD in response to neural tubes of different ages. UPM from E9.5 (20-24 somite) Myf5a2+/+ embryos was cultured with caudal neural tubes from E9.5 (squares) or from E10.5 (circles) wild-type embryos. At the indicated periods, cultures were double stained with an anti-β-galactosidase monoclonal antibody (closed symbols) and with anti-MyoD polyclonal antibody (open symbols) and the number of positive cells was recorded in at least 5 separate cultures from individual embryos. Error bars, standard error of the mean.

Space and time in myogenic commitment
An important point raised by this work is that myoblast commitment, like terminal differentiation, occurs asynchronously. Only a subset of cells in the medial paraxial mesoderm activate Myf5 in response to neural tube signals (ex. Wnt1) to form the epaxial myotome. Similarly, dorsal ectoderm (ex. Wnt7a) promotes myogenesis later only in a subset of cells in the lateral paraxial mesoderm. Apart from the question of threshold levels for signaling molecules, differences between cells may be related to their capacity to respond to mitogens such as FGF which prevent differentiation (see Marcelle et al., 1995), as well as to diverse signaling systems. Notably, the epaxial dermomyotome lip expresses noggin (Hirsinger et al., 1997), a BMP antagonist, and also Wnt11 (Marcelle et al., 1997) which may act as a relay accelerating the response of these cells.

In conclusion, these data reveal that different myogenic pathways can be activated by different Wnt signaling molecules and point to the need for a clearer phenotypic definition of the various myogenic precursors in the embryo in order to understand the spatiotemporal control of skeletal muscle development.

This work was supported by grants from Telethon n. A.67, Fondazione Cenci Bolognetti and from MURST to G. C., and grants from the AFM, ARC and MENESR as well as the Pasteur Institute.
and CNRS to M. B. and by an EC grant Biotech2 CT-0228-95 to M. B. and G. C.; R. K. was supported by an EC fellowship under the EC Biotech grant. U. B. was supported by EMBO short-term and Development Travelling Fellowships.

REFERENCES


