Intrinsic signals regulate the initial steps of myogenesis in vertebrates

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Summary

In vertebrates, despite the evidence that extrinsic factors induce myogenesis in naive mesoderm, other experiments argue that the initiation of the myogenic program may take place independent of these factors. To resolve this discrepancy, we have re-addressed this issue, using short-term in vivo microsurgery and culture experiments in chick. Our results show that the initial expression of the muscle-specific markers *Myf5* and *MyoD* is regulated in a mesoderm-autonomous fashion. The reception of a Wnt signal is required for *MyoD*, but not *Myf5* expression; however, we show that the source of the Wnt signal is intrinsic to the mesoderm. Gain- and loss-of-function experiments indicate that *Wnt5b*, which is expressed in the presomitic mesoderm, represents the *MyoD*-activating cue. Despite *Wnt5b* expression in the presomitic mesoderm, *MyoD* is not expressed in this tissue: our experiments demonstrate that this is due to a Bmp inhibitory signal that prevents the premature expression of *MyoD* before somites form. Our results indicate that myogenesis is a multistep process which is initiated prior to somite formation in a mesoderm-autonomous fashion; as somites form, influences from adjacent tissues are likely to be required for maintenance and patterning of early muscles.

Key words: MyoD, Myf5, Wnt5b, Shh, Bmp, Somite, Presomitic mesoderm

Introduction

Vertebrates skeletal muscle of the body and the limb derive from segmentally organized mesodermal structures named the somites (reviewed by Christ and Ordahl, 1995; Brand-Saberi et al., 1996; Marcelle et al., 2002). Where and how myogenesis is initiated in the embryo has been the matter of intense debate. The first molecular sign of initial muscle differentiation is the expression of the muscle regulatory factors (MRFs) *Myf5* and *MyoD*. Although early expression studies performed in amniotes had suggested that these molecules initiate their expression in somites, improved in situ hybridization in chick, and analyses of *Myf5/lacZ* heterozygote mice, have recently shown that *Myf5* is expressed prior to somitogenesis, within the presomitic mesoderm (Cossu et al., 1996b; Hirsinger et al., 2000; Kiefer et al., 2001). Strong MRF expression is then observed in the medial wall of newly formed somites. Although in mice, this region of the somite strongly upregulate *Myf5* expression (Ott et al., 1991), in chick, it is *MyoD* that is expressed in this tissue (Pownall and Emerson, 1992; Borycki et al., 1997). Because lineage studies performed in chick indicate that cells of the medial somite later become integrated into the myotome, it is likely that this region of the somite contains the first lineage-restricted muscle progenitors (Denetclaw et al., 1997; Denetclaw and Ordahl, 2000; Kahane et al., 1998).

A number of studies have addressed the molecular mechanisms that regulate myogenesis. In vitro and in vivo studies have shown that the neural tube, the surface ectoderm and the notochord/ventral neural tube have the ability to promote myogenic differentiation (Borman and Yorde, 1994; Buffinger and Stockdale, 1994; Christ et al., 1992; Kenny-Mobbs and Thorogood, 1987; Rong et al., 1992; Stern et al., 1995; Teillet and Le Douarin, 1983; Vivarelli and Cossu, 1986; Cossu et al., 1996a; Kuratani et al., 1994; Buffinger and Stockdale, 1994; Stern and Hauschka, 1995; Münsterberg et al., 1995; Münsterberg and Lassar, 1995). The search for the molecular signals that mediate the activities of these tissues has led to the identification of Sonic Hedgehog (Shh, expressed in the notochord and the neural plate) and Wnt family members (expressed in the dorsal neural tube and ectoderm). The combination of Wnt and Shh activates robust muscle marker expression in presomitic mesoderm explants, whereas Shh alone has no effect and Wnt alone has a low level effect (Fan and Tessier-Lavigne, 1994; Kos et al., 1998; Munsterberg et al., 1995; Tajbakhsh et al., 1998; Stern et al., 1995; Maroto et al., 1997; Reshef et al., 1998; Munsterberg and Lassar, 1995). These data led to the proposal that largely naive cells adopt myogenic fate through tissue induction mediated by the combined action of Wnt and Shh emanating from surrounding tissues (Munsterberg et al., 1995). Clearly, this is the most popular model for initiation of myogenesis in vertebrates.

Despite the evidence that Shh and Wnt can induce the

myogenic program in naive mesoderm, other experiments argue that the initiation of myogenesis may take place independent of these factors. For example, removal of the notochord in vivo fails to alter activation of myogenesis in somites, despite the fact that this operation results in the removal of the source of Shh (Bober et al., 1994). Moreover, analyses of the zebrafish and mouse Shh and Smoothened knockouts, and experimental studies performed in chick have shown that myogenesis is initiated, but not maintained, in the absence of Hedgehog signaling, suggesting that it might be dispensable for the initiation of myogenesis (Chiang et al., 1996; Krüger et al., 2000; Duprez et al., 1998; Fan et al., 1995; Marcelle et al., 1999; Teillet et al., 1998; Zhang et al., 2001; Coutelle et al., 2001). More surprising is the observation that myogenesis is the preferred pathway of presomitic mesoderm cells, when they are dissociated to produce a single cell suspension and cultured in serum-free medium (George-Weinstein et al., 1996; George-Weinstein et al., 1997). These data support a model where the initiation of myogenesis is at least partially independent of environmental cues. Such a model is difficult to reconcile with the inductive model mentioned above.

Apparently contradictory findings are often due to differing experimental protocols. Experiments which have shown that myogenesis is regulated by environmental cues typically analyzed the myogenic differentiation of rostral presomitic mesoderm explants (considered as naive tissue) placed in the presence of putative inductive tissues or factors for 24, 48 and sometimes 72 hours. However, it is now clear that in the embryo, inductive processes take place in hours or less: during embryogenesis the rostral presomitic mesoderm (i.e. the tissue that was tested in these experiments) initiates myogenesis as somites form, only hours later. It is thus possible that past experiments have identified molecules implicated in later, rather than earlier stages of myogenesis. Therefore, it seemed important to re-examine the initial steps of vertebrate myogenesis both in vivo and in vitro. The chick embryo is particularly well-suited for these experiments, as embryonic microsurgery and ectopic expression of various molecules can be readily evaluated within hours of manipulation. Here, we first show that the activation of *Myf5* and *MyoD* expression is mesoderm-autonomous. Reception of a Wnt signal is required for the initiation of *MyoD*, but not *Myf5* expression. However, ablation experiments demonstrate that the source of the endogenous Wnt signal is neither the ectoderm nor the dorsal neural tube but the mesoderm itself, where *Wnt5b* is expressed. Our data indicate that *Wnt5b* is likely to be the *MyoD* activating signal, suggesting that the activation of *MyoD* expression is a mesoderm 'auto-induction'. Expression of *Wnt5b* precedes that of *MyoD* by several hours and this delay is controlled by a Bmp signal that inhibits *MyoD* expression in the rostral presomitic mesoderm. Although the initial activation of *MyoD* expression does not require extrinsic signals, we show that *Shh* emanating from axial structures is able to enhance MyoD expression. We believe our results reconcile previously conflicting data by showing that myogenesis is a complex process that is initiated prior to somite formation in a mesoderm-autonomous fashion; extrinsic influences are likely to be required for the enhancement and/or maintenance of these initial events, thereby leading mesodermal cells further along the myogenic differentiation pathway.

