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A molecular mechanism enabling continuous embryonic muscle growth – a balance between proliferation and differentiation

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SUMMARY

Embryonic muscle growth requires a fine balance between proliferation and differentiation. In this study we have investigated how this balance is achieved during chick development. Removal of ectoderm from trunk somites results in the down-regulation of *Pax-3* expression and cell division of myogenic precursors is halted. This initially leads to an up-regulation of *MyoD* expression and to a burst in terminal differentiation but further muscle growth is arrested. Locally applied bone morphogenetic protein-4 (BMP-4) to somites mimics the effect of the ectoderm and stimulates *Pax-3* expression which eventually results in excessive muscle growth in somites. Surprisingly, BMP-4 up-regulates expression of *noggin* which encodes a BMP-4 antagonist. This suggests that the proliferation enhancing activity of BMP-4 can be limited via up-regulation of *noggin* and that myogenic cells differentiate, as an intrinsic property, when deprived of BMP-4 influence. In contrast to BMP-4, Sonic hedgehog (Shh) locally applied to somites arrests muscle growth by down-regulation of *Pax-3* and immediate up-regulation of *MyoD* expression. Such premature muscle differentiation in somites at tongue and limb levels prevents myogenic migration and thus tongue and limb muscle are not formed. Therefore, precise limitation of differentiation, executed by proliferative and *Pax-3* promoting signals, is indispensable for continuous embryonic muscle growth.

Key words: Chick embryo, Muscle development, Somite development, Ectoderm, BMP-4, Sonic hedgehog

INTRODUCTION

The formation of skeletal muscle in vertebrates begins during early embryogenesis and extends into adult life. However, during the embryonic phase the organism produces innumerable muscle cells and a diversity of different muscle which during fetal life are used as a matrix for secondary myotube development (Duxson and Sheard, 1995). Disturbance of embryonic muscle growth results in a deficit of muscle in perinatal and adult life when extracellular or intracellular signalling pathways are altered (e.g. Detwiler, 1926; Tajbakhsh et al., 1997). Therefore it is imperative that the organism produce the appropriate number of embryonic muscle cells since any deficiencies are unlikely to be compensated for during later development.

Skeletal muscle of trunk, limbs and tongue originates from paraxial mesenchyme, but muscle cells do not emerge before the paraxial mesenchyme is segmented into somites (reviewed by Christ and Ordahl, 1995). Somitic cells are the source of several cell lineages and only a subset are committed to a muscle fate. Cells in the ventral half of the somite undergo an epithelial-mesenchymal transition and form the sclerotome which gives rise to the axial skeleton and ribs. The dorsal half of the somite remains epithelialised and forms a highly proliferative compartment called the dermomyotome which is the source of the muscle and connective tissue of the dermis of the back (Christ et al., 1983). Cells at the edges of the dermomyotome de-epithelialise and ingress under the dermomyotome and form the myotome – a sheet of differentiating and postmitotic muscle cells (Denetclaw et al., 1997; Williams and Ordahl, 1997, Kahane et al., 1998b). Unlike the ventral portion of the somite where all the cells rapidly delaminate and are committed to a sclerotomal fate, myotomal cells are recruited from the dermomyotome continually over many days. Therefore myotomal precursors are maintained in an undifferentiated state in the dermomyotome until influenced by environmental cues to differentiate (Hirsinger et al., 1997; Marcelle et al., 1997; Borycki et al., 1998; Reshef et al., 1998).

Interestingly, muscle cells which originate from the dorsomedial part of the somite differentiate rapidly and form epaxial muscle (intrinsic back muscle). In contrast, myogenic cells which originate from the ventrolateral somite quarter and form hypaxial muscle (limb, tongue and ventral body wall muscle) are restrained from differentiation for almost 2 days (Ordahl and Le Douarin, 1992; reviwed by Christ and Ordahl,

1995). During this period myogenic cells delaminate from the ventrolateral lip of the dermomyotome and migrate as undifferentiated precursors at limb and tongue level and populate their respective muscle primordia (Christ et al., 1977; Schemainda, 1979).

The onset of myogenic differentiation occurs with the expression of the Myogenic Regulatory Factor (MRF) genes (Weintraub et al., 1991). In birds, *MyoD* is the first member of this family of transcription factors to be expressed, which subsequently induces other MRFs and eventually results in expression of muscle-specific proteins (Pownall and Emerson, 1992). Another transcription factor, *Pax-3*, is expressed in a wide range of neural tissues and also in paraxial mesoderm, where its expression is considered to specifically mark myogenic precursors (Strachan and Read, 1994; Williams and Ordahl, 1994). Mutations in the *Pax-3* locus in *Splotch* mice lead to an absence of limb and tongue muscle and a reduction in trunk muscle mass (Franz et al., 1993; Tajbakhsh et al., 1997). The developmental mechanism which leads to the muscle defect in the *Splotch* mutant is not fully understood. The *Splotch* phenotype in the limb may simply result from a failure to activate myogenic migration, since Pax-3 induces expression of the receptor tyrosine kinase *c-met* (Epstein et al., 1996), which upon activation by its ligand, Scatter Factor, enables myogenic precursors to migrate from the somites into limb and tongue primordia (Bladt et al., 1995; Brand-Saberi et al., 1996). Additionally, *Pax-3* might regulate cell proliferation, since over-expression of this gene results in cells becoming oncogenic (reviwed by Dahl et al., 1997) and a gain-of-function *Pax-3* mutation results in alveolar rhabdomyosarcoma, a highly proliferative cancer (Shapiro et al., 1993; Fredericks et al., 1995). In contrast to a putative role in regulating proliferation, recent work has suggested that *Pax-3* acts up-stream of *MyoD* and can induce muscle differentiation (Maroto et al., 1997). Since *MyoD* is expressed in the trunk of *Splotch* mice these data suggest the existence of other muscle lineages. Indeed, mutation in the *Myf-5* gene as well as *Pax-3* is required to eliminate almost all skeletal muscle from trunk and limbs (Tajbakhsh et al., 1997). However, other pathways leading to *MyoD* induction exist since head muscle expresses *MyoD* even in *Pax-3/Myf5* mutants (Tajbakhsh et al., 1997) and dispersed epiblast cells expresses *MyoD* without having expressed either *Pax-3* or *Myf-5* by this stage (George-Weinstein et al., 1996).

In this study we address the question of how muscle precursors of the dermomyotome are maintained in an undifferentiated and proliferating state with the ultimate aim of determining the tissues responsible for this action and identifying candidate factors. We have previously shown (Amthor et al., 1998) that muscle precursors of the limb are maintained in an undifferentiated, *Pax-3*-expressing state by signals originating from the ectoderm. Limb ectoderm ablation leads to a down-regulation of *Pax-3* and cells become post-mitotic and express *MyoD* as an indication of myogenic differentiation. Premature differentiation following ectoderm ablation eventually leads to a decrease in limb muscle mass presumably through the exhaustion of the muscle precursor pool. We demonstrated that low levels of BMPs mimic the effect of limb ectoderm and maintain *Pax-3* expression. Furthermore, increase in ectodermal *Bmp* expression by local application of Shh to limb mesenchyme is linked to an excessive muscle growth.

In this paper we report that dorsal trunk ectoderm maintains muscle cells of the dermomyotome in an undifferentiated proliferating precursor state. Furthermore, we provide evidence that the identity of the proliferative ectodermal signal is BMP-4. Local application of BMP-4 to somites primarily enhances *Pax-3* expression and secondary *MyoD* expression which eventually leads to excessive muscle growth. Remarkably, simultaneously with *MyoD* induction, BMP-4 up-regulates *noggin*, a BMP-4 antagonist (Zimmerman et al., 1996). These data demonstrate that trunk muscle generation, like limb muscle development, can occur through a default pathway. We suggest that cells are maintained in a proliferative and undifferentiated precursor state by ectodermal signals. Muscle cells only differentiate after escaping the influence of a proliferative signal either by displacement or through the action of an antagonist. We provide evidence that Shh can also antagonise proliferative signals and induce premature muscle differentiation which arrests further muscle growth in the trunk and prevents the population of limbs and tongue with muscle.

MATERIALS AND METHODS

Preparation of chick embryos

Fertilised chicken eggs were incubated at 38°C, and the embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). Experiments were performed on embryos at stage 13 to 24, which were re-incubated at 38°C and then killed and processed for whole-mount in situ hybridisation or antibody staining.

BMP and Shh bead preparation

BMP-4 was provided by the Genetics Institute, Cambridge Massachusetts. Shh was a gift from Professor Andy McMahon (Boston, Massachusetts). All proteins were applied to 80-120 μm beads. Shh was applied to Affigel beads (Sigma, UK) and BMP-4 was applied to heparin acrylic beads (Sigma, UK). Shh was used at 3-14 mg/ml and BMP-4 at 1-10 μg/ml. Proteins were loaded onto beads as described by Cohn et al. (1995).

