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Sonic hedgehog controls epaxial muscle determination through *Myf5* activation

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SUMMARY

Sonic hedgehog (Shh), produced by the notochord and floor plate, is proposed to function as an inductive and trophic signal that controls somite and neural tube patterning and differentiation. To investigate Shh functions during somite myogenesis in the mouse embryo, we have analyzed the expression of the myogenic determination genes, *Myf5* and *MyoD*, and other regulatory genes in somites of *Shh* null embryos and in explants of presomitic mesoderm from wild-type and *Myf5* null embryos. Our findings establish that Shh has an essential inductive function in the early activation of the myogenic determination genes, *Myf5* and *MyoD*, in the epaxial somite cells that give rise to the progenitors of the deep back muscles. Shh is not required for the activation of *Myf5* and *MyoD* at any of the other sites of myogenesis in the mouse embryo, including the hypaxial dermomyotomal cells that give rise to the abdominal and body wall muscles, or the myogenic progenitor cells that form the limb and head muscles. Shh also functions in somites to establish and maintain the medio-lateral boundaries of epaxial and hypaxial gene expression. *Myf5*, and not *MyoD*, is the target of Shh signaling in the epaxial dermomyotome, as *MyoD* activation by recombinant Shh protein in presomitic mesoderm explants is defective in *Myf5* null embryos. In further support of the inductive function of Shh in epaxial myogenesis, we show that Shh is not essential for the survival or the proliferation of epaxial myogenic progenitors. However, Shh is required specifically for the survival of sclerotomal cells in the ventral somite as well as for the survival of ventral and dorsal neural tube cells. We conclude, therefore, that Shh has multiple functions in the somite, including inductive functions in the activation of *Myf5*, leading to the determination of epaxial dermomyotomal cells to myogenesis, as well as trophic functions in the maintenance of cell survival in the sclerotome and adjacent neural tube.

Key words: Sonic hedgehog, Muscle determination, Mouse, Myf5 activation

INTRODUCTION

Sonic hedgehog (Shh) produced by the notochord has been identified as a developmental signaling molecule involved in the regulation of *MyoD* expression and somitic lineage determination in avian and zebrafish embyros (Johnson et al., 1994; Munsterberg et al., 1995; Blagden et al., 1997; Borycki et al., 1998). In the mammalian embryo, a role for Shh in somite myogenesis has been questioned, based on observations that *MyoD* and *Myf5* are expressed in *Shh* null embryos (Chiang et al., 1996). Recent genetic and gene expression studies, however, have revealed that activation of *Myf5* and *MyoD* in the mouse embryo is subject to complex temporal and spatial regulation during myogenesis in the embryo. Gene targeting studies reveal that the myogenic regulatory genes, *Myf5* and *MyoD*, are partially redundant in their myogenic determination functions (Rudnicki et al., 1992; Braun et al., 1994), but together, these genes are essential for determination of myogenic progenitors throughout the mouse embryo (Rudnicki et al., 1993). During embryonic development, *Myf5* and *MyoD* are activated at different times in somites (Tajbakhsh and Buckingham, 1999). *Myf5* is activated first in E8.0 embryos in the medial, epaxial somite progenitors that give rise to the deep back muscles, and later is activated at E9.75 in the lateral hypaxial somite cell progenitors that give rise to the limb, diaphragm and body wall muscles (Ott et al., 1991; Sporle et al., 1996; Tajbakhsh et al., 1996a). *MyoD* activation in the epaxial and hypaxial dermomyotomal cells follows that of *Myf5* activation, most notably in the epaxial domain (Sassoon et al., 1989; Buckingham, 1992), but *MyoD*

is activated nearly simultaneously with *Myf5* in the limb and head muscle progenitors (Buckingham, 1992; Goldhamer et al., 1992). Finally, in vitro studies in the mouse have established that determination of epaxial and hypaxial progenitors is controlled by distinct signals from the axial notochord and neural tube and from the surface ectoderm (Cossu et al., 1996). In particular, Wnts can induce myogenesis in mouse presomitic mesoderm explants, acting either synergistically with Shh or through an independent myogenic signaling pathway (Tajbakhsh et al., 1998).

