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Sonic hedgehog is a survival factor for hypaxial muscles during mouse development

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SUMMARY

Sonic hedgehog (Shh) has been proposed to function as an inductive and trophic signal that controls development of epaxial musculature in vertebrate embryos. In contrast, development of hypaxial muscles was assumed to occur independently of *Shh*. We here show that formation of limb muscles was severely affected in two different mouse strains with inactivating mutations of the *Shh* gene. The limb muscle defect became apparent relatively late and initial stages of hypaxial muscle development were unaffected or only slightly delayed. Micromass cultures and cultures of tissue fragments derived from limbs under different conditions with or without the overlaying ectoderm indicated that Shh is required for the maintenance of the expression of myogenic regulatory factors (MRFs) and, consecutively, for the formation of differentiated limb muscle myotubes. We propose that *Shh* acts as a survival and proliferation factor for myogenic precursor cells during hypaxial muscle development. Detection of a reduced but significant level of Myf5 expression in the epaxial compartment of somites of *Shh* homozygous mutant embryos at E9.5 indicated that *Shh* might be dispensable for the initiation of myogenesis both in hypaxial and epaxial muscles. Our data suggest that *Shh* acts similarly in both somitic compartments as a survival and proliferation factor and not as a primary inducer of myogenesis.

Key words: Shh, Myogenesis, BMP, Mouse, Hypaxial muscle

INTRODUCTION

The majority of skeletal muscles is derived from transient mesodermal structures, the somites (for recent reviews, see Arnold and Braun, 2000; Brand-Saberi and Christ, 2000; Tajbakhsh and Buckingham, 2000). Within the somite, two different myogenic cell populations have been identified by various means (Ordahl and Le Douarin, 1992; Rong et al., 1992). One cell population resides in the medial dermomyotome and generates epaxial muscles (e.g. intrinsic back muscles), the other one originates from the lateral dermomyotome and produces hypaxial muscles of the body wall and limbs. The epaxial population is dependent on signals from axial structures, notochord and neural tube, which induce and maintain myogenic factor expression (Teillet et al., 1998). The hypaxial population apparently receives different signals from the dorsal ectoderm, the lateral mesoderm and the forming limb buds, which induce myogenic determination/differentiation, inhibit myogenesis and stimulate migration of myogenic precursor cells, respectively (Ordahl et al., 2000).

Muscle development requires a combination of signals that initiate, activate and maintain myogenesis. Besides instructive signals that specifically control myogenesis permissive signals exist that enable myogenic cells to proliferate and survive. A number of contradictory results concerning specific functions of signals that eventually contribute to the formation of epaxial muscles may be explained by difficulties to pinpoint the exact stage of cell development and/or myotube formation on which the signal acts. For example, the striking absence of differentiated axial muscles after ablation of the neural tube and notochord was originally interpreted as indicative of the presence of a myogenesis-inducing factor in axial structures (Rong et al., 1992). However, later studies showed that expression of *MyoD* (*Myod* – Mouse Genome Informatics) and *myogenin* was initiated normally when the neural tube was removed at the level of the unsegmented paraxial mesoderm. A few days after neural tube extirpation the expression of *MyoD* and *myogenin* gradually disappeared and eventually became undetectable leading to the absence of epaxial muscles (Bober et al., 1994a). From these observations it was concluded that the neural tube is not absolutely required for the generation of the skeletal muscle cell lineage, but may support the proliferation or maintenance of myogenic cells (Bober et al., 1994a; Teillet et al., 1998).

A number of candidate molecules have been proposed to

account for the effects of surrounding tissues on somite development and myogenesis. Ectopic expression in limbs of chicken embryos has indicated an important role for members of the TGFβ superfamily of growth factors, namely bone morphogenic protein (BMP) 2, BMP4 and BMP7 in this process (Pourquie et al., 1986). BMP2 and BMP4 expand the number of *Pax3*-expressing proliferative muscle precursor cells that, after deprival of proliferative signals, differentiate and form enlarged muscles (Amthor et al., 1998). The effects of BMPs were shown to be dose-dependent: low doses maintained the proliferative population while high doses prevented expression of *Pax3* and *MyoD* and induced apoptosis. Expression of BMPs can be induced and is possibly controlled by Shh, since implantation of Shh-soaked beads in chicken limb buds results in an induction of BMP2 and BMP7, and subsequent excessive muscle growth. In addition, Shh, which is expressed in the notochord and the floor plate, has been demonstrated to induce, synergistically with Wnts, the expression of myogenic bHLH genes and myogenic differentiation markers in unspecified somites (Münsterberg et al., 1995). In more recent experiments, retroviral expression of Shh in the limb bud was shown to sequentially induce an extension of the expression domains of *Pax3*, *MyoD* and myosin heavy chain (MyHC) genes concomitant with an increase in the proliferation of myoblasts in vitro suggesting that Shh enhances the proliferation of already committed myoblasts (Duprez et al., 1998; Teillet et al., 1998). Although these data indicate a role for Shh in maintenance/growth/survival of the myogenic lineage, similar to its role during sclerotome development (see Dockter, 2000; Monsoro-Burq and Le Douarin, 2000 for recent reviews), it has been suggested that *Shh* might act as an inducer of epaxial muscle development (Borycki et al., 1999). This hypothesis is mainly based on the analysis of *Shh* mutant mice (Chiang et al., 1996), which lack epaxial muscle formation (Borycki et al., 1999).

The interpretation of the exact role of Shh, however, is rather difficult as target genes of Shh in the somite are not known. Thus, Shh might have effects on the proliferation/survival/maintenance of myogenic precursor cells that are distinct from the activation of *Myf5* or *MyoD* genes. In line with this, somite growth is decreased in *Shh* mutants (Borycki et al., 1999) and overexpression of *Shh* increases proliferation of committed skeletal muscle cells in the chick limb. In the latter case, *Shh* primarily extends the expression domain of Pax3. The subsequent activation of MyoD and MyHC genes that eventually leads to muscle hypertrophy (Duprez et al., 1998) seems to be a secondary effect of *Shh* overexpression.

To analyze the physiological role of *Shh* in the control of proliferation and differentiation of limb muscle precursor cells, we took advantage of two available *Shh* mutant strains that lack expression of *Shh*. In Shh$^{-/-}$ mutants, *Shh* was inactivated through recombinant stem cell technology (Chiang et al., 1996). The other mutant, short digits (*Dsh*), was radiation induced, and has, when crossed to homozygosity (*Dsh/Dsh*), a phenotype identical to that described for the *Shh*$^{-/-}$ mutant (Selby et al., 1993). In spite of the large number of affected organs and tissues, not a single significant difference between homozygous *Shh* and *Dsh* mutants was identified. Instead identical changes in the skeleton, the kidney, the heart, the lungs, the gut, the genitalia and the brain were found in *Shh* and *Dsh* mutants (S. M., unpublished observations). Subsequent experiments showed that *Dsh/Dsh* embryos do not express *Shh*, and that *Dsh* is allelic to *Shh* (S. M., unpublished observations). Despite an identical phenotype in the homozygous state phenotypes of heterozygous *Dsh* and *Shh* mutant mice differ slightly, possibly owing to different levels of residual Shh expression in heterozygous mice.