Materials and methods

Isolation of chicken Frizzled

Total mRNA was isolated from somites of 2- and 3-day-old embryos. Reverse transcriptase reaction was performed using a 5′-GGAT-CCGCNGCYARRAACCAVAT-3′ downstream primer containing conserved sequences of the third transmembrane segment of Frizzled receptors. A first cycle of PCR was then performed using the downstream primer described above and an upstream primer: 5′-GAATTCTAYCCNGARMGVCCVAT-3′ containing conserved sequences present in the second transmembrane segment of Frizzled. A second PCR reaction was then carried out using as a template 5 μl of the first PCR reaction, the same upstream primer and a downstream primer (5′-GGATCCGWVAGDATNACCCACCA-3′) located in a more internal region of the third transmembrane segment of Frizzled. With isolated fragments, a 2- to 3-day-old chick embryo library was screened (kindly provided by Dr D. Wilkinson). From this screen three Frizzled expressed in somites were obtained and cloned in the pGemT vector (Promega).

Construction of a secreted form of Fz7, a full size Wnt5b and a dominant negative form of Wnt5b

The extracellular region of the chick Frizzled 7 was amplified by PCR, using the full size Fz7 clone as template, and the primers 5′-GGATTCACCATGGGGCCCGCGGCGGGAGAAGCG-3′ and 5′-GTCGACCGGCTGTCGGCTGCGCCGTG-3′. The amplified fragment was fused to a 6× Myc Tag sequence, subcloned into the Slax13 shuttle vector, and finally transferred into the RCAS-BP(A) retroviral vector (Hughes et al., 1987). The human alkaline phosphatase gene cloned into the RCAS-BP (A) vector (Morgan and Fekete, 1996) served as control.

Dominant-negative Wnt5b was constructed by a deletion of the C terminus of the mouse Wnt5b protein. PCR amplification of amino acids 1 to 301 (including the signal peptide) was performed using a cDNA clone for full size mouse *Wnt5b* as template (kindly provided by A. McMahon) and the primers 5′-CTCGAGCCACCATGGTG-GTCCCAGGGCAT-3′ that includes a consensual Kozak's sequence and a 5′-GAATTCTCAGCAGTAGTCAGGACTGGG-3′ containing a TCA stop codon after cysteine residue 301 from the mouse *Wnt5b* coding sequence. The first amino acid of the mouse *Wnt5b* was changed from a valine to a leucine to conserve a Kozak consensus sequence. This fragment was then transferred into the pCLAG2 electroporation vector (Marics et al., 2002).

The full coding region of mouse *Wnt5b* (kindly provided by A. McMahon) was also inserted into RCAS-BP(B) retroviral vector, as described for S-Fz7.

Cell transfection and injection

Chicken embryonic fibroblast cell line UMNSAH/DF-1 (Himly et al., 1998) were transfected with the lipofectamine reagent (Gibco BRL) according to the manufacturer's instructions. Five to 7 days after transfection, cell infection was tested by immunohistochemistry reaction against the viral core protein p27 (using a rabbit anti p27 polyclonal antibody: SPAFAS). We verified that infected cells efficiently secreted S-Fz7-Myc by an immunohistochemistry reaction against the Myc tag (using a monoclonal mouse anti-human Myc) (Santa Cruz Biotechnology); *Wnt5b* expression was detected using in situ hybridization on transfected cells in culture. The infected fibroblasts stably produced the S-Fz7 and mouse Wnt5b molecules for at least 2 months in culture. Bmp4-producing cells were obtained in a similar way by transfection of UMNSAH/DF-1 fibroblasts with a mouse Bmp4 construct cloned into RCAS(A), provided by Dr P. Brickell.

Transfected cells were injected into embryos. To inhibit endogenous Fz7 signaling, S-Fz7 expressing cells were pressure-injected into somites IV, rostral, middle and caudal PSM. These embryos were analyzed after overnight incubation. Injections were

performed using a Picospritzer pressure injector (General Valve Corporation) and glass needles. As *MyoD* expression was abolished after S-Fz7 injection, this served as a positive control when the effect of S-Fz7 on *Myf5* expression was analyzed. Injections of mixed cells were prepared as follows: S-Fz7- and mouse Wnt5b-transfected cells were trypsinized, counted and mixed at a ratio 1:5, then injected as described before. Alkaline phosphatase-transfected cells were used as control and mixed in the same ratio with S-Fz7. Noggin cell (kindly provided by Dr R. Harland) were injected in the middle presomitic mesoderm and embryos were analyzed 6-8 hours later, CHO dhfr$^-$ cells were used as control.

Surgery

Fertilized chick eggs were obtained from a commercial source. Glass needles were used to separate ectoderm from paraxial mesoderm; contact was permanently impeded by placing a Tantalum foil of appropriate size between the two tissues (GoodFellow Cambridge Limited). To extirpate the dorsal part of the neural tube, an incision in the ectoderm was made using a glass needle, then neural tube and paraxial mesoderm were pulled apart and the dorsal region of the right side of the neural tube was sectioned out. Embryos were re-incubated for 3-5 hours.

Presomitic mesoderm explants and cell dissociation

Presomitic mesoderm cultures were prepared as follows: dispase solution (1 mg/ml) was briefly applied in ovo onto presomitic mesoderm of 13 HH stage embryos where ectoderm has been mechanically removed. The presomitic mesoderm, starting at the Hensen's node until its rostral most part, was isolated and cultured for 3-4 hours in the following media: L-15 Leibovitz medium (Life and Technology) with 10% fetal calf serum, 2 ng/ml of bFGF (Sigma), 50 μg/ml sodium bicarbonate (Gibco Life and Technology).

For cell dissociation, presomitic mesoderms were isolated as described, and incubated in dispase during 10 minutes. Mechanical dissociation was performed through a Pasteur pipette and cells were cultured, in the media described above, for 6 hours. To test the effect of Bmp4 on MyoD activation, dissociated presomitic mesoderm cells were cultured in the presence of supernatant from Bmp4-transfected UMNSAH/DF-1 cells. The supernantant was collected from subconfluent cultures grown ON in the L-15 medium described above. This medium was concentrated 7× on Ultrafree-15 Millipore filters (molecular weight limit: 10K). As control, 7× concentrated medium collected from non-transfected UMNSAH/DF-1 cells was used. To test the effect of Shh on isolated presomitic cells, bacterially produced Shh protein (R&D) was added to the cell culture medium to a concentration of 3 μg/ml. While concentrations of Shh ranging from 1 to 50 μg/ml have been used in the literature, 3 μg/ml corresponds to the average concentration that was found to be efficient in most experimental conditions (Lai et al., 2003; Detmer et al., 2000; Norris et al., 2000; Nakamura et al., 1997; Kanda et al., 2003).

In situ hybridization

Whole-mount in situ hybridization on chick embryos were performed as described (Henrique et al., 1995). The probes used in this study were: a chick *MyoD* probe corresponding to the complete 1518 bp *MyoD* cDNA (Wright et al., 1989); chick *Myf5* corresponding to 336 bp spanning the 5′UTR to the 5′ coding region (Kiefer and Hauschka, 2001); chick *Wnt4* and *Wnt5b* probes corresponding to 400 bp of the coding region (Hollyday et al., 1995); and chick *Delta*-1 probe corresponding to 900 bp of the coding sequence (Henrique et al., 1995) (kindly provided by Dr D. Henrique).