In order to define the functions of Shh in epaxial and hypaxial myogenesis in the somite as well as in myogenesis at other sites in the mouse embryo, we have examined the regulation of the myogenic regulatory genes, *Myf5* and *MyoD*, in somites of *Shh* null embryos. Using this genetic approach, we have assessed whether Shh functions during myogenesis as an inducer of myogenic regulatory genes or as a trophic factor in the control of proliferation and apoptosis, as proposed previously (Teillet et al., 1998). In this study, we now report that Shh is essential for *Myf5* and *MyoD* activation in the determination of epaxial muscle progenitors, but not in the determination of hypaxial myogenic progenitors, which must be specified by independent signaling pathways. We also show that Shh is essential for medio-lateral patterning of epaxial and hypaxial gene expression in somites. In vitro studies reveal that *Myf5* is the primary target of Shh induction in explants of presomitic mesoderm associated with surface ectoderm. *MyoD* activation requires Myf5 function and, therefore, is a downstream regulator of epaxial myogenesis. Finally, our results establish that Shh is not essential for survival or proliferation of epaxial somite cells, although Shh function is essential specifically for the survival of ventral somite sclerotomal cells and for motorneuron and neural crest cells in the adjacent ventral and dorsal neural tube. We conclude, therefore, that Shh has an inductive function in epaxial myogenic determination and a trophic cell survival function in the sclerotome and the neural tube.

MATERIALS AND METHODS

Explant culture

Presomitic mesoderm (PSM) with overlying surface ectoderm (SE) was dissected from E9.5 CD1 (Charles River) or *Myf-5* null (kindly provided by M. Rudnicki) mouse embryos. When indicated, the axial notochord/neural tube complex (NC+NT) was dissected together with the PSM, or SE was removed following an incubation for 45 seconds in a 1× dispase solution (Boehringer Mannheim). In all explants, the lateral plate was removed. Explants were transversally cut into anterior and posterior halves, transferred without dissociation of cells onto gelatin-coated 48-well plates, and cultured for 3 days in a humidified CO_2 incubator at 37°C in 0.5 ml of DMEM/F-12 medium (Gibco-BRL) containing 15% FCS (Gibco-BRL), 1% penicillin/ streptomycin (Gibco-BRL) and 2 ng/ml bFGF (Sigma). Note the absence of a feeder layer in these cultures, which distinguishes this assay from the explant assay described previously (Cossu et al., 1996), in which Shh alone in the absence of SE did not produce a response (Tajbakhsh et al., 1998). Anterior and posterior explants were shown to have identical myogenic potential. Addition of 2 ng/ml bFGF to the culture medium did not affect *MyoD* activation, but was required for cell survival. Purified baculovirus-produced N-Shh (kindly provided by Ontogeny) was added to the culture medium at the final concentration of 200 ng/ml. For cell counts, explants were fixed and DAPI-stained after culture and nuclei were counted using a Leica video camera.

Immunohistochemistry

Cultures were fixed for 30 minutes in 4% paraformaldehyde, washed with PBS, and incubated for 1 hour at room temperature in a blocking solution containing 0.1% Triton X-100 and 2% FCS in PBS. Cultures were then incubated overnight at 4°C with primary antibody (anti-mouse MyoD 5.8A, Novocastra) at a dilution of 1:25, followed by a 30-minute incubation with the secondary antibody (biotin-conjugated anti-mouse IgG, Vector Labs) at a dilution of 1:200. Peroxidase staining to reveal MyoD-positive cells was performed using the Vectastain Kit (Vector Labs) according to the manufacturer's instructions. MyoD-positive cells/explant were counted using an inverted Leitz-DMIRB microscope.