In this study we demonstrate that *Shh* mutant mice fail to form the bulk of differentiated muscle fibers, although initial stages of hypaxial muscle development are unaffected or only slightly delayed. In addition, the effects of *Shh* on micromass cultures and cultures of tissue fragments derived from limbs of wild-type mice under different conditions, with or without the overlaying ectoderm, were analyzed. Our data suggest that *Shh* is required for the maintenance of the expression of myogenic regulatory factors (MRFs) in hypaxial musculature and, consecutively, for the formation of differentiated limb muscle myotubes.

MATERIALS AND METHODS

Mice: genotyping

Shh mutant mice have been described previously (Chiang et al., 1996). Genotyping of *Shh*$^{+/-}$ mice was performed by PCR using the primers 5′-TCTGGATTCATCGACTGTGG-3′, 5′-GATCCCCTCAGAAGA-ACTCGT-3′, which amplify the neomycin resistance gene. Homozygous *Shh* and *Dsh* embryos were readily identified by their abnormal head shape. In previous experiments we have shown that *Dsh/Dsh* mice do not express *Shh* and that *Dsh* is allelic to *Shh* (S. M., unpublished observation). All experiments were carried out in duplicate with *Dsh*/*Dsh* and *Shh*$^{-/-}$ embryos.

Whole-mount in situ hybridization and immunohistochemistry

Whole-mount in situ hybridization with digoxigenin-labeled antisense cRNA probes, embedding in gelatin and sectioning of stained embryos were performed as described previously (Kaul et al., 2000; Braun and Arnold, 1995). Immunohistochemistry on paraffin sections was performed using antibodies against desmin and fast MyHC (Sigma).

Micromass cultures

For micromass cultures, limb buds from E10.5 mouse embryos were dissected in cold Hepes-buffered saline (HBS). Isolated limb buds were digested in 0.1% collagenase, 0.1% trypsin for 20 minutes at 37°C with occasional shaking. Cells were plated in DMEM, 2% FCS in 20 μl drops at 2×10^7 cells/ml in six well primaria dishes (Falcon). For ectoderm removal, limb bud pieces were incubated for 10 minutes in 2% trypsin in HBS at 4°C, washed and the ectoderm removed with tungsten needles. The mesenchyme cells were gently dissociated to produce a single cell suspension and plated in 20 μl drops at 2×10^7 cells/ml. Cells were allowed to attach for 1 hour and then overlaid with 2-3 ml of culture medium. Culture medium was supplemented with supernatants from *Shh* virus-infected primary embryonic fibroblasts, with mock-infected supernatants or with purified growth factors purchased from R&D. The following concentrations were used: BMP2, 120 ng/ml (low concentration) and 400 ng/ml (high concentration); and BMP4, 80 ng/ml (low concentration) and 300 ng/ml (high concentration). Shh-containing supernatant was concentrated 20-fold at 4°C using a Millipore centrifugal filter (No.: UFV2BGC10).

Myotubes were detected by immunohistochemical staining with the MF20 anti-MyHC antibody as described (Braun et al., 1992). Micromass cultures were stained with Alcian Blue for chondrogenic nodules.