Bead application and microsurgical procedures

For bead implantation, somites were punctured with a electrolytically sharpened tungsten needle, and a bead was inserted into the punctured mesenchyme using a blunt glass needle. For ectoderm removal, the ectoderm was stained with Nile Blue in ovo using a blunt glass needle coated with 2.5% agar containing 2% Nile Blue. The ectoderm was peeled from the mesenchyme immediately after staining. At the thoracic level, ectoderm was removed from stage 21-23 embryos extending from somites 21 to 26 and from the lateral side of the neural tube at a dorsomedial limit down to the abdominal wall at a ventrolateral limit. Beads were inserted as described less than 5 minutes following ectoderm removal. Ectoderm removal from stage 13/14 embryos extended either over the whole unsegmented paraxial mesoderm or from somite I-XII (somite staged as described by Ordahl, 1983). Lateral plate was separated from unsegmented paraxial mesoderm by cutting a sagittal slit through all three germ layers laterally of the unsegmented paraxial mesoderm.

Whole-mount in situ hybridisation

All chick embryos were washed in PBS and then fixed overnight in 4% paraformaldehyde at 4°C. Anti-sense RNA probes were labelled with either digoxigenin or fluorescein, and whole-mount in situ hybridisation was performed as described by Nieto et al. (1996). The following probes were used in this study: *Bmp-2*, PCR-cloned fragment (nucleotides 1-797); *Bmp-4*, PCR cloned fragment (nucleotides 1-953,); full-length *Bmp-7* (1.1 kb; gift from Dr Antony Graham); *Follistatin*, full length 1.1 kb fragment; *MyoD*, clone CMD9 full 1.5 kb length fragment (gift from Bruce Patterson); *Noggin*, full length clone – approximately 700 bp; *Patched*, 900 bp PCR fragment

(gift from Dr Cliff Tabin); *Pax-3*, 645 bp fragment corresponding to nucleotides 468-1113 (gift from Dr Martin Goulding). *C-Sim-1* full length clone was a gift from Dr J. Cooke. *Shh* full length clone was a gift from Dr Jane Dodd. Whole-mount embryos were partly cryo-sectioned for further histological examination.

Cell death assay

Embryonic trunks were dissected free of all unwanted tissue and then incubated in Acrydine Orange (100 ng/ml in PBS) at 37°C for 30 minutes. Specimens were then washed 2× 2 minutes in PBS and then flattened on a microscope slide under a coverslip (Weil et al., 1997). Samples were photographed immediately using fluorescence illumination.

BrdU labelling

30 minutes before fixation, 100 µl of 40 mM 5-bromo-2′-deoxyuridine (BrdU, Sigma) dissolved in water was added on the vitelline membrane and embryos were re-incubated at 38ºC. Embryos were fixed overnight in Serra's solution (60% absolute ethanol, 30% formaldehyde, 10% glacial acetic acid) at 4°C, dehydrated, wax-embedded and sectioned. Antibody staining was preceded by hydrolysis for 30 minutes with 2 N HCl. Immunohistochemistry was performed by the indirect immunoperoxidase method with monoclonal anti-BrdU antibody (DAKO) and peroxidase-conjugated goat anti-mouse IgG (Sigma) as second antibody. DAB was used as chromogen, a weak counter-staining was performed with true red.

Immunohistochemistry on serial sections

Embryos were fixed in Serra's solution, wax embedded and serially sectioned at 8 µm. Sections were labelled with either a desmin monclonal antibody (DAKO, 1:100) or with a muscle specific actin monoclonal antibody (Sigma, 1:5000). The secondary antibody was a peroxidase-conjugated goat anti-mouse Ig antibody (Sigma, 1:300). Diaminobenzidine (DAB) was used as a chromogen.

Immunohistochemistry on whole-mounts

Embryos were fixed overnight in 4% paraformaldehyde (PFA), dehydrated in 100% methanol, incubated for 1 hour in 5:1 methanol/H_2O_2, washed in PBT (PBS containing 0.5% Tween), incubated up to 45 minutes in 20 µg/ml proteinase K (in PBT), re-fixed in 4% PFA (in PBT), incubated for 1 hour in 1% horse serum (in PBT), incubated overnight with an anti-desmin monoclonal antibody (DAKO, 1:100, in horse serum/PBT), washed in PBT, incubated overnight in secondary antibody (peroxidase-conjugated goat anti-mouse Ig antibody, Sigma, 1:300, in horse serum/PBT), washed in PBT, washed in AP-buffer (Nieto et al., 1996), incubated for 5-10 minutes in colour reagent (4.5 µl NBT and 3.5 µl BCIP in 1 ml AP-buffer; Boehringer Mannheim), washed in AP-buffer, cleared overnight in di-methylformamide at 4°C and stored in 4% PFA.

Resin and Feulgen histology

Embryos were fixed in 0.12 M sodium cacodylate solution containing 3% glutaldehyde and 2% formaldehyde. They were embedded in epon resin (Serva) using standard procedures and sectioned at 0.75 µm using a Leitz-Ultra-Cut-S microtome. For Feulgen staining, wax-embedded sections (8 µm) were stained according to standard procedures (light green and Schiff's Reagent).

RESULTS

Spatial relationship of the muscle markers *Pax-3/MyoD* and the signalling molecules *Bmp-4* and *Noggin* in the embryonic trunk

The spatial distribution of muscle precursors and muscle cells was correlated with cellular proliferation within the somite. In situ hybridisation was used to detect RNA transcripts of *Pax-3* (a marker for undifferentiated myogenic cells; Williams and Ordahl, 1994) and *MyoD* (a marker for differentiating muscle

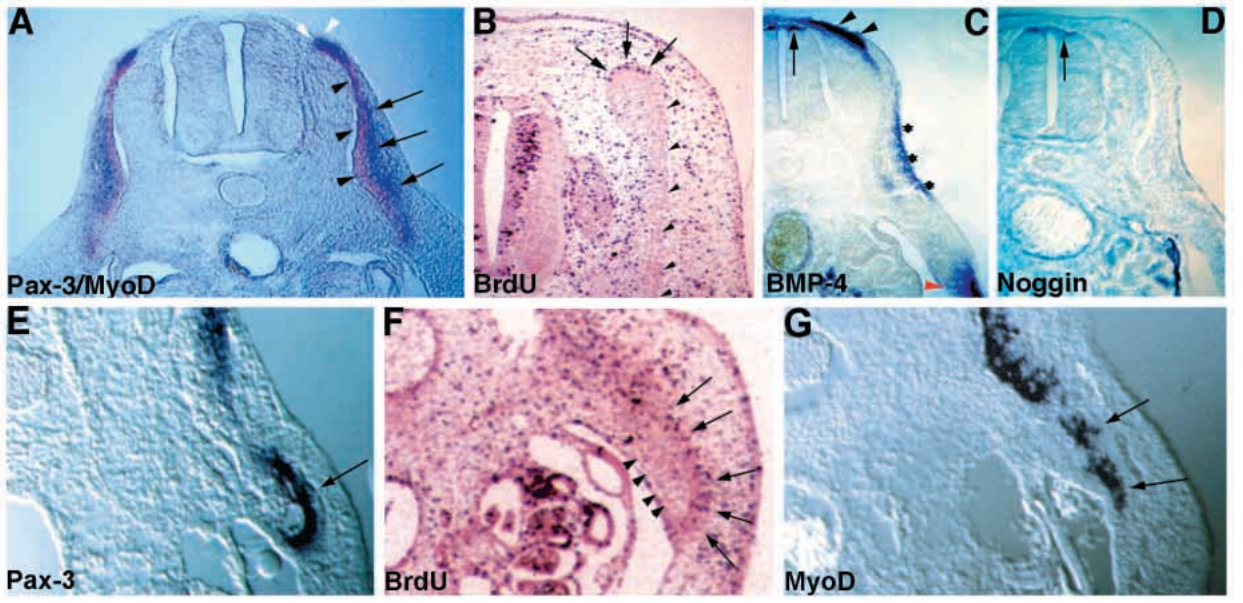

Fig. 1. Expression pattern of *Pax-3*, *MyoD*, *BMP-4* and *Noggin* and cell proliferation during trunk muscle development. Transverse sections of trunk at thoracic level at stage 25. (A) Two colour in situ hybridisation shows that *Pax-3* (blue, arrows) is expressed close to the ectoderm and with highest expression at the dorsomedial lip of the dermomyotome (white arrowheads). *MyoD* (red, black arrowheads) is expressed medially of *Pax-3*. (B) BrdU incorporation is highest in the dorsomedial lip of the dermomyotome (arrows) and lowest in the myotome (arrowheads). (C) *BMP-4* is expressed in dorsal neural tube (arrow), dorsal ectoderm as well as in adjacent sub-ectodermal tissue (arrowheads), in ectoderm and subectodermal mesenchyme laterally (asterisks) and mesenchyme ventrally to somites (red arrowhead). (D) *Noggin* is expressed in the dorsal limit of the neural tube (arrow). (E) High level of *Pax-3* expression in the ventrolateral lip of the dermomyotome (arrow). (F) BrdU is incorporated in the ventrolateral lip of the dermomyotome (arrows) but not in the myotome of the hypaxial domain (arrowheads). (G) *MyoD* expression in the myotome of the hypaxial domain (arrows).

cells; Olson, 1992) and these were compared with the distribution of BrdU-labelled nuclei on transverse sections of thoracic somites of stage 25 chick embryos. *Pax-3*-expressing cells were located in the dispersed derivative of the dermomyotome between the ectoderm and the *MyoD*-expressing myotome (Fig. 1A). The highest level of *Pax-3* expression was found in the dorsomedial and ventrolateral lips of the dermomyotome (Fig. 1A,E). At these lips the dermomyotome remained epithelialised and partially encircled the MyoD-expressing myotome (Fig. 1A,E,G and data not shown). Moderate BrdU incorporation was detected in the dermomyotome where *Pax-3* was expressed as well as in the sclerotome (Fig. 1B). There was almost no BrdU incorporation in the myotome, neither in the epaxial nor in the hypaxial domain (Fig. 1B,F). However, the greatest amount of *Pax-3* expression and highest level of BrdU incorporation coincided in the dorsomedial and ventrolateral lips of the dermomyotome (Fig. 1A,B,E,F).