Double in situ hybridization was carried out in the same way as single in situ, but fluorescein-labeled and digoxigenin-labeled probes were added. The first probe was revealed by an anti-fluorescein alkaline phosphatase-conjugated antibody (Roche) and the INT/BCIP substrate (Roche) giving a red precipitate. Embryos were fixed in 4% formaldehyde PBS overnight. Anti-fluorescein antibody was inactivated by incubating embryos in EDTA 100 mM in MABT buffer (Henrique et al., 1995) at 65°C for 1 hour. Embryos were then incubated with anti-digoxigenin alkaline phosphatase-conjugated antibody (Roche), and revealed with NBT/BCIP reagent (Gibco Life Technologies).

In vivo electroporation

In vivo electroporation were performed as described (Marics et al., 2002). Briefly, Qiagen EndoFree purified *DN-Wnt5b* plasmid was injected into the lumen of the neural tube of stage 13 HH embryos. Electrodes were placed on both sides of the embryo and pulsed five times at 80V, 20 mseconds length with a Intracell TSS 10 electroporator. By placing the positive electrode on the right side of the embryo, we electroporated this half of the neural tube. Embryos were then re-incubated for 8-10 hours.

Results

MyoD induction is independent of Wnt signals emanating from surrounding tissues

To gain further insight into the molecular mechanisms regulating myogenesis, we first examined the role of Wnt-expressing tissue in this process. It is generally believed that Wnts expressed in the dorsal neural tube and in the ectoderm play important functions in the induction of *MyoD* in somites. If any of the Wnt molecules expressed in these tissues were regulating *MyoD* expression, removing these tissues should abolish *MyoD* expression.

We first tested whether the neural tube regulates *MyoD* expression. We removed the dorsal half of the neural tube at the rostral level of the presomitic mesoderm of 2.5-day-old embryos (stage 13 HH) (Hamburger and Hamilton, 1992). At the level of the microsurgery, *MyoD* expression is not detectable by in situ hybridization. Embryos were allowed to form four or five additional somites (incubation of 6-8 hours) after ablations. Among the Wnts expressed in the neural tube (*Wnt1*, *Wnt3a*, *Wnt4*), *Wnt4* extends most ventrally. Therefore, a *Wnt4* probe was incorporated in the in situ hybridization to ensure that we had ablated the entire Wnt-expressing domain of the neural tube. No difference in *MyoD* expression was observed between the operated and the control side (Fig. 1A-C), indicating that the neural tube containing *Wnt1*, *Wnt3a* and *Wnt4* is not required for the initial induction of *MyoD* expression.

As the chick ectoderm expresses *Wnt6* (García-Castro et al., 2002; Marcelle et al., 2002; Schubert et al., 2002), we analyzed the possibility that ectodermal Wnt might regulate *MyoD* expression by separating the ectoderm from the presomitic mesoderm via an impermeable barrier. The experiment was performed at the rostral level of the presomitic mesoderm of stage 13 HH embryos, which were allowed to develop two or three additional somites. No difference in *MyoD* expression was observed after such manipulation (Fig. 1D-F), suggesting that the ectoderm and, by inference *Wnt6*, do not participate in the initiation of *MyoD* expression.

Finally, to exclude the possibility that Wnts from the neural tube and the ectoderm provide synergistic signal(s) to activate *MyoD* expression, we ablated both structures simultaneously using the same protocol described above. Again, *MyoD* expression was not altered in the absence of ectoderm and neural tube (Fig. 1G-I). These experiments indicate that the initiation of *MyoD* expression is independent of the presence

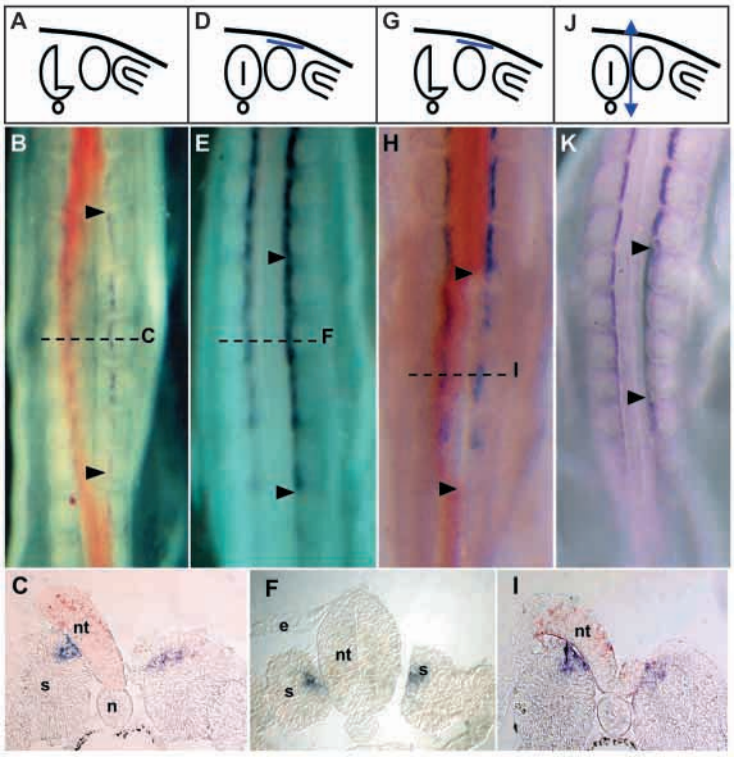

Fig. 1. *MyoD* expression is independent from neural tube and ectodermal signals. (A,D,G,J) Schematic representation of the experimental procedures. Whole-mount in situ hybridization (B,E,H,K) and transversal sections (C,F,I). (B,C) *Wnt4* (red) and *MyoD* (blue) expression after dorsal neural tube ablation. In these conditions, 91% of the embryos showed normal *MyoD* expression (*n*=10/11). (E,F) *MyoD* expression after ectoderm isolation. All of the embryos showed normal *MyoD* expression (*n*=11), compared with the contralateral, unoperated side, or with *MyoD* expression observed rostral and caudal to the membrane (indicated by arrowheads). (H,I) *Wnt4* (red) and *MyoD* (blue) expression after both, dorsal neural tube and ectoderm ablation. Ninety-three percent of the embryos showed normal *MyoD* expression (*n*=14/15). (K) *MyoD* expression 1 hour after separation of somites from axial structures. All of the embryos failed to show *MyoD* expression (*n*=8). Broken lines indicate the level of the sections. n, notochord; nt, neural tube; e, ectoderm; s, somite.

of dorsal neural tube and ectoderm. The possibility remained, however, that the *MyoD* expression we observed after surgery is the consequence of an inductive event mediated by surrounding tissues prior to surgery. To address this possibility, it was important to test the stability of *MyoD* mRNA in paraxial mesoderm. When *MyoD*-expressing somites are surgically separated from axial structures, which normally maintain its expression (Marcelle et al., 1999), *MyoD* expression disappears. We repeated these experiments, monitoring the time that it takes for *MyoD* expression to disappear from a somite that already expresses this molecule. We observed that *MyoD* was undetectable in less than 1 hour (Fig. 1J). This indicates that *MyoD* mRNA is degraded very rapidly in vivo, and suggests that the expression we observed after ablation of the dorsal neural tube and/or ectoderm is a de novo mRNA production resulting from a continuous activation process that took place in the presomitic mesoderm during the time of the experiment.