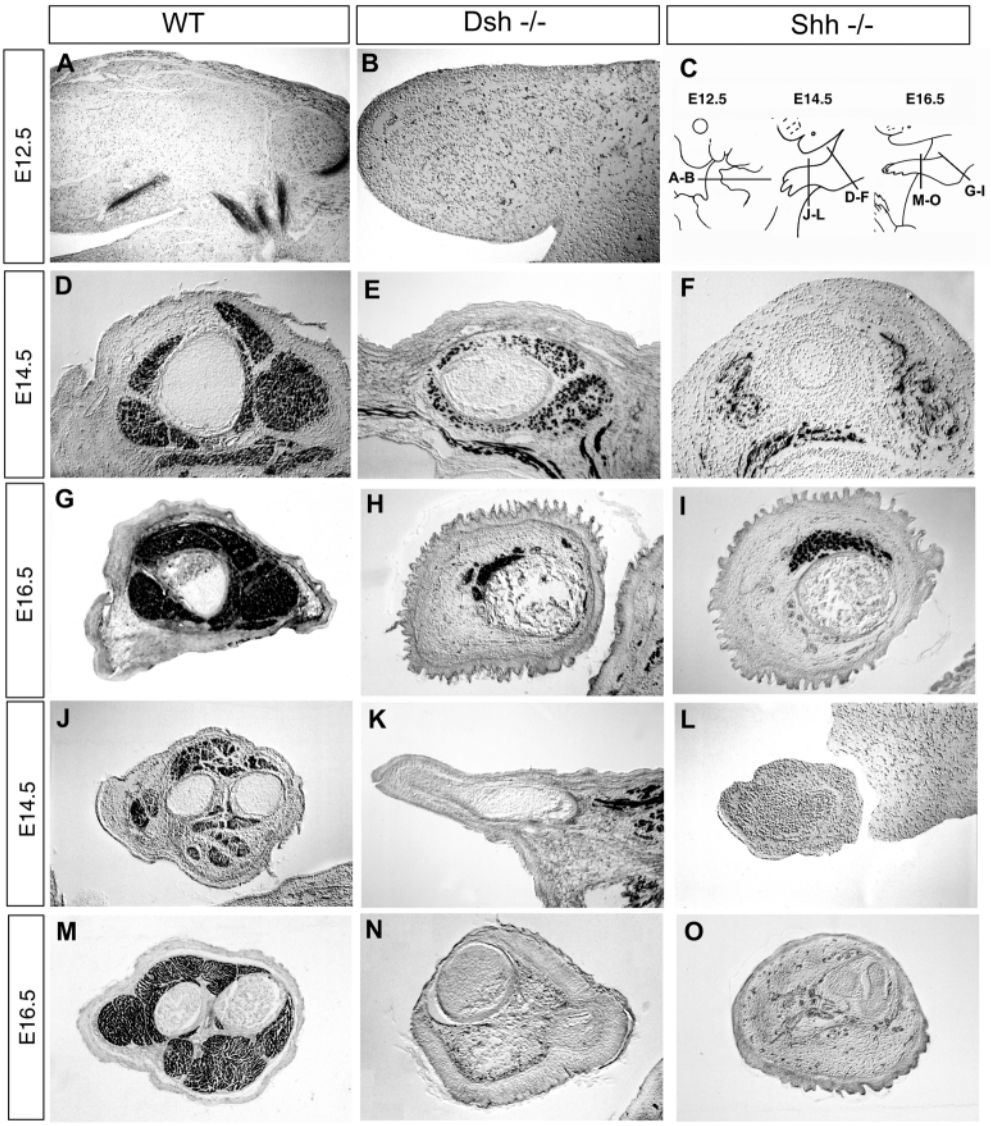

Fig. 1. Seriously reduced limb muscle formation in *Shh*$^{-/-}$ and *Dsh/Dsh* mutant mice between embryonic day 12.5 and E16.5. Comparable transverse and sagittal sections of forelimbs of wild-type (A,D,G,J,M), *Dsh*/*Dsh* (B,E,H,K,N) and *Shh* homozygous mutant mice (F,I,L,O) were stained with antibodies against MyHC and desmin (A,B) or MyHC antibodies alone (D-O). Sections were taken at various levels proximally (D-F,G-I) and distally (J-L,M-O) through the limb as indicated in C. Only very few scattered myocytes were visible in *Dsh*/*Dsh* mutants at E12.5 (B). At E14.5, formation of muscles is greatly diminished in *Dsh* and *Shh* mutants in proximal areas of the forelimb (E,F) and completely absent in distal regions (K,L). At E16.5, only in the dorsal part of the limb (where the extensor muscle are located) are reduced amounts of myotubes present proximally in *Dsh* and *Shh* mutants (H,I). No myotubes are detectable distally in mutant limbs (N,O).

Explant cultures

Limb bud fragments were dissected from E10.5 old embryos and cultured in collagen matrix as described (Guthrie and Lumsden, 1994; Münsterberg et al., 1995). Cultures were supplemented with supernatants from cultures infected with a *Shh*-expressing retrovirus or an alkaline phosphatase-expressing retrovirus. Cultures of primary embryonic fibroblasts, viral infection and harvesting of supernatants were done as described (Maroto et al., 1997; Fekete and Cepko, 1993). Retroviral titers ranged between 5×10^7 and 3×10^9 pfu/ml.

RT-PCR

RT-PCR analysis of explant cultures was essentially done as described previously (Münsterberg et al., 1995). cDNA was amplified for 25-30 cycles using appropriate primer pairs for the genes of interest in the presence of α-(P^{32})dCTP to visualize the reaction products. The following primer pairs were used (Patapoutian et al., 1995; Cornelison and Wold, 1997): Myf-5, 5′-TGCCATCCGCTACATTGAGAG-3′ and 5′-CCGGGGTAGCAGGCTGTGAGTTG-3′; MyoD, 5′-GCCCGCG-CTCCAACTGCTCTGAT-3′ and 5′-CCTACGGTGGTGCGCCC-TCTG-3′; MyHC (embryonic), 5′-GCAAAGACCCGTGACTT-CACCTCTAG-3′ and 5′-GCATGTGGAAAAGTGATACGTGG-3′; GAPDH, 5′-GTGGCAAAGTGGAGATTGTTGCC-3′ and 5′-GATGATGACCCGTTTGGCTCC-3′.

RESULTS

Homozygous *Shh* mutant mice show a severe deficiency of hypaxial muscle formation

To explore the effects of the lack of *Shh* on hypaxial muscle formation, homozygous *Shh* and *Dsh* mutant mice were bred and analyzed at various stages of development. Although homozygous mutant *Shh* mice showed an increased lethality at late fetal stages, sufficient numbers of mutant embryos could be recovered. Sections were taken at various levels of the limbs (Fig. 1C) and investigated using histological and immunohistochemical techniques.

By E12.5 muscle masses of wild-type limbs contain primary myotubes that express various striated muscle-specific proteins of the contractile apparatus such as α-cardiac actin, α-skeletal

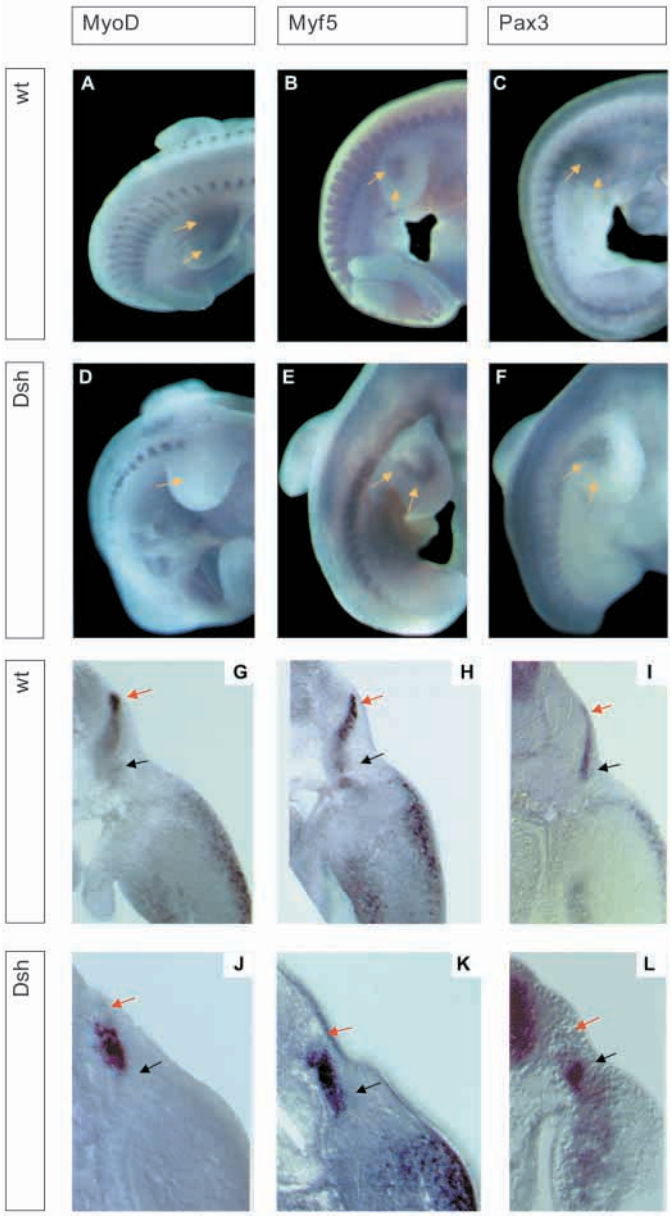

Fig. 2. Migration of limb muscle precursor cells and initial steps of myogenesis are normal in *Shh*$^{-/-}$ and *Dsh/Dsh* mutant mice. Whole-mount preparation (A-F) and sections (G-L) of in situ hybridization with MyoD (A,D,G,J), Myf5 (B,E,H,K) and Pax3 (C,F,I,L) probes in wild-type (A-C,G-I) and *Dsh* mutant embryos (D-F,J-L) at E10.5. Migration of limb muscle precursor cells are virtually normal in *Dsh* mutant mice as indicated by the presence of Pax3-expressing cells (yellow arrows in F). Expression of Myf5 in limbs indicated a normal initiation of myogenesis (E,K). Expression of MyoD normally occurs after induction of Myf5 and was slightly delayed in *Dsh* mutants (D,J). Note the absence of Pax3 expression at this stage in the epaxial domain of the dermomyotome of *Dsh* mutants (red arrow in L) together with MyoD (red arrow in J) and Myf5 (red arrow in K). In contrast, strong expression of MyoD, Myf5 and Pax3 is detectable in the hypaxial domain (black arrows in J-L).

actin, myosin light chains and MyHCs (Ontell et al., 1993; Fig. 1A). In *Dsh* mutant embryos, however, formation of Desmin and MyHC-positive myotubes was severely compromised. Only very few scattered myocytes were detectable (Fig. 1B).