Semi-quantitative PCR

RNA from explant cultures was prepared and reverse-transcribed for 1 hour at 37°C using 10 ng of random hexamer primers and Superscript II (Boehringer Mannheim). Semi-quantitative PCR was carried out in a 50 μl reaction volume in the presence of 1.5 mM $MgCl_2$, 200 μM dNTP, 1 μM of each primer (GAPDH + Myf5), 0.25 μl [α-^{32}P]dCTP (3000 Ci/mmole, NEN) and 0.5 μl Taq Polymerase (Promega). *GAPDH* primers were f-GTGCCAGCCTCGTCCCGTAG and r-CCAAAGTTGTCATGGATGACC, and *Myf5* primers were f-TGAAGGCTCCTGTATCCCCTCAC and r-ATAGTTCTCCACC-TGTTCCCTCAGC. After denaturation for 5 minutes at 94°C, amplification was performed for 28 cycles (94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute) with both primers, followed by 5 minutes at 72°C. PCR products were resolved by electrophoresis on a 5% acrylamide gel, analyzed by autoradiography with a phosphoimager Storm 860 (Molecular Dynamics), and radioactivity quantitated using a program ImageQuant (Molecular Dynamics). Levels of *Myf5* expression were normalized to the level of *GAPDH* expression in each culture condition.

In situ hybridization

Whole-mount in situ hybridization was carried out essentially as previously described (Henrique et al., 1995). RNA probes were synthesized using restriction enzymes and RNA polymerase as follows: *MyoD*: *Mlu*I and T3; *Myf5*: *Hin*dIII and T7; *Pax3*: *Xho*I and T7; *Sim1*: *Eco*RI and T7. Transverse sections (100 μm) of whole-mount stained embryos were performed with a Vibrotome 1000 and photographed using DIC optic system (Leica).

TUNEL assay

E9.5 and E10.5 wild-type and *Shh* null embryos were embedded in paraffin, and 10 μm transverse sections were collected. Sections were treated for 10 minutes at room temperature with Proteinase K (20 μg/ml), washed in PBS, and incubated for 2 hours at 37°C in 100 μl of 1× TdT buffer containing 2 ng Biotin-dCTP (Boehringer Mannheim) and 30 i.u. TdT enzyme (Promega). Following a 30-minute incubation at room temperature in PBS containing Streptavidin-Texas Red (Vector Labs; 1:50), slides were mounted in Vectashield (Vector Labs), and photographed using a video camera (Leica).

Proliferation assay

Pregnant mice from Shh +/− heterozygous crosses were injected with 100 μg/g body mass of BrdU into the peritoneal cavity 1 hour before killing. E10.5 wild-type and *Shh* null embryos were embedded in paraffin, and 8 μm transverse sections were collected. Following de-waxing, slides were incubated at 37°C for 50 minutes in 2N HCl, neutralized in 0.1M sodium borate, pH 8.5, for 10 minutes, and incubated for 1 hour at 37°C with an anti-BrdU antibody (1:10; Boehringer Mannheim) in PBS containing 1% BSA and 0.1% Tween 20. Following incubation for 1 hour at 37°C with a secondary

biotinylated anti-IgG antibody (1:200, Vector Labs), labeling was revealed using the ABC detection kit (Vector Labs) and DAB/peroxidase staining. Slides were mounted in Aquamount, and photographed with a video camera (Leica).

RESULTS

Shh is essential for *Myf5* and *MyoD* activation in the epaxial somite muscle progenitors

To investigate the function of Shh in the specification of epaxial and hypaxial myogenic cells in the mouse embryo, we used a whole-mount in situ hybridization assay to examine the expression of *Myf5*, *MyoD* and other somite-expressed genes in *Shh* null embryos (Figs 1, 2). In newly formed somites of E9.75 wild-type mouse embryos, *Myf5* activation occurs exclusively in the dorso-medial compartment, specifying the progenitor cells of epaxial muscles (Figs 1A,B, 2A). In contrast, *Myf5* expression is absent from the medial epaxial domain of newly-formed somites in E9.75 *Shh* null embryos (Figs 1C,D, 2B). Interestingly, at this stage, somites of *Shh* null embryos retain an epithelial morphology, and formation of the dermomyotome is clearly observed (Fig. 2A,B). In interlimb somites of E9.75 wild-type and *Shh* null embryos, *Myf5* expression initiates on schedule in the hypaxial domain (Fig. 1A-D), showing that Shh-independent signaling processes regulate the activation of this gene in the hypaxial somite muscle progenitors.