Frizzled receptors are expressed in somites at the time that myogenesis is initiated

For Wnt signaling to play a role in myogenesis, members of the Wnt signaling pathway must be present in the myogenic region of the somite at the time and place where *MyoD* expression is initiated. Wnts mediate their activities through seven transmembrane domain Frizzled receptors (Fz), which in turn activate β-catenin-dependent and/or -independent signaling pathways (reviewed by Huelsken and Birchmeier, 2001). Using mRNA extracted from somites, we have amplified by RT-PCR fragments of Fz receptors (see Materials and methods). Three of the amplified fragments, corresponding to chick *Fz1*, *Fz2* and *Fz7* display an expression patterns consistent with a possible role during myogenesis. The three genes display similar expression patterns in the paraxial mesoderm: they are observed in most of the presomitic mesoderm; their expression increases as somites form (Fig. 2A-C). In sections, we observed that the three genes are expressed throughout the epithelial undifferentiated somites, overlapping the region where *MyoD* expression is first observed (i.e. the medial wall of the somite; Fig. 2D). Therefore, *Fz1*, *Fz2* and *Fz7* expression patterns, which precede and overlap that of *MyoD*, have the proper spatiotemporal distribution pattern to play a role in the initiation of its expression.

In vivo, the initiation of *MyoD* but not of *Myf5* expression is dependent upon *Fz* signaling

To test whether Wnt signaling is required for *MyoD* expression in vivo, we blocked Wnt signaling using a soluble (Myc-tagged) form of the Fz7 receptor (S-Fz7) containing the CRD (cysteine-rich domain) region of Fz7. Such constructs have been previously shown to act as competitive inhibitor molecules that bind to their cognate ligand and titrate it out from the endogenous receptor (Hsieh et al., 1999). S-Fz7 was inserted into an RCAS retroviral vector and transfected into a chick fibroblast cell line. We verified that the infected cells efficiently secrete S-Fz7 in the culture medium (see Materials and methods). Cell pellets were injected at multiple sites (caudal, middle and rostral level) into the presomitic mesoderm and early (third or fourth newly formed) somites of 2-day-old embryos (stage 11 HH). Pellets injected in the presomitic mesoderm could therefore exert their action before *MyoD* expression is initiated. The next day, while embryos injected with control, alkaline phosphatase-transfected cells displayed normal *MyoD* expression (Fig. 3A), somites that formed in the presence of S-Fz7-secreting cells did not express *MyoD* (Fig.

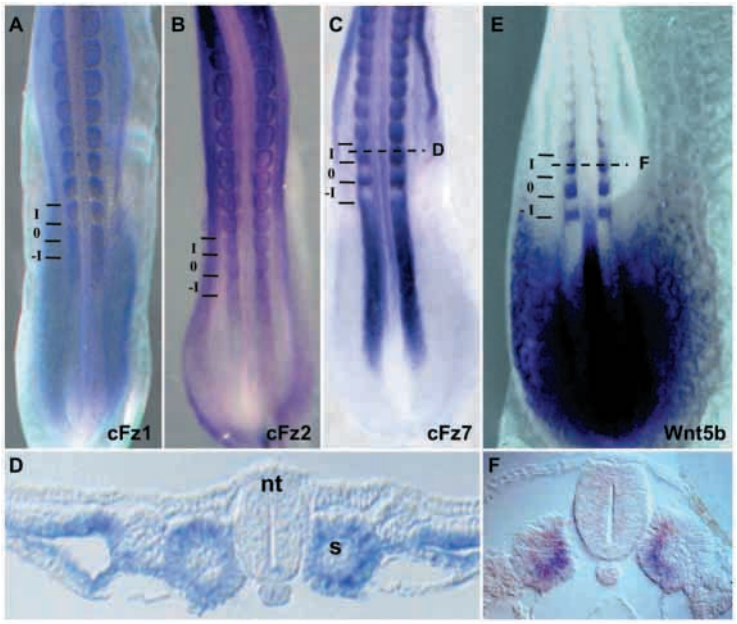

Fig. 2. Fz receptors and *Wnt5b* expression patterns. Whole-mount in situ hybridization of (A) *Fz1*; (B) *Fz2*; (C) *Fz7* and (E) *Wnt5b*. (D) Section of C at the level of the first somite. (F) Section of *Wnt5b* (blue) and *MyoD* (red) double in situ hybridization at the level of the first somite. Sections levels are indicated by broken lines. Somite numbers are indicated by roman numerals. s, somite; nt, neural tube.

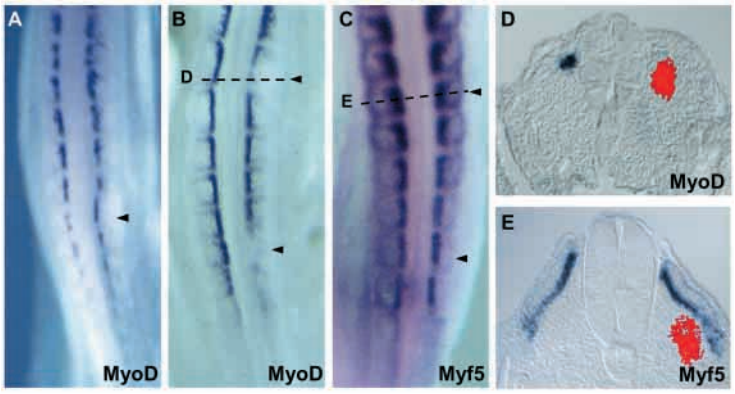

Fig. 3. Fz7 signaling is required for *MyoD*, but not for *Myf5* initiation. (A) *MyoD* expression in an embryo injected with control alkaline phosphatase-transfected cells. Eighty-eight percent of the embryos presented normal *MyoD* expression (n=23/26). (B) *MyoD* expression in an embryo injected with S-Fz7-transfected cells. S-Fz7-secreting cells were injected at the caudal, middle and rostral presomitic level, as well as in the third or fourth newly formed somite. Seventy-seven percent of the embryos presented an inhibition of *MyoD* expression in somites formed in the presence of S-Fz7-transfected cells (n=23/30). Inhibition was more pronounced around S-Fz7-expressing cells injected in the caudal and middle presomitic mesoderm (shown here). MyoD inhibition was sometimes observed when cells were injected in the rostral presomitic mesoderm, although this effect was seldom observed when they were injected in somites which already expressed MyoD. (C) In sharp contrast, embryos (87%) injected with S-Fz7-transfected cells presented normal *Myf5* expression in injected somites (n=20/23). (D) Transverse section of B. (E) Transverse section of C. Section levels are indicated by broken lines. Injected cells were stained with the fluorescent dye DiI and observed under UV illumination. (D,E) Composites of a bright-field image of transverse sections and a fluorescent image showing the DiI-labeled S-Fz7-expressing cells. Arrowheads indicate the position of the injected cells.

3B,D). In sharp contrast, *Myf5* expression was unaffected in the same experimental conditions (Fig. 3C,E). These results indicate that, in vivo, a Wnt signal, antagonized by S-Fz7, is necessary for the initiation of *MyoD*; this signal is not required for *Myf5* expression.