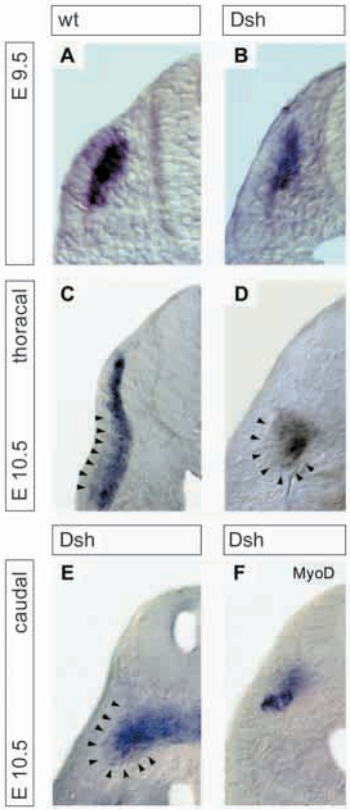

Fig. 3. Expression of *Myf5* is reduced but not absent in the epaxial domain of somites of *Dsh/Dsh* mice. Vibratome sections of whole-mount hybridization of E9.5 (A,B) and E10.5 (C-F) wild-type (A,C) and *Dsh*/*Dsh* mutant (B,D-F) embryos probed with Myf5 (A-E) and MyoD (F). At E9.5, *Myf5* expression is clearly detectable in the epaxial domain of dermomyotomes of *Dsh*/*Dsh* mutant mice (B); 1 day later only the expression in the hypaxial part of the somite is left (D-F). At this stage (E10.5), the dermomyotomal epithelium is severely distorted in *Dsh* mutant embryos both in the thoracic (D) and in the caudal parts of mutant embryos (E-F). Note the extension of *Myf5* expression towards the notochord into the sclerotome in the caudal part of the embryo (E). The abnormal position of the dermomyotomal epithelium is indicated by arrowheads in D,E. The normal localization of the dermomyotome is shown by arrowheads in C. Expression of *MyoD* was observed in the hypaxial but never in the epaxial domain of mutant somites (F).

At E13.5 differences between mutant and wild-type embryos were easily recognizable (not shown) and at E14.5 when secondary myotube formation is about to start virtually no MyHC-positive cells were found in distal positions of limbs of *Dsh* and *Shh* mutant mice (Fig. 1K,L). At the same stage, *Dsh* and *Shh* mutant embryos contained only a greatly reduced amount of MyHC-positive cells in presumptive muscle areas of the proximal limb (Fig. 1E,F). At E16.5 Hemotoxylin and Eosin stained tissue sections and sections reacted with MyHC antibodies showed a complete absence of differentiated muscle tissue in the distal portion of *Dsh* and *Shh* mutant limbs (Fig. 1N,O). In the proximal limbs only a small part of extensor muscles immediately adjacent to the humerus was left (Fig. 1H,I). The ventral muscle masses were completely missing. We generally did not observe any major differences in the muscle phenotype of *Shh* and *Dsh* mutant mice at this or earlier developmental stages.

Migration of limb muscle precursor cells and initial expression of myogenic factors is virtually normal in *Shh* mutants

We next investigated the pattern of migration of myogenic precursor cells into the limb buds in *Shh* mutant mice using a Pax3 probe (Bober et al., 1994b). After cells had reached the limb buds, initial steps of muscle cell differentiation were analyzed using probes for the myogenic factors *Myf5* and *MyoD*. Whole-mount in situ hybridization and sections of

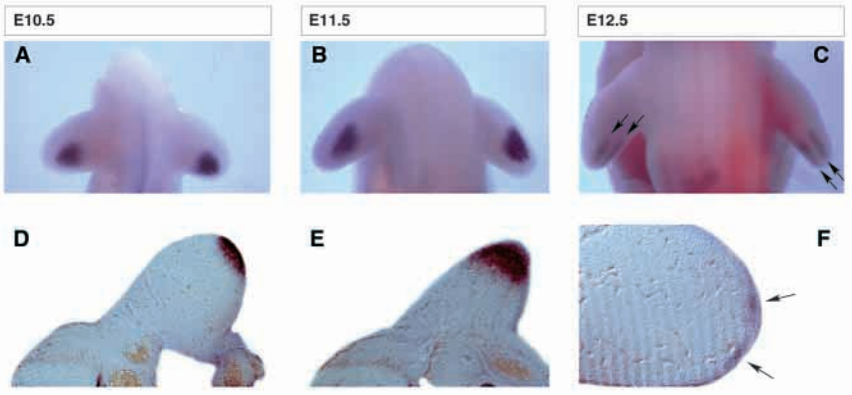

Fig. 4. Expression of *Shh* in limb buds of mouse embryos between E10.5 and E12.5. Whole-mount preparations (A-C) and sections (D-F) of wild-type embryos at E10.5 (A,D), E11.5 (B,E), and E12.5 (C,F) reacted with a probe for *Shh* mRNA. The initial expression domain in the ZPA of limb buds (A,D) increases in size and covers a large area of the limb at E11.5 (B,E). 1 day later, two stripes of *Shh*-expressing cells are visible that are located at the posterior margin of the limb bud (arrows in C and F).

stained embryos revealed that the migration of limb muscle precursor cells took place normally in *Dsh* (Fig. 2) and *Shh* (data not shown) mutant mice. Likewise, the expression of myogenic factors in limb buds of *Dsh* (Fig. 2E) and *Shh* mutant mice (data not shown) occurred in a pattern that was virtually indistinguishable from wild-type embryos, although a slight delay in the activation of the *MyoD* gene was noted (Fig. 2D). At this stage the expression of *Pax3*, *Myf5* and *MyoD* in the hypaxial domain of somites of mutant mice was easily identified (black arrows in Fig. 2J-L) in contrast to the lack of expression in the epaxial domains (red arrows in Fig. 2J-L).