By E10.5 in wild-type embryos, *Myf5* is expressed in the ventro-lateral domain of interlimb somites where hypaxial muscle progenitor cells arise (Figs 1E,F, 2C). At this stage, *MyoD* in interlimb somites is expressed at higher levels in the hypaxial dermomyotome than in the epaxial dermomyotome (Fig. 1I,J). Most notably in the E10.5 *Shh* null embryos, neither *Myf5* nor *MyoD* expression is detected in the epaxial dermomyotome, although normal expression of both genes is observed in the hypaxial dermomyotome (Figs 1G,H,K,L, 2D). Noticeably, although both epaxial and hypaxial dermomyotome is clearly visible in posterior somites (Figs 2I-L, 3E,F), by the interlimb region, the epithelial organization of the epaxial dermomyotome is lost while the hypaxial dermomyotome remains, including the ventral lateral lip (Figs 2C-H, 3G). This suggests that a secondary defect due to the lack of *Shh* signalling is the loss of the dorso-medial lip of the dermomyotome. In *Shh* heterozygous mutant embryos, we did not observe defects in *Myf5* or *MyoD* expression in somites (data not shown). These observations, therefore, establish that Shh is essential specifically for *Myf5* and *MyoD* activation in the myogenic progenitors of the epaxial dermomyotome, but not in the progenitors of the hypaxial dermomyotome.

Because of the dramatic and highly specific defects in epaxial gene expression in the *Shh* null embryos, we examined *Myf5* and *MyoD* expression at other myogenic locations. We found that expression of *Myf5* and *MyoD* in limb buds and hypoglossal chord, precursor of tongue, pharynx and shoulder muscles, is unaffected in *Shh* null embryos, and is detected but delayed in the branchial arches (Fig. 1G,K). Taken together,