These results show that the developing trunk musculature is divided into two layers. There is a *Pax-3*-expressing layer of undifferentiated and proliferating cells situated close to the ectoderm and a *MyoD*-expressing layer of differentiating and non-proliferating cells positioned more centrally. Thus in the embryonic trunk there is a spatial organisation of myogenic cells which is reminiscent of the myogenic organisation described in the limb (Amthor et al., 1998).

It has been proposed that BMPs are responsible for maintenance of *Pax-3* expression in muscle precursors of the limb (Amthor et al., 1998). The expression of *Bmp-2*, *-4* and *-7* in the trunk of stage 25 chick embryos was analysed by in situ hybridisation. Only *Bmp-4* expression was detected in the ectoderm and subectodermal mesenchyme dorsomedially and laterally of the dermomyotome as well as in the dorsal neural tube and in the mesenchyme of the prospective abdominal wall (Fig. 1C). Thus transcripts were localised close to *Pax-3* expression. At this late stage, *noggin* was strongly expressed in the dorsal neural tube (Fig. 1D). In the somitic mesenchyme *noggin* was expressed only at very low levels.

Thus, as during the development of limb muscle, *Pax-3*-expressing cells in the trunk are flanked by *Bmp-4*-expressing cells.

Ectoderm maintains proliferation of myogenic cells in the trunk

To determine if trunk ectoderm, like limb ectoderm, was a source of a proliferation signal during embryonic trunk muscle growth, embryos were analysed following ectoderm removal. Stage 21-23 embryos (n=19) had the ectoderm mechanically removed in the thoracic region, and *Pax-3* expression, BrdU incorporation, *MyoD* expression and terminal differentiation markers were analysed at various time thereafter. 20 hours after ectoderm removal, *Pax-3* expression was no longer detected in the dorsomedial lip and ventrolateral lip of the dermomyotome but was only slightly reduced in the part of the dermomyotome which is situated between the dorsomedial and ventrolateral lips (Fig. 2A1 and 2B1). This loss of *Pax-3* expression was linked to a marked decrease in the number of BrdU-labelled nuclei in the mesenchyme located between the ectoderm and the myotome and in the dorsomedial and ventrolateral lips of the dermomyotome 17 hours after ectoderm removal (Fig. 2B4 and compare with Fig. 1B and 1F). We followed the fate of the myogenic cells and detected an increase in *MyoD* expression in the myotomes as early as 11 hours after ectoderm removal and transverse sections showed thicker myotomes on the operated side compared to the contralateral (data not shown). After 20 hours, the up-regulation of *MyoD* was more prominent and myotomes were still thicker but shorter in mediolateral extension than on the contralateral side (Fig. 2A2a and Fig. 2B2). After 48 hours, *MyoD* expression in the myotomes was still considerably up-regulated and the myotomes were only half as long mediolaterally as contralateral myotomes (Fig. 2A2b). 24 hours after ectoderm removal, the cells of the myotome completed the myogenic programme and expressed markers of terminal differentiation (n=18) such as desmin (Fig. 2A3, B3) and actin (data not shown). The myotome appeared thicker but shorter in ventrolateral extension when compared to the contralateral side (Fig. 2B3). Ectoderm healed in over ablated areas over several days (data not shown).

These data suggest that the ectoderm maintains closely situated myogenic cells in a proliferating and *Pax-3*-expressing state and limits muscle differentiation to the myotome. If the ectoderm is removed, *Pax-3*-expressing cells immediately differentiate. This exhausts the reservoir of proliferating muscle precursors and ultimately arrests further growth of trunk muscle. Therefore, ectodermal signals ensure continuous embryonic muscle growth in the trunk by limiting differentiation of myogenic cells which is similar to the situation during limb muscle development (Amthor et al., 1998).

Withdrawal of *MyoD* repressing signals initiates premature muscle differentiation in early somites

Ablation of limb and dorsal trunk ectoderm accelerated *MyoD* expression. We determined whether the withdrawal of proliferation signals could initiate premature muscle differentiation in somites at stages where *MyoD* is not normally expressed. The ectoderm overlying somites I-XII at stage 14 was removed (n=12). This resulted in *MyoD* being expressed prematurely one somite more caudally than on the unoperated side following a re-incubation of only 4 hours (Fig. 2C1). Transverse sections showed that *MyoD* expression was up-regulated in the proper dorsomedial compartment (Fig. 2C2). After 24 hours of re-incubation only a few *MyoD*-expressing cells were found next to the neural tube whereas *MyoD* was expressed throughout the myotome on the contralateral side (Fig. 2C3). At these young stages we noted that ectoderm regeneration occurred over ablated areas within 24 hours after operation.

Pourqui et al. (1996) have found that lateral plate mesoderm is a strong inhibitor of *MyoD* expression and inducer of *Pax-3* expression in somites. We repeated this experiment and separated somites I-IV of stage 13/14 embryos (n=6) from the intermediate mesoderm by cutting a sagittal slit laterally of the somites and inserted a gold-leaf. 24 hours after such an operation we found *MyoD* expression spread over the entire mediolateral extent of the somites at the operated side (data not shown). We varied this experiment and separated the segmental plate mesoderm (SPM) from the lateral plate as well as removed the ectoderm overlying the SPM (posteriorly of somite I) of stage 13/14 embryos (n=12). After 8 hours of re-incubation, the SPM at the anterior level of the operation site had formed 5 new somites, of which somite II to IV expressed

MyoD in their medial aspect, unlike the normal somites at the corresponding contralateral level (Fig. 2C4). After 24 hours of re-incubation *MyoD* expression spread over the entire mediolateral extent of the somites at the operated side (data not shown). This indicates that the lateral somite half has been medialised after separation of lateral structures. However, these manipulations did not force *MyoD* expression in unsegmented paraxial mesoderm (data not shown).

Therefore myogenic precursors prematurely expressed *MyoD* and entered the differentiation programme after having been released from the suppressing influence of the ectoderm and the lateral plate. This eventually prevents myotome formation presumably through the exhaustion of the proliferative pool. These results are consistent with the observation of arrest in muscle growth after ectoderm removal from advanced somites or from wing buds (Amthor et al., 1998).

BMP-4 rescues *Pax-3* expression after ectoderm removal in the trunk

We demonstrated that *Pax-3*-expressing cells of the dermomyotome are flanked by *Bmp-4*-expressing cells. BMPs have been implicated in maintaining *Pax-3* expression during limb muscle development as well as during early somite development (Pourquié et al., 1996; Amthor et al., 1998). To test if BMP-4 can up-regulate *Pax-3* expression in the embryonic trunk, beads soaked in 10 μg/ml of BMP-4 were applied to somites at stages 20-22 and the effect on *Pax-3* and *MyoD* expression was determined (n=10). Implantation of BMP-4 beads into thoracic somites in the presence of ectoderm induced strong *Pax-3* expression after 18 hours (data not shown). By 38 hours, *Pax-3* was still up-regulated (Fig. 3A1) but intriguingly, we simultaneously found an up-regulation of *MyoD* (Fig. 3A3). Frontal sections of such an embryo revealed an enormous expansion of the myotomal layer and neighbouring myotomes were fused (Fig. 3A4). When beads were soaked in lower concentrations of BMP-4 (1 μg/ml) and applied to trunk somites at stage 23 (n=7) no change in *Pax-3* or *MyoD* expression was observed (data not shown). Beads soaked in high concentration of BMP proteins (100 μg/ml BMP-2 or in 1 mg/ml BMP-7) and applied to trunk somites lead to loss of *Pax-3* and *MyoD* as demonstrated (Amthor et al., 1998).

We then determined whether BMP-4 could substitute for the ectoderm signal. BMP-4 beads were inserted into the somitic mesenchyme of stage 23 embryos after ectoderm removal (n=5). 16 hours after re-incubation we found that the high *Pax-3* expression in the dorsomedial part of the dermomyotome was maintained near the bead whereas neighbouring somites lost this expression (Fig. 3A2 and compare with Fig. 2A1).