Myf5 expression is reduced but not absent in the epaxial domain of somites of *Shh* mutant mice at E9.5

Whole-mount in situ hybridization revealed that the level of *Myf5* mRNA was significantly reduced in the somites of homozygous *Dsh* mutant embryos at E9.5. In the most mature, cranial somites, virtually no expression of *Myf5* was detectable. In newly formed somites, however, in which *Myf5* activation occurs exclusively in the dorsomedial quadrant and in somites at the interlimb level, *Myf5* mRNA was detectable. To allow better spatial resolution of *Myf5* expression in different domains of somites, vibratome sections of thoracal somites of mutant and wild-type embryos stained for *Myf5* expression were prepared. As shown in Fig. 3B, *Myf5* mRNA was readily detectable in the epaxial domain of mutant embryos, although the expression level was reduced compared with wild-type controls. One day later, at E10.5, when the expression of *Myf5* has expanded into the hypaxial domain, the expression of *Myf5* in the epaxial domain has faded and only a strong but distorted expression in the hypaxial domain was left (Fig. 3D).

At E9.5 no significant change in the morphology of somites from wild-type and mutant embryos was observed. In contrast, at E10.5, somites of mutant embryos displayed significant malformations. The axis of the somite seemed to have shifted towards the midline, generating the appearance of laying on its side. In the dorsomedial region next to the neural tube, where the epaxial part of the dermomyotome should have been located, no epithelium was discernible. Whether this morphological alteration corresponded to a specific loss of the dorsomedial lip or resulted from a change in the proliferation rate of somitic cells is hard to distinguish. Interestingly, in the caudal parts of *Dsh* mutant embryos an extension of the *Myf5* expression domain towards the notochord and into the sclerotome was observed (Fig. 3E). The initial expression of *Myf5* in the epaxial domain of thoracic somites and its consecutive loss during somite maturation strongly resembled the picture seen in limb buds where an initial expression of *Pax3*, *Myf5* and *MyoD* was followed by a loss of myogenic cells and a virtual complete absence of individual skeletal muscles.

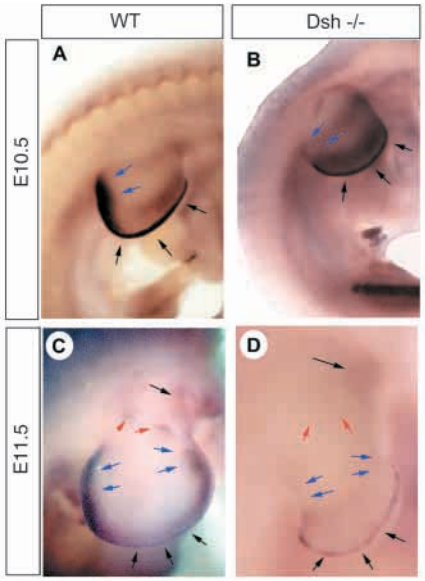

Fig. 5. *Shh* is essential for normal *Bmp4* expression in limb buds between E10.5 and 11.5. Whole-mount in situ hybridization of wild-type (A,C) and *Dsh/Dsh* mutant (B,D) mouse embryos at E10.5 (A,B) and E11.5 (C,D) with a Bmp4 RNA probe. At E10.5, a strong *Bmp4* expression domain at the posterior margin of the limb bud is virtually absent in *Dsh* mutants (blue arrows in A and B), while the expression in the progress zone below the AER is not severely affected (black arrows in A,B). At E11.5, only the expression in the progress zone is unaffected in *Dsh* mutant embryos (black arrows in C,D). No expression is detectable at the anterior and posterior margin of *Dsh* mutant limb buds and in the low-level expression domains proximally in the limb (red arrows in C and D) while another low-level expression domain proximally in the limb (single black arrow) is not severely downregulated.

Altered expression of BMPs in *Shh* mutant limbs

In order to control myogenic cell proliferation and maintenance Shh needs to be present at the right time and in the right place.

We therefore analyzed the expression of *Shh* mRNA in limb buds of wild-type mice between E10.5 and E12.5 (Fig. 4). At E10.5, *Shh* mRNA is expressed mainly at the posterior margin in the zone of polarizing activity. At E11.5, the expression domain has broadened, now covering a fairly large area at a dorsal and posterior position within the limb. This expression decreased considerably within 1 day, so that at E12.5 only two stripes of *Shh* mRNA-expressing cells were visible in the posterior margin that runs in a proximal to distal direction (Fig. 4C,F). Accepting the notorious difficulties to explain long-range biological activities of *Shh* (Christian, 2000) this expression profile would be compatible with the defects seen in *Shh* mutants. Alternatively, Shh may exert its functions indirectly by initiating the expression of other signaling molecules such as BMPs.

It has been shown that BMP2, BMP4 and BMP7 rescue *Pax3* expression after removal of the limb ectoderm and that the effect of Shh in inducing *Pax3* and, consecutively, *MyoD* might be indirect and mediated by BMPs (Amthor et al., 1998). We found that expression of *Bmp4*, which in wild-type mice is strongly expressed in limb buds between E10.5 and E11.5 (Heymer and Ruther, 1999; Barna et al., 2000) is severely downregulated in *Dsh* mutant mice. In wild-type embryos, high levels of *Bmp4* expression are present in the anterior and posterior margin of the limb bud including the ectoderm and the underlying mesenchyme, and in the apical ectodermal ridge (AER) and the progress zone (black and blue arrows in Fig. 5A,C). In addition, a weaker expression was seen at E11.5 in a proximal location in the limb, which might correspond to the developing joints (red arrows and single long black arrow in Fig. 5A,C). In *Dsh* mutant mice at E10.5, the expression in the posterior margin of the limb bud, which is the strongest expression domain in wild-type mice at this time point, was virtually absent (blue arrows in Fig. 5B). In contrast, expression in the AER and the progress zone was apparently not affected (short black arrows in Fig. 5B). At E11.5 expression in both the anterior and posterior marginal zone was absent in *Dsh* mutant mice (blue arrows in Fig. 5D). Some of the comparatively weak expression domains in the proximal location of the limb were also reduced at E11.5 in *Dsh* mutant mice (red arrows in Fig. 5D), while others were not (single long black arrow in Fig. 5D).

Shh and BMPs stimulate myogenic differentiation in micromass cultures of limb bud mesenchyme

To gain a better control of signals that direct limb bud mesenchyme development we used a micromass culture system to cultivate mesenchyme isolated from E10.5 limb buds, either with or without the overlaying ectoderm or additional growth factors (Grass et al., 1996).