Wnt5b expression in paraxial mesoderm precedes the initiation of *MyoD*

As the experiments described above show that Wnt signaling is necessary for *MyoD* induction, but does not emanate from surrounding tissues, we explored an alternative source of Wnt signal within paraxial mesoderm that could account for its myogenic activity.

Developing chick embryos were screened with a number of Wnt probes (*Wnt1*, *Wnt3a*, *Wnt4*, *Wnt5a*, *Wnt5b*, *Wnt6*, *Wnt7a*, *Wnt7b*, *Wnt8c*, *Wnt11*); of these, we found that *Wnt5b* is expressed in the paraxial mesoderm prior to initiation of *MyoD* expression. In the rostral presomitic mesoderm, *Wnt5b* is expressed in two transversal bands which correspond to the prospective caudal borders of somite 0 and –I (Fig. 2E). Therefore, *Wnt5b* expression is initiated ~3 hours before *MyoD* expression in somites. Soon after somites have formed, *Wnt5b* expression gradually shifts medially, such that from somite stage II onwards, the medial border of all somites expresses *Wnt5b* (Fig. 2E). As somites differentiate into a ventral sclerotome and a dorsal dermomyotome, *Wnt5b* expression becomes restricted to the dorsomedial lip (not shown), which contains progenitors for epaxial myotomal cells (Denetclaw et al., 2001; Denetclaw et al., 1997). Double in situ hybridization with *MyoD* and *Wnt5b* indicate that cells within the medial somite and the medial lip co-express both genes (Fig. 2F). Altogether, these data indicate that *Wnt5b* is expressed in a pattern compatible with a role in *MyoD* induction.

Wnt5b likely represents the MyoD activating cue

To be a candidate in the initiation of *MyoD* expression, *Wnt5b* should oppose the effect of S-Fz7, which we showed above inhibits *MyoD*. To test this, pellets of cells expressing the secreted form of Fz7, mixed with control cells or with cells expressing a mouse *Wnt5b* construct, were injected into the paraxial mesoderm. S-Fz7 cells mixed with control (i.e. alkaline phosphatase-transfected) cells (1:5) inhibited *MyoD* induction (Fig. 4A). By contrast, injection of S-Fz7 cells mixed with *Wnt5b*-expressing cells (1:5) rescued normal *MyoD* expression (Fig. 4B), demonstrating that *Wnt5b* is able to counter the *MyoD*-inhibiting activity of secreted Fz7. As *Fz7* and *Wnt5b* are co-expressed in the chick presomitic mesoderm and somites, the rescue of the phenotype obtained with S-Fz7 by *Wnt5b* further indicates that *Wnt5b* mediates its activity, at least in part, through *Fz7*.

We next constructed an inhibitory form of *Wnt5b* and asked whether this molecule impedes the initiation of *MyoD* expression. Truncation of the C terminus, cysteine-rich domain (CRD) of *Xenopus Wnt8* and *Wnt11* have been shown to act as dominant-negative (DN) forms of these molecules (Hoppler et al., 1996; Tada and Smith,

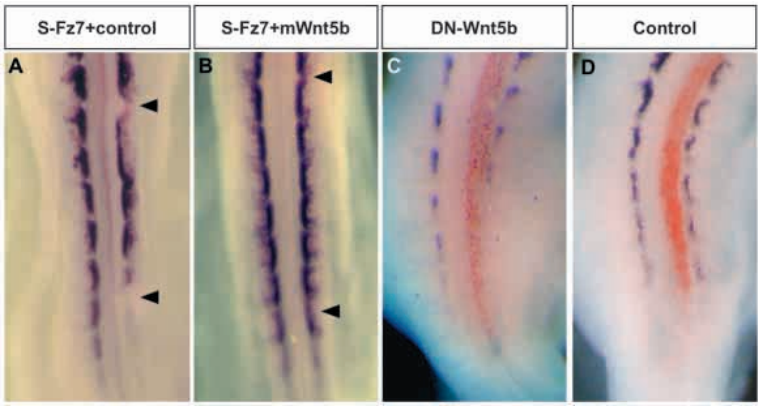

Fig. 4. *Wnt5b* signal regulates *MyoD* expression. (A,B) Rescue of the *MyoD*-inhibitory effect of S-Fz7 by *Wnt5b* co-injection. (A) *MyoD* expression in an embryo co-injected with S-Fz7 transfected cells mixed with control cells at a ratio of 1:5. Seventy-one percent of these embryos present an inhibition of *MyoD* expression (n=27/38). (B) Rescue of *MyoD* expression by co-injection of S-Fz7 transfected cells mixed with *Wnt5b*-expressing cells at a ratio of 1:5. In 57% of embryos, *MyoD* expression was rescued to normal levels (n=25/44). (C) Inhibition of *MyoD* initiation in DN-Wnt5b electroporated embryos. Mouse *Wnt5b* (red) and *MyoD* (blue) expression in embryos electroporated with DN-Wnt5b in the caudal neural tube. 80% of the embryos presented inhibition of *MyoD* initiation (n=15/18). (D) *GFP* (red) and *MyoD* (blue) expression in embryos electroporated with a GFP cDNA in the caudal neural tube. All of the embryos had normal *MyoD* expression (n=12).

2000). Although these experiments indicated that the inhibition elicited by each *DN*-Wnt is specific, and does not alter the activity of unrelated Wnt molecules, clearly more experiments are needed in order to probe that this is the case for all DN Wnt constructs. We inserted a mouse *Wnt5b* molecule lacking the CRD (*DN-Wnt5b*) into an electroporation plasmid. Because in our hands, PSM electroporation results in poor expression, the *DN-Wnt5b* construct was electroporated in one half of the caudal neural tube; from where it is secreted into the presomitic mesoderm, and counteracts the activity of the endogenous *Wnt5b*. After 8-10 hours, we observed that *MyoD* expression was not initiated in the first somite, as it was on the control side, but more rostrally, roughly at the level of the third somite (Fig. 4C). Normal *MyoD* expression was observed in control embryos electroporated with a GFP construct (Fig. 4D). Together, these results indicate that *Wnt5b* (probably through *Fz7*), mediates the initiation of *MyoD* expression.

Expression of *MyoD* in the presomitic mesoderm is repressed by a Bmp signal

Although the data described above suggest a likely role for *Wnt5b* in the induction of *MyoD* expression, it is paradoxical that expression of *Wnt5b* in the presomitic mesoderm precedes that of *MyoD* by at least 3 hours. Such a developmental delay is consistent with the possibility that initiation of *MyoD* expression may be temporarily inhibited in the presomitic mesoderm. A candidate for an inhibitor is Bmp4. Bmp4, expressed in the lateral plate mesoderm, is believed to repress *MyoD* expression in the lateral somite, thereby delaying hypaxial (i.e. lateral) muscle formation; epaxial (i.e. medial) muscles, which are further away from the Bmp source, develop earlier (Pourquié et al., 1996).