Recent work has shown that over-expression of BMP-4 in the pre-somitic mesoderm leads to lateralisation of the somite (Tonegawa et al., 1997). We were unable to detect lateralisation (e.g. ectopic expression of *cSim-1*) after the implantation of BMP-4 beads into the trunk somites at stage 20 (data not shown) probably because our implants were done well after the somite compartmentalisation had been established. Recently, we demonstrated that BMP-4 beads (10 μg/ml) could induce cell death in limb mesenchyme which we visualised in vivo by staining with Acridine Orange (Amthor et al., 1998). However, we were unable to detect cell death following implantation of BMP-4 beads into thoracic somites (data not shown). Heparin beads soaked in PBS did not disrupt the normal expression pattern of either *Pax-3* or *MyoD* (Fig. 3B1,B2,B3). Histological examination (using Feulgen-stained wax sections and toluidine-blue-stained semi-thin sections) of tissue architecture after implantation of beads soaked in PBS in trunk somites revealed no changes in morphology and beads were well integrated in the somitic environment (Fig. 3B4 and data not shown).

Thus local application of BMP-4 induces an expansion of *MyoD* expression by amplifying the *Pax-3*-expressing precursor pool and even maintains *Pax-3* expression after ectoderm removal. This is reminiscent to the role of BMPs during limb muscle growth (Amthor et al., 1998).

BMP-4 induces *Noggin*, the gene encoding its own antagonist

It was striking that 36 hours after BMP-4 bead implantation both *Pax-3* and *MyoD* expression were up-regulated despite previous findings which indicated that BMPs are only strong inducers of *Pax-3* (Pourquié et al., 1996; Amthor et al., 1998; Reshef et al., 1998). One possibility for this difference is that BMP-4 could induce its own antagonist which would inhibit proliferation and support muscle differentiation through a default pathway (see Discussion).

We performed a series of experiments in which we inserted BMP-4 beads into somites of stage 22 embryos, re-incubated for 18 hours and determined the distribution of *noggin* transcripts (n=4). We found that BMP-4 up-regulated *noggin* expression in somites (Fig. 3C1). Transverse sections of the thorax revealed up-regulation of *noggin* in the mesenchyme over a distance of approximately 100 μm from the bead (data not shown). These results suggest that BMP-4 not only maintains *Pax-3* expression and cell proliferation but also indirectly initiates muscle differentiation by induction of its antagonist *noggin* (Zimmermann et al., 1996).

Next we asked if such a cascade of gene regulation could initiate the first *MyoD* expression in the somites. Previous work has identified the dorsal neural tube as being able to induce muscle differentiation in paraxial mesoderm (Stern et al., 1995; Spence et al., 1996). However, it seems contradictory that BMP-4 is expressed in the dorsal neural tube at the time of first *MyoD* transcription in adjacent somitic tissue (Pourquié et al., 1996). We applied a BMP-4 bead on top of the neural tube at the level of caudal SPM of stage 14 embryos (n=3). This resulted in a marked up-regulation of *noggin* expression in the neural tube after 24 hours (Fig. 3C2 and 3C4; bead dislodged during photography) compared to a non-treated neural tube (Fig. 3C3). Heparin beads soaked in PBS did not induce *noggin* expression in somites or in neural tube (data not shown).

These results indicated that muscle differentiation may not be induced by the dorsal neural tube as previously suggested, but that Noggin expressed by the neural tube antagonises the proliferative BMP signals and myogenic cells differentiate through a default pathway.

Shh forces myogenic cells within somites into premature differentiation preventing further muscle growth

Our data suggest that withdrawal of proliferative signals leads to muscle differentiation through a default pathway. In contrast,

others have suggested that Sonic hedgehog can up-regulate *MyoD* expression (Johnson et al., 1994; Borycki et al., 1998). This supports the model of muscle differentiation as an inductive process (reviwed by Cossu et al., 1996a). In the embryonic trunk, Shh is expressed in ventral axial structures (notochord and floor plate; Marti et al., 1995) that are closer to *MyoD*-expressing cells than to *Pax-3*-expressing cells.

To test if muscle differentiation can be induced by Shh, beads soaked in 14 mg/ml Shh protein were inserted into mature somites in which cells were committed to a muscle fate (*n*=29). Application of Shh beads to somites of a wide range of developmental stages (18-23) in which the myotome had formed resulted in complete down-regulation of *Pax-3* expression (Fig. 4A1 and Fig. 4B1) and simultaneous up-regulation of *MyoD* expression (Figs 4A2, 5B3) as early as 12 hours after manipulation. Examination of transverse sections showed that Shh caused an expansion of the *MyoD*-expressing myotome to now include the former *Pax-3* domain (4B2). Furthermore, the muscle cells terminally differentiated and expressed desmin (Fig. 4A5). However, Shh did not induce *MyoD* expression elsewhere e.g. sclerotome (Fig. 4B2,B3,B4). After 32 hours exposure to Shh, myotomes next to the bead were smaller in their medio-lateral extension compared to the opposite side (Fig. 4A3,B3) and after 2 days, less myotomal