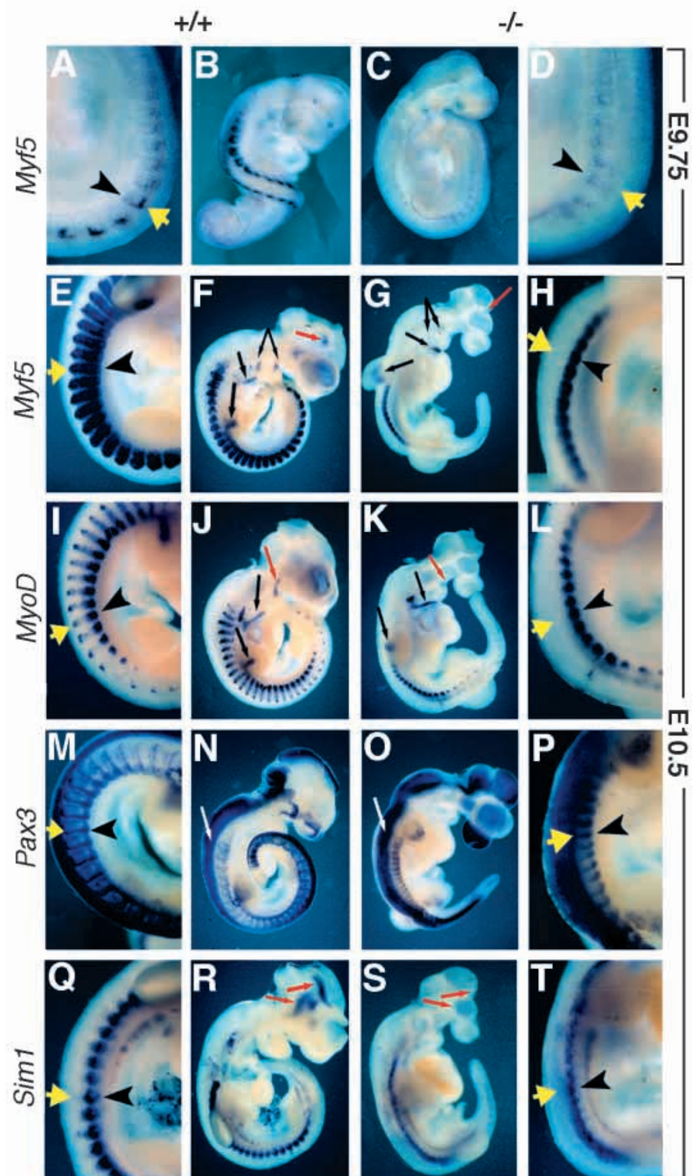

Fig. 1. Sonic hedgehog is essential for *Myf5* and *MyoD* activation in the epaxial domain of somites in vivo. Gene expression in wild-type (A,B,E,F,I,J,M,N,Q,R) and *Shh* null (C,D,G,H,K,L,O,P,S,T) embryos was analyzed by whole-mount in situ hybridization. (A-D) *Myf5* expression in E9.75 wild-type (A,B) and *Shh* null embryos (C,D). In *Shh* null embryos, *Myf5* is not activated in the epaxial domain of somites (yellow arrow) although activation in the hypaxial domain (black arrowhead) occurs on schedule. (E-L) *Myf5* (E-H) and *MyoD* (I-L) expression in E10.5 wild-type (E,F,I,J) and *Shh* null (G,H,K,L) embryos. *MyoD* and *Myf5* expression is missing in the epaxial somite domain of *Shh* null embryo (yellow arrow). In contrast, *MyoD* and *Myf5* expression at other sites, such as hypaxial somite domain (black arrowhead), limbs, hypoglossal chord and branchial arches, is unaffected or delayed (black arrows), demonstrating the specific requirement of Shh for epaxial myogenesis. Red arrows indicate that *Myf5*, *MyoD and Sim1* expression is missing in the head of *Shh* null embryos, likely because of midbrain defects in these embryos (Chiang et al., 1996). (M-P) *Pax3* expression in E10.5 wild-type (M,N) and *Shh* null (O,P) embryos. Note that *Pax3* expression is observed in somites and in the presomitic mesoderm of *Shh* null embryos, indicating that cells are viable. White arrows indicate ventral expansion of *Pax3* in the neural tube of *Shh* null embryos. (Q-T) *Sim1* expression in E10.5 wild-type (Q,R) and *Shh* null (S,T) embryos. Magnification, ×15 (B,F,J,N,R), ×20 (C,G,K,O,S) and ×40 (A,D,E,H,I,L,M,P,Q,T).