To test the possibility that Bmp might act on the presomitic mesoderm as well, we injected cells expressing the Bmp-antagonist Noggin at the level of the middle presomitic mesoderm of developing embryos. In 91% of the embryos, we observed 6-8 hours later that *MyoD* was prematurely expressed in the presomitic mesoderm as one, and sometimes two, transverse bands (Fig. 5B-D), in an expression pattern reminiscent to that of *Wnt5b*. In addition, *MyoD* was ectopically expressed at the posterior border of newly formed somites. As observed previously (Marcelle et al., 1997), Noggin acts at a considerable distance, as its effect is observed on the contralateral, uninjected side of the embryo. On sections, we observed *MyoD* expression in the dorsal region of the rostral presomitic mesoderm (Fig. 5D, arrows) and of the epithelial, undifferentiated somite (Fig. 5C, arrows). This observation indicates that rostral presomitic mesoderm cells and the dorsal portion of undifferentiated somites are competent to initiate *MyoD* expression, but are repressed to do so by a Bmp signal. The expression pattern of *MyoD* after ectopic expression of Noggin supports the hypothesis that *MyoD*-activating signals are initially localized at the caudal border of the segments, constituting additional supporting evidence for a role of *Wnt5b* in *MyoD* activation. The observation that MyoD expression was never expressed further caudally (i.e. more than two transverse bands) in the presomitic mesoderm indicates that the competence to express MyoD is

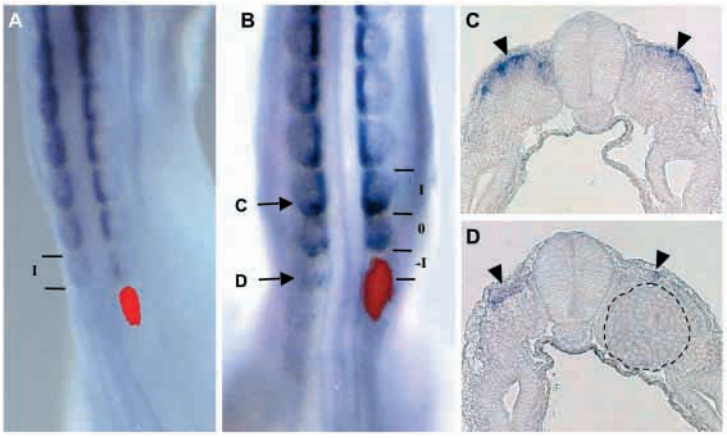

Fig. 5. *MyoD* expression is repressed by a Bmp signal in the presomitic mesoderm. (A) *MyoD* expression after injection of control cells. All of the embryos showed normal *MyoD* expression (n=25). (B) *MyoD* expression after injection of Noggin-expressing cells. Ninety-one percent of the embryos showed ectopic expression of *MyoD* in the presomitic mesoderm and first somites (n=42/46). (C) Sections at the level of newly formed somite. (D) Sections at the level of presomitic mesoderm. Arrowheads in C and D indicate the ectopic expression of MyoD, on the injected and contralateral sides of the embryo. The broken line indicates the position of noggin-expressing cells. Sections levels are indicated by arrows in B. (A,B) Composite of a bright-field image of whole-mount or transverse section and a fluorescent image showing the DiI-labeled control and noggin-expressing cells. Somite numbers are indicated by roman numerals.

acquired by presomitic mesoderm cells between somite levels –I and 0.

Mesoderm-autonomous initiation of *MyoD* expression in vitro

The experiments described above indicate that, in vivo, the initiation of *MyoD* expression in the somite requires a *Wnt5b* signal, probably mediated by Fz7. It was, however, possible that *Wnt5b* and/or *Fz7* are themselves regulated by external cues. To determine this, we have cultured presomitic mesoderm isolated from surrounding structures for 3-4 hours. Under these experimental conditions, somite border formation does not occur, but normal segmentation is observed (which can be recognized by the metameric expression of molecular markers such as Delta1) (Fig. 6A) (Palmeirim et al., 1998; Palmeirim et al., 1997; Sosic et al., 1997). We observed that the expression of *Wnt5b* and *Fz 7* was initiated normally in the segments that formed during the incubation time (Fig. 6C,D), suggesting that the initiation of their expression is not regulated by signals emanating from surrounding tissues. However, the medial restriction of *Wnt5b* expression that are observed in vivo was not observed in the explants, indicating that their medial restriction is regulated by extrinsic cues.

Because the in vivo microsurgery and explant culture experiments described above indicate that myogenic signals and their mediators are independent from external cues, we then assayed the expression of *Myf5* and *MyoD* in presomitic mesoderm explants cultured in isolation from surrounding tissues. After 3-5 hours incubation, the two or three newly formed segments expressed *Myf5*, demonstrating that the initiation of its expression in the rostral presomitic mesoderm and in newly formed somites is mesoderm-autonomous (Fig. 6B). As shown for *Wnt5b*, *Myf5* expression was observed as transversal bands in the segments that formed in vitro. Thus, the restriction of *Myf5* expression to the medial region of the somite (observed in vivo) is also regulated by extrinsic cues. Perhaps because the Bmp inhibitory activity that we uncovered is tightly bound to presomitic mesoderm cells, *MyoD* expression was not observed in these conditions. A similar situation is observed in *Xenopus* animal pole tissue, where Bmp bound to cell membranes inhibits the activation of neural markers. This inhibition can be removed by cell dissociation (Godsave and Slack, 1989; Grunz and Tacke, 1989; Sato and Sargent, 1989). To test whether a similar situation is encountered here, we dissociated rostral presomitic mesoderm cells and placed them in culture. After 6 hours, *MyoD* expression was now observed (Fig. 6F). Conversely, dissociated cells cultured in conditioned medium from Bmp4-secreting cells did not express MyoD (Fig. 6N); this supports the hypothesis that Bmp bound to presomitic mesodermal cells