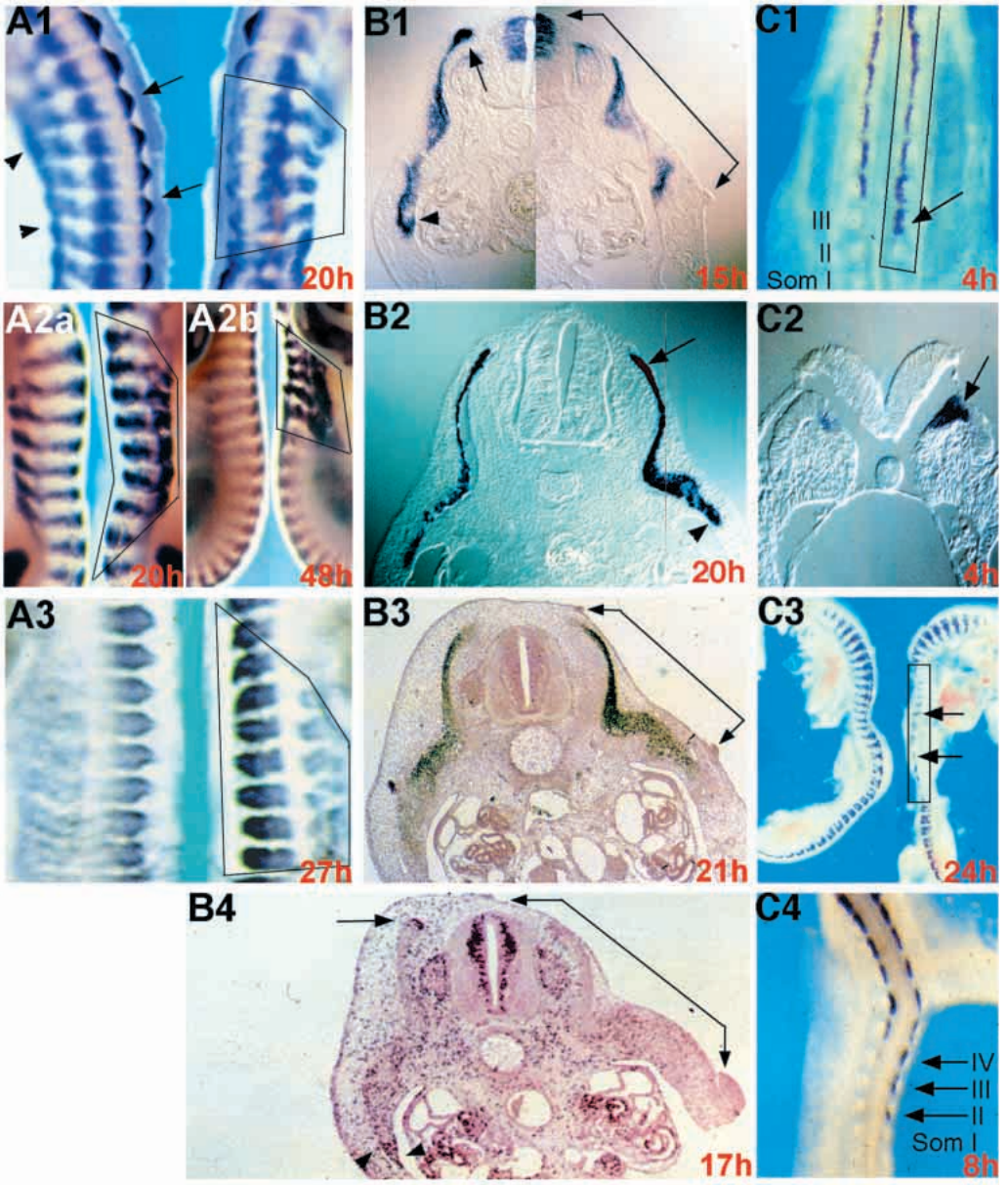

Fig. 2. Influence of ectoderm on gene expression and cell proliferation in somites. Boxed regions mark the extent of ectoderm removal. Re-incubation periods are indicated. (A1) Dorsomedial lips (arrows) and ventrolateral lips (arrowheads) of the dermomyotome express high levels of *Pax-3* which are down-regulated after removal of ectoderm overlying thoracic somites at stage 22 (boxed region). (A2a and A2b) After removal of thoracic ectoderm at stage 21, *MyoD* expression is enhanced throughout the somites (boxed region) but the mediolateral extension of the myotomes is less than in the contralateral region. (A3) Desmin is up-regulated following ectoderm ablation (boxed region). (B1) Transverse section shows *Pax-3* expression at trunk level after ectoderm removal. *Pax-3* is expressed at high levels in the dorsomedial lip (arrow) and ventrolateral lip (arrowhead) of the dermomyotome on the non-operated side whereas it is down-regulated on operated side (extent of ectoderm removal marked by bracket). (B2) Transverse section shows *MyoD* expression at trunk level after ectoderm removal. Although expression is up-regulated (arrow) there is a decrease in ventrolateral extension of expression (arrowhead). (B3) Removal of trunk ectoderm results in muscle cells terminally differentiating as shown by the expression of desmin. Ventrolateral extension is also reduced. (B4) High level of BrdU incorporation in the dorsomedial (arrow) and ventrolateral (arrowhead) lips of the dermomyotome on the non-operated side of a stage 23 embryo. Ectoderm removal decreases amount of BrdU incorporation under site of operation compared to contralateral side. Subectodermal mesenchyme appears condensed after ectoderm removal. (C1) Removal of ectoderm overlying the somites at stage 14 leads to premature up-regulation of *MyoD* in somite III (arrow). (C2) Transverse section of embryo in C1 at level of somite III shows up-regulation of MyoD. Ectoderm has not regenerated (arrow). (C3) 24 hours after this procedure *MyoD* expression does not mark complete myotomes, but some *MyoD*-positive cells reside next to the neural tube (arrows). (C4) Separation of unsegmented paraxial mesoderm from lateral plate and removal of overlying ectoderm at stage 14 leads to premature up-regulation of *MyoD* expression in newly formed somites II-IV (arrows).

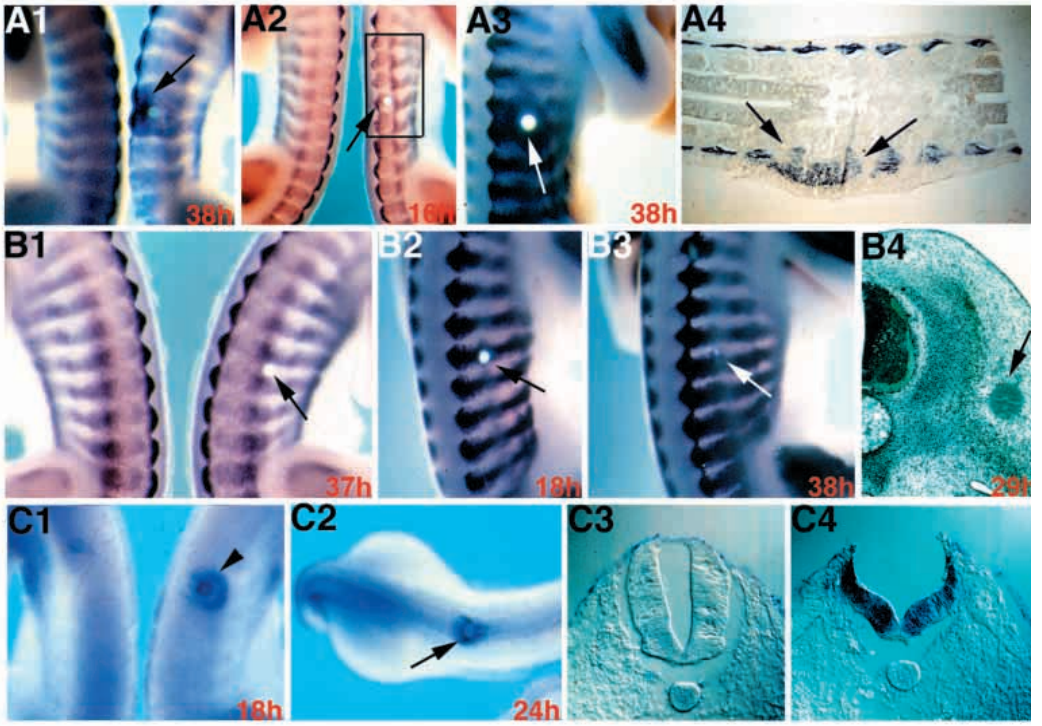

Fig. 3. Effect of BMP-4 beads on trunk muscle development. (A1) BMP-4 beads applied to thoracic somites at stage 20 up-regulates *Pax-3* expression (arrow). (A2) Application of BMP-4 bead to thoracic somites after ectoderm removal at stage 23 (boxed area) maintains *Pax-3* expression near the bead (especially in dorsomedial aspect, arrow, and compare to Fig. 2A1). (A3) BMP-4 application to thoracic somites at stage 21 results in up-regulation of *MyoD* expression (arrow). (A4) Frontal section of embryo in A3 shows that 3 myotomes are fused and enlarged in size (arrows). (B1) Control bead does not influence *Pax-3* expression in thoracic somites. (B2 and B3) Control beads do not influence *MyoD* expression either after 18 hours or after 38 hours when implanted at stage 20/21. (B4) Feulgen stained transverse section of thoracic region shows normal somite morphology after control bead insertion at stage 25. (C1) Application of beads soaked in BMP-4 to thoracic somites at stage 22 locally up-regulates *noggin* expression (arrowhead). (C2) BMP-4 applied between ectoderm and neural tube at stage 14 at the caudal level of unsegmented paraxial mesoderm results in local up-regulation of *noggin* in the neural tube (arrow). (C3) Transverse section of unoperated embryos shows very little *noggin* expression in the neural tube. (C4) Induction of *noggin* expression in the neural tube by BMP-4 bead at stage 14 (section of wholemount in C2, bead which was situated in the lumen of the neural tube dislodged during photography of the wholemount).

muscle had developed, as visualised by *MyoD* expression (Fig. 4A4,B4) or desmin expression (Fig. 4C5). Shh beads did not interfere with *Pax-3* expression of prospective ventrolateral body wall muscle when inserted in presumptive flank of HH-stage 20 embryos (Fig. 4A1,B1). Shh beads soaked in 3 or 7 mg/ml of protein solution had the same effect on *Pax-3* and *MyoD* expression as when soaked in 14 mg/ml (data not shown). Affigel beads soaked in PBS did not disrupt the normal expression pattern of *Pax-3* or *MyoD* (data not shown).

Thus Shh clearly can initiate muscle differentiation and its action is opposite of ectoderm activity. This indicates that Sonic hedgehog may counteract proliferative signals such as BMPs during muscle development and release myogenic cells from their *Pax-3* state which causes an up-regulation of *MyoD* and muscle differentiation. Premature differentiation eventually results in a reduction in the number of muscle cells through exhausting the pool of mitotically active myogenic cells.

Previously, we have shown that Shh acts during limb muscle development via induction of secondary signalling molecules such as BMP-2 and -7 (Amthor et al., 1998). However, Shh when applied to thoracic somites did not to induce *Bmp-2*, *-4* or *-7* in thoracic ectoderm or mesenchyme (data not shown).

Shh has been implicated only to act as a short range signal (Yang et al., 1997). The distance over which cells can respond to Shh signalling can be analysed by the expression of *patched*, which encodes the Shh receptor (Marigo et al., 1996a). At stage 20, *shh* was expressed in the notochord and floorplate (Fig. 4C1) and *patched* expression was only detected in sclerotomal tissue near the notochord but not in other parts of the somite such as myotome, dermomyotome or subectodermal mesenchyme (Fig. 4C2,C3). However, implantation of Shh beads in the mesenchyme of advanced somites induced *patched* expression over the same distance as muscle development was influenced following 25 hours of re-incubation (n=3, data not shown and see Fig. 5h in Amthor et al., 1998). Furthermore, transverse sections showed up-regulation of *patched* throughout the sclerotome and within mesenchyme between the myotome and ectoderm but the myotome was free of *patched* expression (Fig. 4C4).

Premature differentiation of myogenic cells in somites prior to migration prevents formation of limb and tongue muscle

The mouse *Pax-3* mutant, *Splotch*, not only has reduced trunk muscle mass but fails to form both tongue and appendicular muscle (Franz et al., 1993; Tajbakhsh et al., 1997). These results have been interpreted as a failure to activate the *MyoD* gene so the *Pax-3*/*MyoD* muscle lineage would be lost. In contrast, our experiments suggest that *MyoD* is in fact up-regulated after down-regulation of *Pax-3*. To understand this contradiction and to highlight the molecular events of *Splotch* development we tried to experimentally produce a Splotch phenotype in a chick embryo. We used Shh as a tool to artificially induce muscle differentiation and to determine whether premature muscle differentiation in the somites prior to lateral migration would prevent muscle formation in limbs and tongue. One marker used to examine the effect of Shh in

this assay was *follistatin* since it is expressed in differentiating muscle of the myotome as well as in migrating muscle precursors (Amthor et al., 1996).