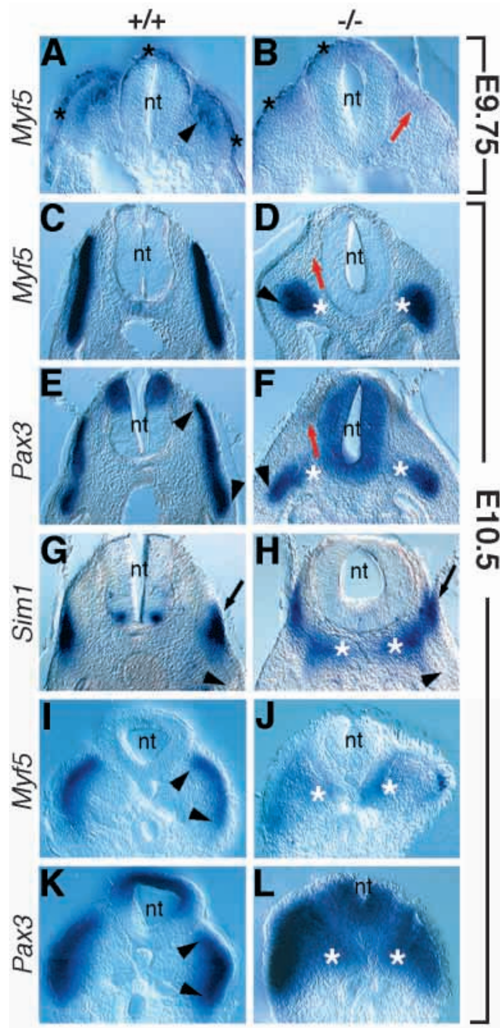

Fig. 2. Expansion of hypaxial gene expression in the ventral somites in E10.5 *Shh* mutant embryos. (A-L) Transverse sections of whole-mount in situ hybridization of E9.75 (A,B) and E10.5 (C-L) wild-type and *Shh* null embryos with *Myf5*, *Pax3* and *Sim1* DIG-labeled RNA probes. (A,B) Transverse sections at the level of the tail of E9.75 wild-type (A) and *Shh* null (B) embryos hybridized with a *Myf5* RNA probe. At this axial level of the wild-type embryo, *Myf5* begins to be expressed in the dorso-medial somite (black arrowhead) and is not yet detected in the ventro-lateral somite. In the homozygous mutant littermate, *Myf5* expression could not be detected even after prolonged staining (red arrow). Black asterisks indicate sites of background staining due to long exposure time of embryos during color development. (C-H) Sections at the level of the interlimb region of E10.5 embryos hybridized with *Myf5* (C,D), *Pax3* (E,F), and *Sim1* (G,H) RNA probes. Black arrowheads indicate the dorso-medial and ventro-lateral lip of dermomyotome in wild-type embryos. Note that the dorso-medial, but not the ventro-lateral lip is missing in these embryos at this axial level. Although *Myf5* and *Pax3* are not detected in the dorsal somite (red arrow), many cells are present attesting of the viability of the dorsal somite. In contrast, *Sim1* expression, which marks the central domain of the dermomyotome, is unaffected (black arrow). Noticeably, *Myf5*, *Pax3* and *Sim1* expression expands medially into the ventral somite (white asterisk), indicating that Shh also functions by preventing medial expansion of hypaxial genes. (I-L) Sections of the tail of E10.5 embryos hybridized with *Myf5* (I,J) and *Pax3* (K,L) RNA probes. Black arrowheads indicate *Myf5* and *Pax3* expression in the epaxial and hypaxial domain of newly formed somites of wild-type embryos. In *Shh* null embryos in which epaxial *Myf5* expression does not occur (see B), hypaxial expression of *Myf5* and *Pax3* expands into the entire somite (white asterisk). Note the ventral expansion of *Pax3,* and the loss of *Sim1* expression in the neural tube (F,H,L). nt, neural tube.

these data establish that Shh signaling is essential for epaxial myogenesis, and that other Shh-independent signaling pathways must control myogenesis at all other sites in the embryo.

Shh is essential for medial-lateral somite patterning

To examine the status of epaxial dermomyotomal cells in *Shh* null embryos, we performed in situ hybridization with *Pax3* (Goulding et al., 1991) and *Sim1* (Fan et al., 1996) probes. In interlimb somites of E10.5 wild-type embryos, *Pax3* is expressed throughout the dermomyotome (Figs 1M,N, 2E), whereas *Sim1* expression is concentrated in the central dermomyotome (Figs 1Q,R, 2G). In interlimb somites of E10.5 *Shh* null embryos, *Pax3* expression is not detected in the dorso-medial domain and is restricted to a ventro-lateral domain (Figs 1O,P, 2F) where hypaxial *Myf5* expression also is localized (Fig. 2D). The lack of *Pax3* expression in the dorso-medial domain of interlimb somites is accompanied by the loss of the dorso-medial lip of the dermomyotome (Fig. 2D,F,H). In contrast, *Sim1* expression, which marks a central dermomyotome territory is relatively unaffected in *Shh* null embryos (Figs 1S,T, 2H). Therefore, *Shh* null embryos are characterized by specific gene expression defects restricted to the epaxial dermomyotomal cells of somites.