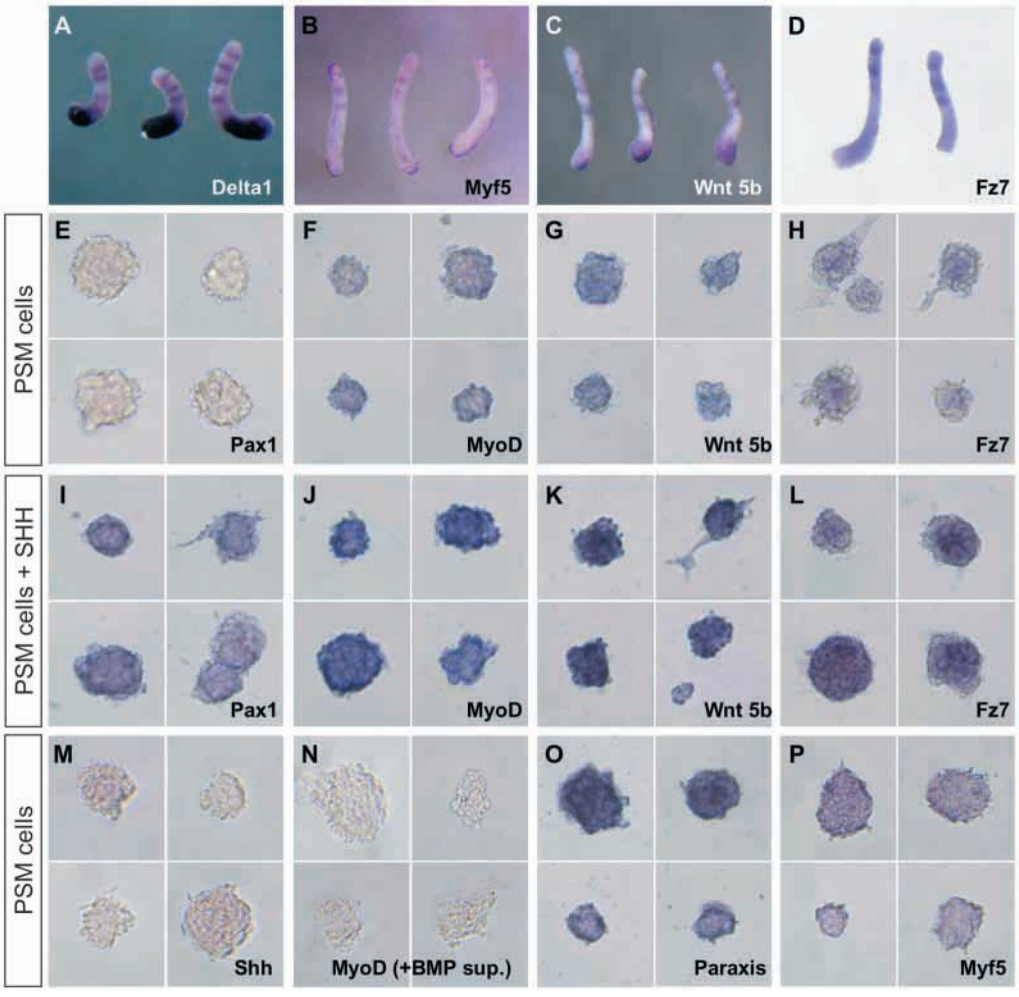

Fig. 6. Initiation of *MyoD* expression in vitro. (A,D) Culture of isolated presomitic mesoderm for 3-4 hours. (A) Delta1 (*n*=30), (B) *Myf5* (*n*=55), (C) *Wnt5b* (*n*=67) and (D) Fz7 (*n*=8) were autonomously expressed in these conditions. (E,P). Culture of dissociated rostral presomitic mesoderm cells. Note that the same results were obtained in six independent experiments. In these experimental conditions, *MyoD* (F), *Wnt5b* (G), *Fz7* (H), *Paraxis* (O) and *Myf5* (P) are expressed. *Pax1* (E) and *Shh* (M) serve as negative controls. When dissociated presomitic mesoderm cells are cultured in the presence of supernatant from Bmp4-expressing cells, initiation of MyoD expression is not observed (N). When Shh is added to dissociated presomitic cells, expression of *MyoD* (J), *Wnt5b* (K) and *Fz7* (L) is strongly enhanced, while *Pax1* (I) expression is activated.

is removed through cell dissociation. As controls, we found that *Wnt5b*, *Fz7* and *Myf5* (Fig. 6G,H,P) were expressed in these conditions, confirming that their expression is mesoderm-autonomous. *Paraxis*, which is normally expressed in the rostral presomitic mesoderm and in dorsal somites, was found to be expressed in dissociated cells (Fig. 6O), indicating that the identity of the presomitic mesoderm cells was not grossly altered during the time of the experiment. *Pax1* (which is not expressed in these culture conditions) and Shh (which is never expressed in the presomitic mesoderm) served as negative controls (Fig. 6E,M). Together, these data confirm the experiments performed in vivo and indicate that the molecular mechanisms which regulate the activation of myogenesis occur mesoderm-autonomously.

As previous studies had shown that *Shh* synergizes with Wnt to activate myogenesis (Munsterberg et al., 1995; Tajbakhsh et al., 1998), we tested whether addition of Shh protein to presomitic mesodermal cells in culture modifies *MyoD* expression. The expression of *Pax1*, which served as a positive control, was induced in these conditions (Fig. 6I). *MyoD* expression was enhanced in presence of Shh protein (Fig. 6J). However, the expression of *Wnt5b* and *Fz7* was increased as well (Fig. 6K,L): it is therefore unclear whether Shh acts directly on *MyoD* expression or indirectly through the activation of its putative activating signal, *Wnt5b* and of the receptor *Fz7*.

Together, these data confirm those obtained in vivo, and suggest that *MyoD* expression is activated in a Wnt-dependent, mesoderm autonomous fashion in the presomitic mesoderm. In the presomitic mesoderm region, external cues are likely to repress, through Bmp signaling, the premature activation of myogenesis. As somites form, Shh, which emanates from axial tissues, is likely to play a role in the enhancement and/or maintenance of inductive events that took place in the presomitic mesoderm.

Discussion

Based on the in vivo and in vitro data exposed above, we propose a model that summarizes the tissues and molecular interactions leading to the initiation of myogenesis in vertebrates (see Fig. 7). The central message in this model is that tissues surrounding the presomitic mesoderm do not participate in the initial steps of myogenesis. This information introduces a new conceptual route into initiation of myogenesis that contradicts the most popular model, which suggested that the presomitic mesoderm is a naïve tissue that awaits environmental cues to undergo myogenesis. Although sclerotome differentiation is likely to be controlled by such a mechanism, being totally dependent upon Hedgehog signaling to arise (Zhang et al., 2001), our results suggest that early muscle differentiation is controlled differently.

We show that Wnt signaling, previously identified as an important player in myogenesis, has a crucial role in the initiation of myogenic gene expression in the presomitic mesoderm. Our data reveal an unexpected paradox: *Myf5* and *MyoD*, two MRFs that can largely compensate each other in null mice, and thus are often thought of as almost interchangeable molecules, are differentially controlled by Wnt. Although we show that *Myf5* expression is independent of any identifiable signal, *MyoD* requires a Wnt signal to be activated. Based on converging evidence, we propose that *Wnt5b*, which is expressed in the presomitic mesoderm, is the Wnt signal required for *MyoD* activation.

The significance of *Myf5* expression for presomitic mesoderm cells is unclear. Although *Myf5* has been previously shown to act as a muscle master gene when overexpressed in cell culture (Braun et al., 1989), this cannot be its role in the presomitic mesoderm, because it is expressed in a large population of cells, not all bound for the muscle lineage (for a review, see Brand-Saberi et al., 1996). In mice mutant for *Myf5* and *MyoD*, it was shown that it is not only the presence, but also the dose of MRF that is important for muscle differentiation (Rudnicki et al., 1993). Therefore, it is possible that the low level expression of *Myf5* confers a bias or a competence of the entire rostral paraxial mesoderm towards the myogenic pathway prior to somitogenesis. The medial restriction of *Myf5* expression that is observed in vivo was not observed when presomitic mesoderm was cultured in isolation from surrounding structures. This indicates that extrinsic cues might restrict its expression medially. At present it is unclear whether, similar to MyoD, Bmp inhibiting signals participate in the medial restriction of Myf5. It is also possible that, as was recently shown in mouse, Myf5 expression in the medial somite would be positively modulated by activating cues, such as Shh, emanating from axial structures (Gustafsson et al., 2002).