We positioned Shh beads in stage 13/14 embryos at the level of occipital somites from which prospective tongue muscle precursors migrate ventrally (*n*=21). To ensure exposure of lateral somite compartments to Shh we inserted the beads dorsal to the intermediate mesoderm. Remarkably, no ventrally migrating, *follistatin*-positive cells were detected on the operated side although expression of *follistatin* in the occipital somites was up-regulated (33 hours re-incubation, Fig. 5A1). Similarly, we found fewer *Pax-3*-positive migrating cells at the ventral side of the occipital somites 36 hours after exposure to Shh compared to the contralateral side (Fig. 5A2). We observed an up-regulation of *MyoD* in occipital somites but the first differentiating tongue muscle cells were missing (40 hours re-incubation, Fig. 5A3). This ultimately lead to a lack of tongue muscle on the operated side as demonstrated by lack of *MyoD* expression 70 hours after Shh bead insertion (Fig. 5A4).

These results were reproducible at the limb level. Shh beads were positioned in stage 14/15 embryos between somites 16 and 21, from which limb muscle precursors are recruited (*n*=14). The *follistatin*-positive migratory population was not detectable but *follistatin* was up-regulated in the somites at limb level after 24 hours (Fig. 5B1). Additionally, we found fewer *Pax-3*-positive cells migrating laterally 36 hours after exposure to Shh (Fig. 5B2). We observed an up-regulation of *MyoD* expression in somites at limb level as early as 12 hours after Shh bead implantation (in this case the bead was inserted at stage 18; *n*=2, Fig. 5B3). When beads were implanted at stage 14 and re-incubated for 72 hours, embryos developed to stage 25 and possessed well-developed limb buds. Remarkably, less MyoD expression was detected in limbs on the operated side than on the contralateral side (Fig. 5B4). However, in the limb the situation was more complex compared to the tongue, because ectopically applied Shh interfered with limb outgrowth. Migration was only prevented in half of the examined cases. Affigel beads soaked in PBS did not disrupt the normal expression of *follistatin*, *Pax-3*, or *MyoD*. Furthermore, placing a bead in the migratory route had no effect on cell movement. Cells appeared to go round the bead to continue their normal path on the other side (data not shown).

Thus in situ differentiation of prospective limb and tongue muscle cells within the somitic environment prevents formation of appendicular and tongue muscle. Therefore cells which under normal conditions would have migrated towards limb and tongue muscle blastemata were not lost but integrated into axial muscle. These results demonstrate that a delay in muscle differentiation is a required mechanism for appendicular and tongue muscle development.

DISCUSSION

Removal of ectoderm overlying somites results in premature differentiation of proliferating myogenic cells and further trunk muscle growth is arrested. Local application of BMP-4 protein mimics ectoderm activity and sustains muscle growth. BMP-4 induces *noggin* expression which suggests that, as a fail safe mechanism, BMP-4 limits its proliferation inducing activity via regulation of its antagonist Noggin. Opposing BMP-4 activity, locally applied Shh protein forces epaxial muscle to differentiate prematurely which inhibits further muscle growth. Thus, when differentiation is not precisely limited muscle fails to grow.

Ectodermal signals sustain muscle growth by limiting differentiation

A striking feature during trunk muscle development is the spatial distribution of cells committed to muscle formation. A layer of *Pax-3*-expressing muscle progenitors is sandwiched between the ectoderm and the myotome. *Pax-3*-expressing cells are therefore situated closer to the ectoderm than the differentiated muscle located in the myotome. Additionally, the degree of muscle differentiation correlates with the proliferation activity. *Pax-3*-expressing tissue is mitotically active whereas differentiating muscle of the myotome is mitotically silent. However, we found a few BrdU-positive cells in the myotome of stage 25 embryos whereas at younger stages the myotome is completely devoid of dividing cells (Sechrist and Marcelle, 1996; Kahane et al., 1998b). This raises the possibility that intramyotomally situated BrdU positive cells of late somite stages could be non-mucle in origin and may be connective tissue cells which invade back muscle prior muscle individualisation.

Ectoderm has been shown to induce *Pax-3* and *MyoD* expression in paraxial mesoderm in vitro and in vivo (Fan and Tessier-Lavigne, 1994; Dietrich et al., 1997 and 1998; Reshef et al., 1998) and to increase muscle formation of explanted unsegmented paraxial mesoderm (Cossu et al., 1996b). However, from these data it is unclear if a single ectoderm-derived signal regulates both the *Pax-3* and *MyoD* gene locus or multiple ectoderm-derived signals regulated each gene independently. We show that removal of trunk ectoderm results in a decrease in proliferation and *Pax-3* expression of the adjacent muscle precursors. Instead of proliferating continuously, muscle precursors suddenly express *MyoD* and differentiate and become integrated into the myotome. This burst in premature differentiation primarily leads to an increase in myotomal muscle. In the long term, however, muscle growth is arrested because the pool of proliferating undifferentiated muscle precursors is exhausted. The first morphological sign of an impaired muscle development is an arrest in medio-lateral growth of the myotome. The arrest in medio-lateral extension of the dermomyotome correlates well to the loss of proliferation and *Pax-3* expression in the dorsomedial and ventrolateral lips of the dermomyotome after ectoderm removal. This confirms observations that the myotome forms by appositional growth at the lips of the dermomyotome (Kaehn et al., 1988; Denetclaw et al., 1997). In contrast, Kahane et al. (1998b) considered the dorsomedial lip being only of minor importance for continuous myotomal growth. The stages when experiments have been performed may account for these differences as Kahane et al. (1998b) worked on stage 15 embryos compared to our work which was performed on stage 21-23 embryos. However, the myotome not only grows as a sheet, but becomes thickened during development. The potential for muscle precursors located between the ectoderm and myotome to join the myotome after ectoderm removal, suggests that the myotome thickens as a result of continuous recruitment of these cells. Interestingly,

myotome formation in the transverse plane can be divided into several stages. A first wave of myogenic cells forms a primary myotome directly underneath the dermomytome. Thereafter (approximately up to day 4) new myogenic cells are added to the side of the myotome which faces the sclerotome – thus the myotome grows in a lateral to medial direction (Kahane et al., 1998a,b). Our results (experiments performed from day 4 onwards) suggest that during further myotomal growth cells at the interface between the dispersed dermomyotome and myotome differentiate and join the myotome when deprived of proliferative signals and hence myotomal growth would be directed from medial to lateral.

Thus, trunk ectoderm maintains the adjacent myogenic cells in a proliferating and undifferentiated state and restricts differentiation to the deeper myotome. We propose that cells from this pool of proliferating muscle precursors continually escape the influence of proliferation signals. These cells spontaneously differentiate and join the myotomal muscle. Therefore, to guarantee continuous trunk muscle growth it is imperative to maintain proliferation and to limit differentiation.

BMP-4 influences proliferation and differentiation of somitic muscle

Local application of BMP-4 protein to somites enhances *Pax-3* expression and maintains *Pax-3* expression after ectoderm removal. This suggests that BMP-4, which is expressed in the ectoderm and mesenchyme flanking the *Pax-3*-expressing tissue, exerts proliferative activity during trunk muscle development. Pourquié et al. (1996) demonstrated that during somite formation over-expression of BMP-4 leads to an expansion of the *Pax-3*-positive cell population at the expense of the *MyoD* population. Furthermore, exposure of explants of somites with overlying ectoderm to Noggin, the antagonist of BMP-4, results in up-regulation of *MyoD* at the expense of the *Pax-3* population (Reshef et al., 1998). We show, however, that implantation of BMP-4 beads lead eventually to an expansion of the *MyoD*-expressing myotome. Thus, exposure to BMP-4 leads to excessive muscle growth via stimulation of *Pax-3* expression.

Whereas BMP-4 bead application in the presence of ectoderm causes excessive muscle growth, BMP-4 beads are unable to induce such a strong response in the absence of ectoderm. Furthermore, mesenchymally expressed BMP-4 is unable to maintain high *Pax-3* expression in the dorsomedial lip of the dermomyotome after ectoderm removal. This suggests that mesenchymally derived BMP-4 is in a different biochemical state from ectodermal BMP-4 and BMP-4 introduced on beads. It is possible that the ectoderm produces a BMP maturation factor. In addition to a direct effect on myogenic cells, BMP-4 could act indirectly via the ectoderm. BMP-4 could activate members of the Wnt family since a number of these genes are expressed in the ectoderm (Parr et al., 1993; Tajbakhsh et al., 1998) and several Wnt proteins have been shown to induce *Pax-3* expression (Stern et al., 1995; Münsterberg et al., 1995; Maroto et al., 1997; Fan et al., 1997) or activate *MyoD* and *Myf5* expression (Tajbakhsh et al., 1998) in explants of paraxial mesoderm.