Although activation of hypaxial gene expression is not affected in *Shh* null embryos, the domain of expression of *Myf5*, *Pax3* and *Sim1* is ventrally expanded in E10.5 interlimb somites (Fig. 2D,F,H). This ventro-medial expansion of hypaxial gene expression is even more dramatic in newly formed somites of E10.5 *Shh* null embryos, where *Myf5* (Fig. 2I,J) and *Pax3* (Fig. 2K,L) expression expands in the whole somite. This observation indicates that in newly formed somites, cells of the medial compartment are largely viable. In agreement with previous data in avian embryos (Hirsinger et al., 1997), this observation also provides direct evidence that, in addition to its function in *Myf5* expression in the epaxial domain of somite, Shh also has negative regulatory functions to restrict hypaxial gene expression to the lateral domain and to establish epaxial and hypaxial somite compartments.

The *Shh* requirement for *Myf5* expression is not the result of a proliferation defect in the epaxial somite

To address whether the deficit in epaxial muscle progenitors in *Shh* null embryos is due to a failure of somite cell proliferation, we performed PCNA labeling at E9.5 (data not shown) and BrdU labeling at E10.5 (Fig. 3) of wild-type and *Shh* null embryos. At E10.5, in the posterior embryo, active mitotic cells are observed throughout the neural tube and newly formed somites of wild-type and *Shh* null embryos, including the cells of the dorso-medial somite (Fig. 3A,E). In somites at the hindlimb level of wild-type embryos, BrdU-labeled cells are detected throughout the dermomyotome (Fig. 3B), including the dorsal and ventral lip where *Myf5* and *MyoD* are being activated (Fig. 1E,I), and become sparse in the ventral somite