Cultures were stopped 72 hours after explantation and stained with the MF20 antibody against MyHC and with Alcian Blue to score muscle and cartilage cell differentiation, respectively. Removal of the ectoderm from the limb bud mesenchyme resulted in a significant decrease of MyHC-positive cells when compared with micromass cultures prepared from complete limb buds (Figs 6, 7). The myocytes in ectoderm-free cultures appeared rather small and short, and rarely showed the elongated morphology characteristic for primary myocytes. The addition of Shh rescued the absence of ectoderm and led to an increase of the number of MyHC-positive cells (Fig. 6C). The numbers of myocytes in cultures with exogenous Shh exceeded those observed in cultures isolated from complete limb buds without additional Shh. It is likely that this phenomenon depended on the concentration of Shh used, as lower concentrations of Shh generated fewer myocytes (data not shown). When exogenous Shh was added to cultures from limb buds containing ectoderm, a further increase in the number of MyHC-positive cells was observed (Fig. 6F). RT-PCR analysis of RNA isolated from micromass cultures confirmed that expression of Shh, BMP2 and BMP4 was reduced in cultures without ectoderm, while the expression of Wnt7a and En1 was absent (data not shown).

We wanted to know whether we could mimic the effect of Shh on muscle cell development by BMP2 or BMP4 in our culture system. Therefore, we added BMP2 alone or in combination with Shh to limb bud-derived mesenchyme in micromass cultures. As shown in Figs 6, 7, exogenous human BMP2 at a low concentration increased the number of MyHC-positive cells compared with a mesenchyme culture without ectoderm and without additives (compare Fig. 6B with 6D). In agreement with the study by Amthor et al. (Amthor et al., 1998) we observed a reverse effect, which was probably due to enhanced apoptosis, when the concentration of BMP2 was raised above a concentration of 250 ng/ml (data not shown). Interestingly, a combination of Shh and BMP2 synergistically increased the number of MyHC-positive cells reaching the same order of magnitude as a combination of ectoderm and additional Shh (compare Fig. 6E with 6F; Fig. 7). Thus, the effects of Shh on muscle cell development may at least in part be mediated by BMPs. A similar but less extensive series of experiments was performed using BMP4 instead of BMP2. In our type of assay, no significant differences were obtained when BMP4 was substituted for BMP2 although it was sometimes necessary to adjust the concentration of BMP4 to achieve the same results (data not shown, see Materials and Methods).

Shh maintains the expression of myogenic factors and enables myogenic differentiation in limb bud organ cultures

We next asked whether Shh is able to control the presence of myogenic cells in intact, ectoderm-free tissue explants, as indicated by the expression of myogenic factors and MyHC. Pieces of mesenchyme tissue were isolated from E10.5 mouse limb buds after a short exposure to dispase to allow separation of mesenchyme and ectoderm (Guthrie and Lumsden, 1994). Single explants were cultured in collagen gel overlaid with medium containing Shh secreted from primary fibroblasts or with supernatant from mock-infected cells for 1, 3 or 7 days. As shown in Fig. 8 we were able to detect transcripts for the myogenic factors *Myf5* and *MyoD* by semi-quantitative RT-PCR 1 day after explantation, regardless of the presence or absence of Shh. However, this picture changed dramatically after prolonged incubation. After 3 days a decrease in the mRNA levels of *MyoD* and *Myf5* was obvious in cultures without Shh when compared with cultures that contained Shh. After 7 days without Shh, the levels of *Myf5* and *MyoD* had dropped further. Only small amounts of the myogenic factors were detectable. In contrast, cultures with exogenous Shh showed robust expression of *Myf5* and *MyoD* with no apparent signs of a reduction of *Myf5* and *MyoD* expression. MyHC (embryonic)

as a marker for terminal differentiation was first seen 3 days after explantation in cultures with Shh, while cultures without Shh were devoid of MyHC (embryonic) even after prolonged incubation. The expression of *Gapdh* in explant cultures remained unchanged in the presence or absence of Shh.

Taken together, our in vitro data from both micromass cultures and collagen gel explants support the view that Shh maintains the expression of myogenic factors. This expression leads to an expansion of the myogenic lineage, and thus eventually enables myogenic differentiation in limb buds.

DISCUSSION

The removal of the neural tube and notochord leads to the absence of epaxial muscles while the hypaxial musculature develops normally. These findings had favored the view that inductive signals, which presumably are responsible for this effect, namely *Shh*, are less important for the development of hypaxial muscles (Rong et al., 1992; Teillet et al., 1998; Borycki et al., 1999). Using a genetic model, we show that the absence of Shh results in a severe deficiency of hypaxial limb muscles in the mouse. As the initial stages of myogenesis in the limb were not affected in *Shh* mutant embryos, we suggest that the lack of Shh leads to a defect in the maintenance and/or expansion of the myogenic lineage. Based on the early expression of the myogenic factor *Myf5* in the epaxial domain of somites in the absence of Shh we propose that no substantial differences exist in the response of epaxial and hypaxial myogenic cells to *Shh*.

The suggested role of *Shh* in muscle development resembles its effect on sclerotome induction (Chiang et al., 1996; see Dockter, 2000; Monsoro-Burq and Le Douarin, 2000 for recent reviews). *Shh* mutant embryos display minimal initial expression of *Pax1*, an early sclerotomal marker, but fail to maintain *Pax1* expression. Since *noggin* mutant embryos show a delayed expression of *Pax1* in somites and noggin alone can induce low levels of *Pax1* expression, it has been speculated that, in vivo, noggin acts to initiate low levels of *Pax1* and that *Shh* augments and maintains the expression of *Pax1* initiated by noggin (McMahon et al., 1998). Thus, *Shh* may have a common function in the expansion and maintenance of various cell lineages.

What is the role of *Shh* for the development of the myogenic lineage?

The function of Shh in the development of muscle cells has been discussed controversially. Some researchers favor the view that Shh directly induces myogenesis possibly via the *Gli*-dependent signal transduction pathway (Borycki et al., 2000) while others postulate a role in the regulation of muscle cell proliferation and lineage maintenance (Duprez et al., 1999). The striking lack of epaxial muscles both in *Shh* mutant mice and in chicken embryos with deleted neural tubes and notochord had focused the attention on epaxial muscles, although some of the data were obtained from limb muscle cells (Rong et al., 1992; Ordahl and Le Douarin, 1992; Borycki et al., 1999).

The main argument for the induction of epaxial myogenesis by Shh was the lack of expression of myogenic factors in the epaxial domain of somites. However, the analysis has concentrated mainly on relatively late stages of mouse development, E9.75 and later, disregarding the possibility that an initial expression of muscle determination factors such as *Myf5* may occur in *Shh* mutant mice. A function of Shh in the regulation of muscle cell proliferation and lineage maintenance was excluded, as no lineage-specific proliferation defects in the neural tube and somites were detected, although overall cell proliferation in E9.5 embryos was decreased. In addition, areas of increased cell death were found in the neural tube and the ventral somite but not in the dorsal somite, which gives rise to epaxial muscles (Borycki et al., 1999). The failure to detect a massive increase in the number of apoptotic cells in the dorsal parts of somites of $Shh^{-/-}$ mice could be due to a number of reasons, which do not necessarily exclude a trophic role of Shh on muscle precursor cells.