We have to consider that in the mammalian embryonic trunk a second muscle lineage exists which is regulated through the *Myf-5* gene and not through *Pax-3* (Tajbakhsh et al., 1996; Maroto et al., 1997; Tajbakhsh et al., 1997). Further work is required to determine if this muscle cell line is regulated in a similar manner to the *Pax-3/MyoD* lineage in chicks.

Evading the action of proliferation signals

BMP-4 beads simultaneously induce *Pax-3* expression and up-regulate *noggin* expression, the latter encoding a BMP-4 antagonist. This suggests that the proliferative activity of BMP-4 is limited by negative feedback. Such a mechanism would explain how, after a period of increased proliferation, myogenic cells finally express *MyoD*, because myogenic cells are withdrawn from BMP-4 influence. Evidence that Noggin regulates the onset of *MyoD* expression during early somite development by restricting *Pax-3* expression further supports this view (Hirsinger et al., 1997; Marcelle et al., 1997; Reshef et al., 1998). It is striking that *noggin* at late embryonic stages is expressed predominantly in the roof plate but only at very low levels at other sites of *Bmp-4* expression. This indicates either that only BMP-4, which is expressed in the roof plate, is capable of inducing *noggin* or that there is a specific responsiveness of the roof plate to express *noggin* following exposure to BMP-4. The ability of a highly expressed proliferation signal (BMP-4) to induce its antagonist (Noggin) could explain how proliferation and differentiation are regulated in a small domain – a situation which is realised in the dorsomedial lip of the dermomyotome. However, other signalling molecules such as Wnt-1 and Shh which both act on myogenic cells have been shown to induce *noggin* expression (Hirsinger et al., 1997; Reshef et al., 1998). This indicates the existence of a complex mechanism which modulates the BMP-4 effect during trunk muscle development.

Another mechanism that could explain how proliferative activity decreases is passive displacement of myogenic cells from a proliferation signal as a consequence of embryonic growth. As the BMP-4-expressing ectoderm and subectodermal mesenchyme is displaced outwards, cells in the *Pax-3* layer between the ectoderm and myotome will continue to proliferate only if they are sufficiently close to the ectoderm. Those cells that find themselves at some distance from the ectoderm will initiate *MyoD* expression and become integrated in the myotome which enlarges the back muscle.

Ectopically applied Shh represses proliferation and initiates differentiation of trunk muscle

Exposure to Shh protein forces muscle precursors of the embryonic trunk to down-regulate *Pax-3* and to differentiate. Although this burst in muscle differentiation primarily enlarges the myotome, the developmental consequence is an arrest in further muscle growth. Thus, the presence of Shh exhausts the reservoir of proliferating myogenic cells which is similar to the effect produced by ectoderm removal. This suggests that in somites, locally applied Shh protein antagonises proliferation activity of endogenous BMP-4 signals. It was recently suggested that Shh might counteract BMP-4 by up-regulating the BMP-4 antagonist Noggin (Hirsinger et al., 1997). Shh-antagonising BMP activity is by no means confined solely to muscle. Monsoro-Burq et al. (1996) have suggested that superficial somitic mesenchyme that forms the vertebral spinous process is induced by BMP-4 and inhibited by Shh. Recently, Shh has been implicated in preventing apoptosis during somite development (Teillet et al., 1998). Our

experiments do not exclude this as a property of Shh. The effect, however, which forces muscle progenitors to differentiate cannot be explained by Shh solely acting as a survival factor.

In the trunk of stage 20 embryos, Shh is only expressed in the notochord and floorplate and thus not in proximity to tissue with a muscle fate. It was reported that Shh up-regulates its own receptor Patched (Marigo et al., 1996a,b) and we found RNA transcripts of *patched* in sclerotomal tissue next to the notochord and in the neural tube but not at sites of *Pax-3* or *MyoD* expression. Additionally, due to its membrane attachment, Shh acts locally rather than as a long range signalling molecule (Yang et al., 1997). This suggests that Shh is unlikely to have any direct influence on the development of trunk muscle in mature somites. However, we do not rule out Shh having an effect on myogenic cells at other stages of muscle development. It was recently shown that Shh can mimic notochord activity to up-regulate *MyoD* expression during early somite development (Borycki et al., 1998). Interestingly, Shh acts directly on myogenic cells as dorsomedially situated cells of epithelial somites expresses *MyoD* concomitantly to the Shh responsiveness genes *Patched* and *Gli*. Marcelle et al. (1997) detected *patched* expression in the dorsomedial lip of the dermomyotome of HH-stage 18 embryos and showed Shh having a modulating effect on the cells of the dorsomedial lip by regulating *Wnt-11* expression. However, a detailed analyses of the Shh responsiveness gene *Gli* at later stages of muscle development could further elucidate a possible role of Shh.

In contrast to the growth arrest in response to Shh, previous studies have indicated that Shh can act to amplify the myogenic population in specific sites of the embryo (Currie and Ingham, 1996, Duprez et al., 1998). Indeed, we have recently shown that Shh application to chick limbs lead to an up-regulation of *Pax-3* and transient down-regulation of *MyoD* expression (Amthor et al., 1998). Thus in the limb, local application of Shh transiently prevents muscle differentiation, which results in excessive muscle growth, but in the trunk, Shh forces muscle to prematurely differentiate, which results in an arrest of further muscle growth. This contradiction can be resolved by taking into account the abilities of Shh to induce secondary signalling molecules. In the limb, Shh induces BMPs which in turn enhances muscle growth whereas in the trunk, Shh is unable to induce *Bmp-2*, *Bmp-4*, or *Bmp-7*.