As somites form, strong *MyoD* expression is observed in the medial somite. Experimental evidence indicate that cells present within the medial somite are committed to the myogenic lineage, as they differentiate into myocytes even when exposed to a challenging environment, i.e. a notochord influence (Williams and Ordahl, 1997). This suggests that the expression of *MyoD* is a crucial step in the molecular mechanisms that lead somitic cells to muscle terminal differentiation. The level of *MyoD* expression observed in dissociated presomitic mesodermal cells were lower than those which are observed in intact embryos. This indicates that

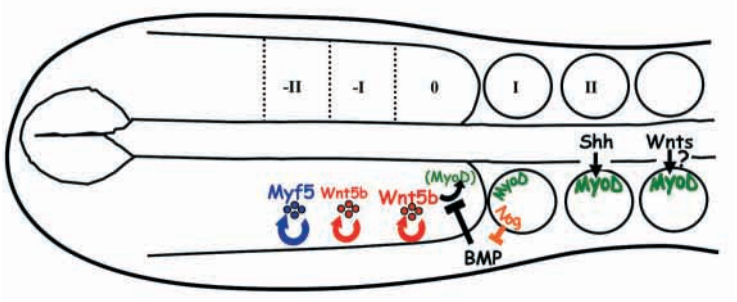

Fig. 7. A model for myogenesis initiation. The expression of *Myf5* is autonomously initiated in the presomitic mesoderm. Soon after, *Wnt5b*, which is autonomously expressed in the presumptive caudal border of somite –I and 0, triggers the activation of *MyoD* expression. However, this effect is inhibited by the action of Bmp. Noggin, expressed in the lateral region of the rostral presomitic mesoderm and the newly formed somites, is likely to release Bmp inhibition. This leads to the expression of *MyoD*. Shh (and maybe Wnts), secreted by axial structures, amplifies and/or stabilizes *MyoD* expression in older somites.

additional factor(s) might be required to obtain a strong expression of *MyoD* in newly formed somites. We show here that the addition of Shh to dissociated presomitic mesoderm cells enhances *MyoD* expression, thereby providing experimental evidence for a role of Shh in the amplification of the mesoderm-autonomous activation of MyoD. Importantly, Shh also enhances the transcription of the putative *MyoD*-activating signal, *Wnt5b* and its receptor *Fz7*, indicating that the enhancing activity of Shh on MyoD might be indirect. In addition, Shh was previously shown to act both as a cell survival and as a maintenance factor during myogenesis (Teillet et al., 1998; Marcelle et al., 1999; Duprez et al., 1998); this emphasizes the complexity of Shh role during this process.

The data presented here suggest that intrinsic Wnt and extrinsic *Shh* signaling are sequentially required to activate a cascade of molecular events that lead the presomitic mesodermal cells towards their terminal myogenic differentiation. Recent evidence indicates that Wnt and Shh signaling might interact directly during myogenesis, as (1) Wnt signaling in the presomitic mesoderm regulates the expression of the *Gli* effectors, which mediate *Shh* activity (Borycki et al., 2000), whereas (2) GSK3, an effector of the β-catenin-dependent Wnt signaling, directly modulates the transcriptional activity of *Gli* (Price and Kalderon, 2002; Jia et al., 2002). Thus, it would be interesting to examine whether *Gli* molecules could serve as a molecular node where Wnt and *Shh* signaling converge and synergize to regulate the expression of *MyoD* in somites.

The observation that ectopic *Wnt5b* opposes the *MyoD*-inhibitory effect of S-Fz7 demonstrates that Wnt5b binds Fz7. Interestingly, *Wnt5b*, its putative receptor *Fz7* and *MyoD* are co-expressed in somitic cells. Although this could suggest that *Wnt5b* mediates its myogenic activity in an cell autonomous fashion, the observation (1) that myogenic differentiation is more conspicuous if presomitic mesoderm cells are cultured at high density (George-Weinstein et al., 1997; George-Weinstein et al., 1996), and (2) that *MyoD* expression was observed in the present work in groups, rather than isolated presomitic cells, suggests on the contrary that *Wnt5b* might act in a non cell-autonomous fashion.

Although the in vitro and in vivo data presented here indicate that *Wnt5b* might play a necessary role in initiation of myogenesis, this does not rule out the possibility that other signaling molecules may also be involved. In *Drosophila*, the *Notch/Delta* pathway has been shown to play a fundamental role in this process (Baylies and Michelson, 2001; Frasch, 1999). In chick, overexpression of *Delta* inhibits *MyoD*, but not *Myf5* expression in differentiated muscle cells (Delfini et al., 2000; Hirsinger et al., 2001). Although the relevance of these observations to the process of muscle determination in vivo is not yet understood, it is clear that there are still a number of avenues to be explored in order to fully understand how mesodermal cells progress towards myogenesis in vertebrates.

An unanswered question is whether Wnts in the neural tube or in the ectoderm have a role in myogenic differentiation. *Wnt1 and Wnt3a* in the neural tube regulate the formation and the maintenance of the dorsomedial lip (DML), a structure that specifically expresses Wnt11, and that drives the growth and the morphogenesis of both the epaxial myotome and the dermomyotome (Marcelle et al., 1997; Ikeya and Takada, 1998; Ordahl et al., 2001). Thus, if the source of Wnt1 and Wnt3a (i.e. the dorsal neural tube) is surgically removed, myogenesis is initiated (as shown in the present work), but the DML is not formed, and therefore muscle growth is arrested. A similar result is obtained if the DML itself is surgically removed (Ordahl et al., 2001). This results in embryos where dorsal muscles are missing, not because they are not induced, but because they do not grow. In mice mutant for *Wnt1* and *Wnt3a*, a similar phenotype is observed (Ikeya and Takada, 1998). This suggests that *Wnt1* and *Wnt3a* from the dorsal neural tube act similar to Shh as maintenance and growth factors for the epaxial myotome. Wnts are also expressed in the ectoderm where they are believed to play a role in the specification of the dorsal compartment of the somite, the dermomyotome (Fan et al., 1997; Maroto et al., 1997; Capdevila et al., 1998; Wagner et al., 2000). As the dermomyotome is the source of muscle cells, it is likely that a change in its specification would lead indirectly to modifications in myotome formation. Thus, neither the mutant mice nor experiments performed in vivo have demonstrated that these or other Wnts secreted from the tissues surrounding the somites play a direct role in myogenesis.

Premature initiation of myogenesis in the presomitic mesoderm is abrogated by Bmp. Lateral plate mesoderm is a plausible source for this Bmp signal (Pourquie et al., 1996). But it is also possible that Bmp-like molecules expressed in other tissues, including the presomitic mesoderm itself, would play this role in vivo. We and others have shown that the initiation of Noggin expression in the presomitic mesoderm, slightly before somites form, is likely to play a role in the release of the Bmp inhibitory activity (Hirsinger et al., 1997; Marcelle et al., 1997; Reshef et al., 1998). Interestingly, Bmp4 in the lateral plate mesoderm was recently shown to activate the expression of its own inhibitor noggin in the presomitic mesoderm, thereby uncovering an unexpected active role of Bmp in the initiation of *MyoD* expression (Sela-Donenfeld and Kalcheim, 2002). Other possible players in antagonising Bmp activity are the Bmp inhibitors Follistatin and Flik (Follistatin-like protein), which are both expressed in the dermomyotome (Amthor et al., 1996; Patel et al., 1996; Zimmerman et al., 1996). Interestingly, Fgf8 expressed in the caudal presomitic mesoderm was recently shown to inhibit myogenesis (Dubrulle et al., 2001). This observation is consistent with a model where myogenesis in the presomitic mesoderm is actively repressed by multiple molecular mechanisms until somites form.

In conclusion, the experiments and observations described here suggest that at the time that somitogenesis is taking place, paraxial mesoderm is not entirely naive, as myogenesis is initiated, but actively repressed, by Bmp signaling. Myogenesis, which has been examined in the past largely as a one-step event, is likely to be the result of a number of intrinsic and extrinsic signals that synergize in a highly organized choreography to form the primitive vertebrate skeletal muscle.

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