Overexpression of *Shh* in chicken limb buds, on the other hand, resulted in an extension of *Pax3* and subsequently *MyoD* expression domains leading to muscle hypertrophy in vivo and activation of muscle cell proliferation in vitro. It has been reasoned that Shh protein produced in the zone of polarizing activity (ZPA) may serve as a signal for the survival and growth of limb muscle precursor cells, and that Shh secreted from the notochord and neural tube most likely also acts as a survival factor on the epaxial muscle lineage (Duprez et al., 1998).

Our own results on *Shh* mutant mouse embryos clearly support the view that *Shh* is mainly involved in maintenance and/or expansion of the myogenic lineage irrespective of its epaxial or hypaxial descent. The loss of the expression of myogenic markers in explant cultures deprived of Shh and the successive reduction of myogenic cells in *Shh* mutants is pivotal in this context. The observation that the expression of *Pax3* is lost in the epaxial domain of somites at later (Fig. 2) adds further evidence. In addition, the dermomyotomes of somites of *Shh* mutant mice show severe morphological abnormalities (Fig. 2) that cannot be explained simply by a lack of initiation of myogenic factor expression, as similar observations were not made in *Myf5*, *MyoD* or *Myf5/MyoD* double mutant mice (Braun et al., 1992; Rudnicki et al., 1992; Kaul et al., 2000). In this context it is interesting to discuss the extension of the Myf5 expression towards the notochord in caudal parts of *Dsh* mutant embryos. The picture resembles the appearance of cells that express myogenic markers in the midline of the zebrafish mutant floating head (*flh*). It has been speculated that the homeobox gene *flh* suppresses myogenic differentiation in the midline thus enabling cells to adopt a notochord fate (Talbot et al., 1995). Obviously, the absence of *Shh* and the accompanied defects in sclerotome formation did yield similar effects, at least in the caudal part of mutant embryos.

The muscle phenotype is not dependent on the lack of innervation in Shh mutant mice

As the formation of motoneurons is absent in *Shh* and *Dsh* mutant mice it was important to exclude a possible indirect effect of *Shh* mutations on muscle formation. In general it is accepted that primary myotubes are virtually insensitive to the lack of innervation, while the correct formation of secondary myotubes depends on a previous muscle-nerve interaction, although there has been some discussion in the past on the role of innervation in primary myotube formation (Duxson et al., 1989; Harris et al., 1989). Depending on the experimental system used, a modest decrease in the number of primary

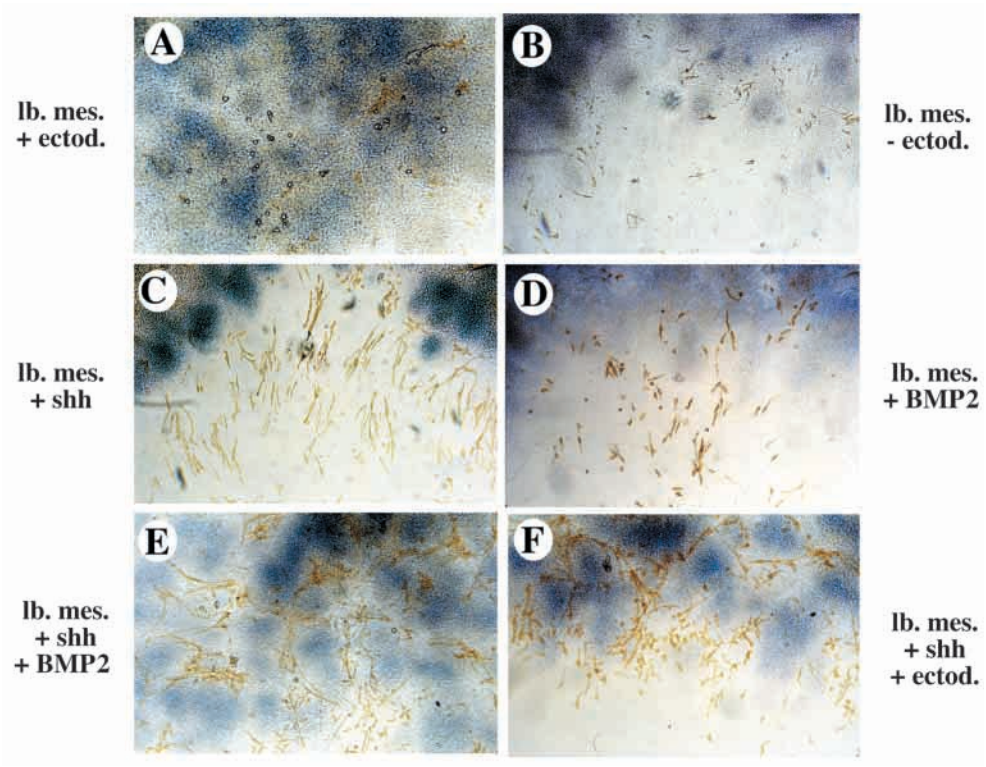

Fig. 6. Ectoderm, Shh and BMP2 stimulate myogenic differentiation in micromass cultures derived from the limb bud mesenchyme. Micromass cultures were established from complete limb buds of E10.5 mouse embryos (A, F) or from the limb bud mesenchyme separated from the ectoderm (B-E) and stained with Alcian Blue and the MyHC antibody MF-20 after incubation with Shh (C,F), BMP2 (D), and Shh and BMP2 (E). Removal of the ectoderm without additional growth factors led to decrease of myocytes (B). Addition of Shh (C) or BMP2 (D) rescued the absence of the ectoderm although the morphology of myocytes treated with BMP2 alone was different (shorter and smaller myocytes than in control cultures) (D). Combined addition of Shh and BMP2 (E) or ectoderm and Shh (F) led to a further increase in myogenic differentiation.

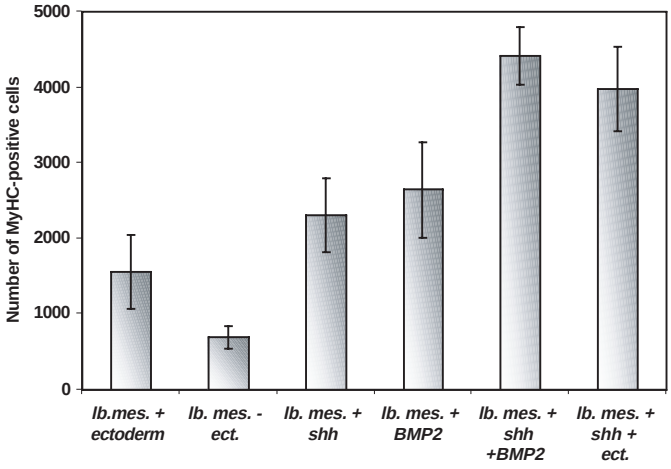

Fig. 7. Shh and BMP2 increase the number of differentiated muscle cells in limb bud-derived micromass cultures (lb. mes.). Myocytes from cultures presented in Fig. 6 were counted after staining with the MyHC antibody MF-20 and plotted. Cumulative data from four independent experiments are shown. Only myocytes that were discernible as individual cells were counted. lb.mes., limb bud mesenchyme.