However, these two extreme experimentally induced

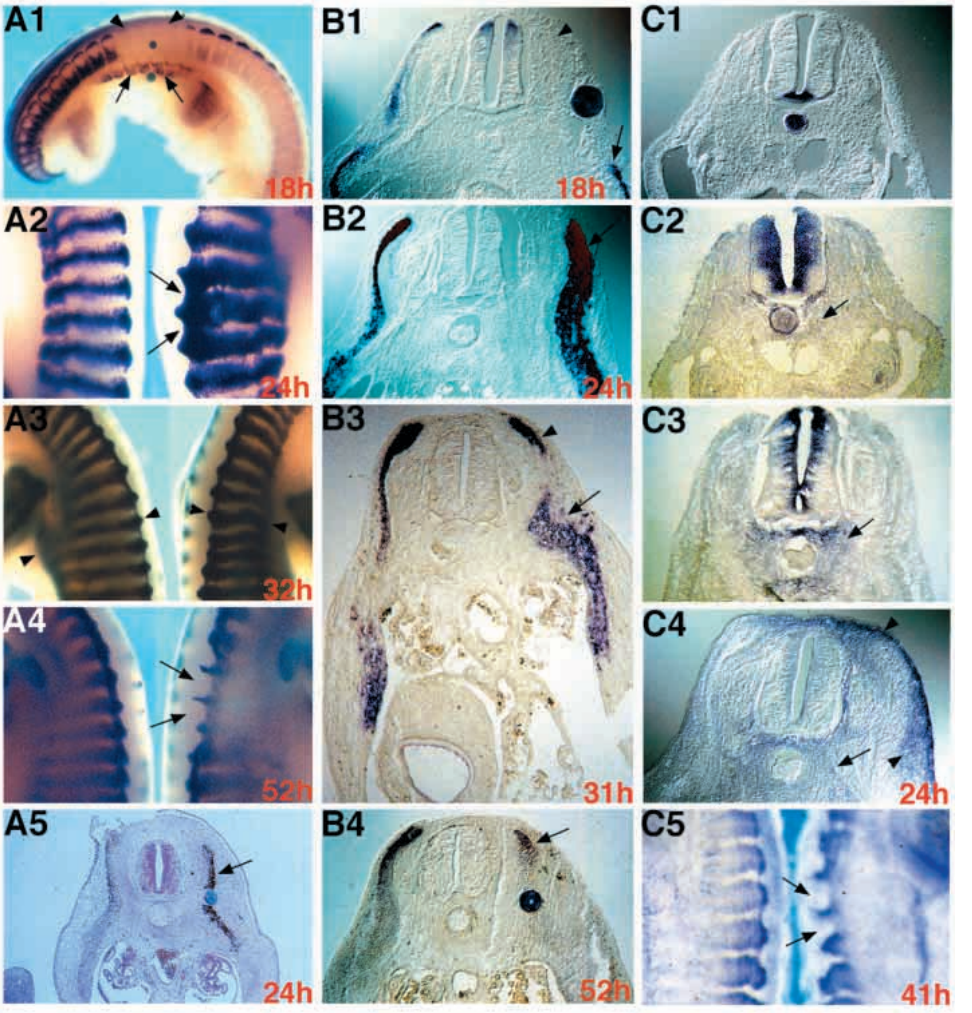

Fig. 4. Effect of ectopic sonic hedgehog on trunk and limb muscle development. (A1) Shh bead implants in somites at stage 20 leads to loss of *Pax-3* expression in epaxial muscle (arrowheads) but expression in hypaxial muscle is maintained (arrows) after 18 hours. (A2) Same procedure shows increased *MyoD* expression in epaxial muscle after 24 hours (arrows). (A3) Shh bead implant in somites at stage 22 results in less extended myotomes after 32 hours (arrowheads mark myotome lengths). (A4) 52 hours after implantation into somites at stage 21, the *MyoD* expression domain of epaxial muscle is reduced in size (arrows). (A5) Shh application at stage 22 resulted in up-regulation of desmin by 24 hours. (B1) Transverse section of embryo in A1 shows loss of *Pax-3* expression in medial tissue (arrowhead) but not in lateral regions (arrow). (B2) Transverse section of embryo in A2 shows up-regulation of *MyoD* expression extending into the former *Pax-3* expression region (arrow). (B3) Transverse section of embryo in A3 shows that *MyoD* expression eventually decreases (arrowhead) and a smaller ventrolateral extension of the myotome (arrow) compared to the unoperated side. (B4) Transverse section of embryo in A4 shows that after 52 hours *MyoD* expression is almost eliminated by the effect of Shh. (C1) *Shh* expression at thoracic level at stage 20 shows expression in the notochord and floor plate. (C2) Expression of *patched* at thoracic level at stage 20 and stage 24 (C3) shows expression in the neural tube and in the sclerotome (arrow). (C4) Application of Shh at stage 20 resulted in an up-regulation of *patched* expression in the sclerotome (arrow) and ectopic expression in subectodermal mesenchyme (arrowheads). (C5) Shh application at stage 21 resulted in reduced desmin expression (arrows) after 41 hours.

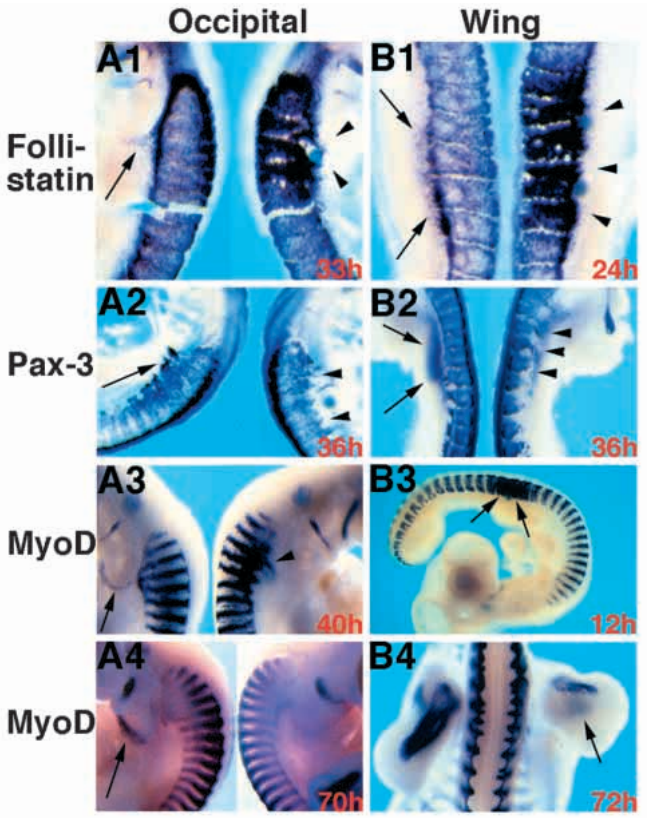

Fig. 5. Sonic hedgehog prevents formation of tongue and limb muscle by pre-mature muscle differentiation in somites before migration. (A1-A4) Shh beads were applied laterally at the level of somites 2-6 at stage 13/14. (A1) *Follistatin*-positive tongue muscle precursors migrate ventrally at the un-operated (arrow) but not at the operated side (arrowheads) but *follistatin* expression is up-regulated in occipital somites in presence of a Shh bead. (A2) Similarly, fewer *Pax-3*-positive myogenic cells migrating ventrally from occipital somites at the operated side (arrowheads) than at the un-operated side (arrow). (A3) *MyoD* is up-regulated in occipital somites (arrowhead) but first differentiating *MyoD*-expressing cells of tongue muscle are missing at the operated side compared to the un-operated side (arrow). (A4) *MyoD* expression is missing in tongue primordia compared to un-operated side (arrow) 70 hours after Shh bead application. (B1-B4) Shh beads were applied laterally at the level of somites 16-21. (B1) Shh bead insertion at stage 15 prevents lateral migration of *follistatin*-positive myogenic cells (arrowheads) compared to the contralateral side (arrows) but up-regulates *follistatin* expression in somites at limb level. (B2) In the presence of ectopic Shh, fewer *Pax-3* positive muscle precursors migrate laterally at limb level (arrowheads) than on the contralateral side (arrows) when bead was applied at stage 13. (B3) Insertion of Shh bead in somites at stage 18 leads to an up-regulation of *MyoD* after 12 hours (arrows). (B4). Insertion of Shh bead in somites at limb level at stage 14 results in a smaller domain of *MyoD* expression in the wing of the operated side after 72 hours (arrow).

conditions – arrest of muscle growth in the embryonic trunk and excessive muscle growth in the limb – emphasise the importance of precise temporally organised proliferation and differentiation during embryonic muscle development. Imbalance to either side must be avoided to obtain appropriately sized muscles.

Delay of muscle differentiation allows limb and tongue muscle development

We have taken advantage of Shh as a tool to artificially force myogenic cells to differentiate prematurely to highlight the importance of temporal co-ordination of muscle differentiation. When Shh is applied to somites at occipital or brachial levels prior to migration of muscle precursors into tongue and limb, muscle precursors prematurely differentiate and remain within the somitic environment. This leads to both the tongue and wing being almost muscleless. Firstly this suggests that the migratory potential of differentiated muscle cells is considerably lower than their undifferentiated precursors. Secondly, an appropriate number of proliferating muscle precursors is required to invade limbs and tongue which is the basis for a appropriate muscle mass development. Thus, delay of muscle differentiation is the required mechanism if paired appendages and tongue are to be populated with muscle.

Strikingly, whereas Shh forced hypaxial muscle at occipital and brachial levels to down-regulate *Pax-3* and to up-regulate *MyoD*, hypaxial muscle at the interlimb level did not respond to such treatment. Hypaxial myogenic cells at interlimb level do not realise their migratory potential but remain epithelised and passively populate the abdominal wall as the hypaxial bud elongates ventrally (reviwed by Christ and Ordahl, 1995). The responsive differences between hypaxial muscle at the neck/limb and interlimb level to Shh are also reflected at the molecular levels and examplified by the expression of *Lbx-1*, a homoebox gene (Dietrich et al., 1998) which is only expressed in prospective migratory populations.

Application of Shh to the somites at neck and limb level in a chick embryo results in a phenotype which resembles that of *Splotch*, a null mutant mouse for the *Pax-3* gene (Franz et al., 1993), in which, similar to our experiment, tongue and limb muscle are not formed. Furthermore, we have shown that following removal of ectoderm overlying young somites, myogenic cells prematurely differentiate, but, subsequently, no myotome forms and only few *MyoD*-expressing cells reside next to the neural tube. This manipulation resembles the situation in *Splotch/Myf5*$^{-/-}$ double homozygotes where a few differentiated muscle cells are found next to the neural tube (Tajbakhsh et al., 1997). These similarities led us to ask, if in *Splotch* and *Splotch/Myf5*$^{-/-}$ myogenic cells differentiated prematurely before any amplification? This hypothesis can be experimentally tested in mice by generating a *Pax-3* null mutant which has a reporter gene (e.g. *lacZ*) inserted at the site of recombination, thereby allowing one to follow the fate of the muscle precursor cells.

A common mechanism enabling continuous muscle growth in limbs and somites

Embryonic muscle growth in trunk and limbs requires continuous signalling which maintains a pool of *Pax-3*-expressing and mitotically active muscle precursors (Amthor et al., 1998). During both trunk and limb muscle development, ectodermally derived BMPs are capable of regulating *Pax-3* expression and thus myogenic proliferation. Muscle cells differentiate spontaneously after escaping proliferation signals – implying that muscle differentiation occurs through a default pathway. One way this could be achieved is by passive displacement of myogenic cells from the source of proliferation signals as the embryo grows. Additionally,

proliferative activity of BMPs may be antagonised by Noggin which is expressed in the dorsal neural tube and in the mesenchymal core of the limb bud. The presence of a proliferation agonist (BMP-4) and antagonist (Noggin) could organise proliferation and differentiation in close juxtaposition. Such a situation is realised in the dorsomedial lip of the dermomyotome as well as in the premuscle masses of the developing limb. Remarkably, BMP-4 can induce *noggin* which suggests that the proliferative activity of BMP-4 can be limited by negative feed back. Thus, continuous growth of somite-derived muscle requires maintenance of proliferation and precise limitation of differentiation and we suggest that both states are regulated by a single type of molecule – the BMPs.

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