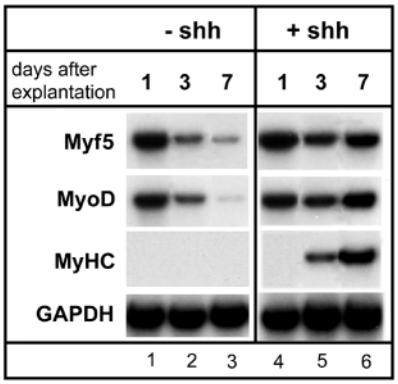

Fig. 8. Shh maintains the expression of myogenic factors in limb bud explant cultures and enables myogenic differentiation. Tissue fragments derived from E10.5 limb buds were incubated in collagen gels without ectoderm for 1, 3 and 7 days with or without the addition of Shh. RNA was isolated and subjected to RT-PCR with primers specific for the markers indicated. The lack of Shh led to a decrease in the expression of *Myf5* and *MyoD* after 3 and 7 days, while cultures in the presence of Shh maintained the expression of *Myf5* and *MyoD*, albeit at a slightly reduced level. Expression of the embryonic isoform of MyHC was only detected when Shh was added to the cultures and the fragments were incubated for at least 3 days.

myocytes has been described (Wilson and Harris, 1993) that is, however, far below what we have observed in *Shh* and *Dsh* mutants. In addition, it has been shown using the mouse mutant peroneal muscular atrophy model that normal numbers of primary myocytes formed in aneural muscles and that secondary myotube formation in the mouse, as in the chick, is not directly dependent on innervation (Ashby et al., 1993a). In this model, secondary myotubes initially appeared in normal numbers but degenerated later during life (Ashby et al., 1993b). Taken together these studies clearly show that it is not very likely that the lack of innervation contributed significantly to the major loss of myotubes observed in *Shh* and *Dsh* mutant mice before E16.5.

Is the effect of *Shh* on myogenic cells direct or mediated indirectly by other cytokines?

Despite the fact that *Shh* in not generally believed to exert its

effects on distantly located cells with the aid of secondary relay signals, numerous examples exist in which diffusible morphogens act downstream of *Shh* signaling to initiate target gene expression. For example, in *Drosophila* wing and leg imaginal disc development, Decapentaplegic (Dpp), the ortholog of *Bmp2* and *Bmp4* functions downstream of *Hh* (Basler and Struhl, 1994). Similarly, it has been shown recently that in vertebrate limb development the secreted BMP antagonist gremlin relays the Shh signal from the ZPA to the AER (Zuniga et al., 1999). However, the relationship between *Shh* and BMPs is still not well established in vertebrate development. Recently, Patel and colleagues demonstrated that Shh beads, when implanted into wing buds of developing chicken embryos upregulated the expression of its receptor, patched, leading to an increase of the area in which *Pax3* and subsequently *MyoD* are expressed (Amthor et al., 1998). Since the pattern of *Pax3* and *MyoD* expression cannot be explained by the direct action of Shh, Patel and colleagues reasoned that the effect of Shh in inducing *Pax3* was indirect and mediated by proliferative signals such as BMP2 and BMP7.

We show that the normal expression of *Bmp4* in limb buds is dependent on Shh and that in particular the strong expression domains of *Bmp4* in the anterior and posterior margin of the limb bud were absent in *Shh* mutant mice. Whether this deficiency contributes to the loss of limb musculature in *Shh* mutants is not absolutely clear, but several lines of evidence support the view that the reduced expression of *BMP4* at least contributes to the muscle phenotype:

(1) Implantation of BMP2 and BMP4-loaded beads into developing chicken limb buds induced *Pax3* expression and subsequently *MyoD* expression at some distance of the bead. Higher concentrations of BMP2 or BMP4, or a closer contact to implanted beads prevented *Pax3* and *MyoD* expression and induced apoptosis (Amthor et al., 1998).

(2) Treatment of micromass cultures derived from mouse limb bud mesenchyme with BMP2 or BMP4 increased the number of myotubes (this study).

(3) Ectopic expression of *Shh* in chicken limb buds induced muscle hypertrophy (Duprez et al., 1998) and at the same time resulted in an induction of *BMP2* expression (Amthor et al., 1998).

In our in vitro system, both the addition of Shh and BMPs increased the number of MyHC-positive myotubes and substituted for the absence of ectoderm. As Shh and BMPs are also present in the mesenchyme, it is unlikely that the effect of ectoderm removal is due only to a reduction of Shh- and BMP-expressing cells. Additional signaling cascades derived from the ectoderm (e.g. based on Wnt7a), which normally contribute to the expression of Shh and BMPs, or their receptors, in limb bud mesenchyme might have been eradicated. (This possibility is in agreement with our findings that removal of ectoderm resulted in an absence of Wnt7a and En1 expression and a reduction of *Shh*, *Bmp2* and *Bmp4* mRNAs in corresponding micromass cultures.) Exogenous addition of BMPs and Shh probably bypasses this influence. The well known stimulation of Shh and BMP receptors by their ligands may also add to this positive-feedback loop. Taken together, a rather complex picture emerges with a number of different feedback loops that contribute to assure the survival and extension of the right amount of muscle tissue both in epaxial and hypaxial structures.

Is there a difference between epaxial and hypaxial muscles?

Classical experiments in chicken embryos involving the excision of the neural tube and notochord have led to the definition of two different muscle cell lineages in somites giving rise to epaxial and hypaxial cells, respectively (Ordahl and Le Douarin, 1992; Rong et al., 1992). This distinction is based on the differential dependence on local signaling molecules, anatomical position and future fate of these cells. Some hypaxial cells undergo a long range migration to supply muscle precursor cells for the limb. These migration events are an invention of athropods, which, owing to their complex limb structure, cannot solely rely on an extension of the myotome that was still sufficient in some fish species to generate muscles of the fin (Grim, 1973; Neyt et al., 2000). Muscle precursor cells of the limb are kept in an undifferentiated state to allow migration and additional proliferation. However, there seems to be no reason why this cell population should have given up the use of the Shh signaling systems to allow maintenance, survival and expansion of muscle cells. Indeed, our results show that hypaxial as well as epaxial cells depend on *Shh*. The main difference is that *Shh* dependence of hypaxial cells becomes apparent later during development, which might be due to the extra steps necessary to generate limb muscle cells. Thus the mechanisms that control development of epaxial and hypaxial muscle precursor cells might be more similar than anticipated.

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