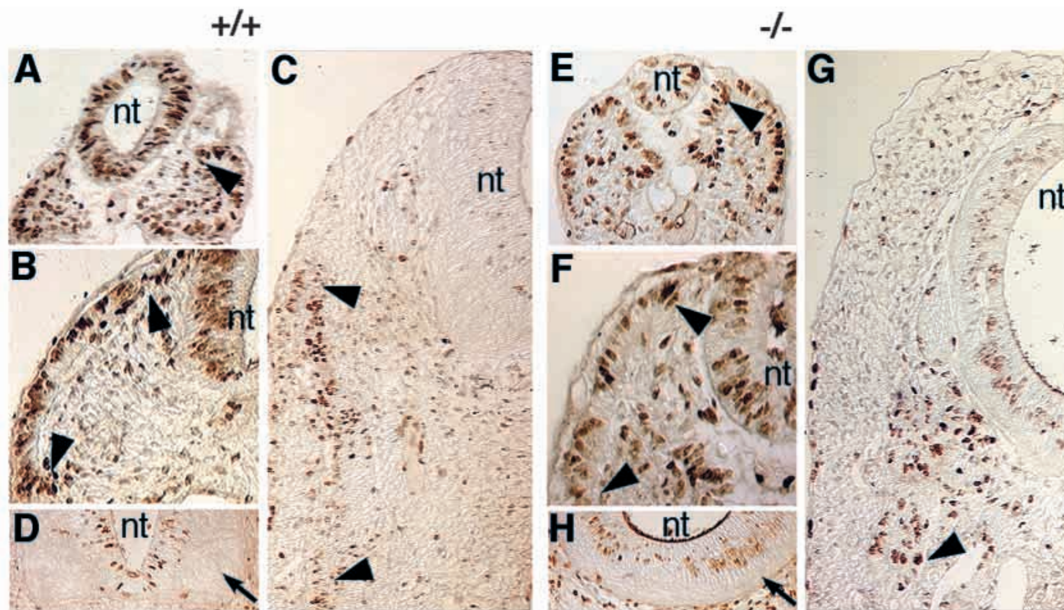

Fig. 3. Cell proliferation in *Shh* null embryos. Transverse sections of E10.5 BrdU-labeled wild-type (A-D) and *Shh* null (E-H) embryos do not show lineage-specific defects in cell proliferation. (A,E) Transverse sections at the level of the tail show mitotic cells in the epaxial domain of somites in +/+ and −/− *Shh* embryos (black arrowheads). Neural tube (nt) in A appears larger because the section is slightly oblique. (B,F) Transverse sections of somites at the level of the hindlimb also show the presence of mitotically active cells in both the epaxial and hypaxial dermomyotome. Black arrowheads indicate the epaxial and hypaxial dermomyotome. (C,G) Transverse sections of somites at the interlimb level show a decrease in cell proliferation in mesenchymal cells, but dermomyotomal cells remain mitotically active (black arrowheads) in wild-type embryos. In *Shh* null embryos, the dorso-medial lip of the dermomyotome is missing at this level, but the hypaxial dermomyotome still proliferates (black arrowhead). (D,H) Proliferation in the ventral neural tube at the interlimb level is unaffected. In *Shh* null embryos, as in wild-type embryos, cells in the ventricular zone are proliferative whereas cells at the periphery of the neural tube withdraw from the cell cycle (black arrow). Note in (A,B,E,F) the presence of the notochord, indicating that it had not yet degenerated. Nt, neural tube.

(Fig. 3B). In *Shh* null embryos, a similar pattern of BrdU labeling is observed throughout the dermomyotome, and cells in the epaxial dermomyotomal lip are clearly present as in the wild-type embryo (Fig. 3F). These data, therefore, indicate that the lack of *Myf5* and *MyoD* activation in the epaxial somite of *Shh* null embryos cannot be attributed to a lineage-specific deficit in cell proliferation in the dorso-medial somite. At E9.5, PCNA-labeled cells are reduced in *Shh* null embryos compared to wild-type embryos (data not shown), which could explain the decreased size of *Shh* null embryos. However, PCNA-labeled cells in *Shh* null embryos are observed in the dorso-medial domain of newly formed somites that fail to activate *Myf5* (Fig. 2B), further supporting the conclusion that the requirement for Shh to activate *Myf5* in the epaxial dermomyotome is not related to defects in the proliferative capacity of epaxial dermomyotomal cells.

Decreased cell proliferation occurs in mesenchymal cells of anterior somites in wild-type embryos, although epaxial and hypaxial dermomyotomal cells remain mitotically active (Fig. 3C). In *Shh* null embryos, in the absence of epaxial dermomyotome, little cell proliferation is observed in the dorsal somite as in wild-type embryos (Fig. 3G). However, the hypaxial dermomyotome remains mitotically active (Fig. 3G). In the neural tube at this stage, cell proliferation in wild-type embryos is restricted to the ventricular zone (Fig. 3C,D) whereas lateral cells have withdrawn from the cell cycle and are differentiating to form motorneurons. Although the differentiation of the ventral neural tube is disrupted in *Shh* null embryos (Chiang et al., 1996), the neural tube of *Shh* null embryos also displays a central zone of proliferating cells and a lateral zone of cells withdrawn from the cell cycle (Fig. 3G,H), indicating that cell proliferation is not selectively disrupted in the neural tube of *Shh* null embryos.

The *Shh* requirement for *Myf5* expression is not the result of a cell survival defect in the epaxial somite

To address whether lack of *Myf5* activation in the epaxial somite domain is due to a cell survival defect, we performed TUNEL assays in E9.5 and E10.5 wild-type and *Shh* null embryos (Fig. 4). At E9.5, when *Shh* null embryos fail to activate *Myf5* in newly formed posterior somites (Fig. 2B), TUNEL assay did not detect cell death in posterior somites of mutant embryos except for occasional apoptotic cells that were also observed in wild-type embryos (Fig. 4A,B). Thus, apoptosis cannot account for failure of epaxial cells in posterior somites to activate *Myf5* and *MyoD* expression in *Shh* null embryos. However, increased numbers of apoptotic cells are detected in the more mature somites of the more anterior interlimb region of E9.5 *Shh* null embryos (Fig. 4D), demonstrating the sensitivity of the TUNEL assay. These apoptotic somite cells are localized primarily to the ventral somite and not in the dorsal somite, which has only a few apoptotic cells (Fig. 4D). These findings explain the progressive loss of *Pax1* expression observed in the sclerotome of *Shh* null embryos as a result of cell death (Chiang et al., 1996). At E10.5, TUNEL-positive cells are evident in the

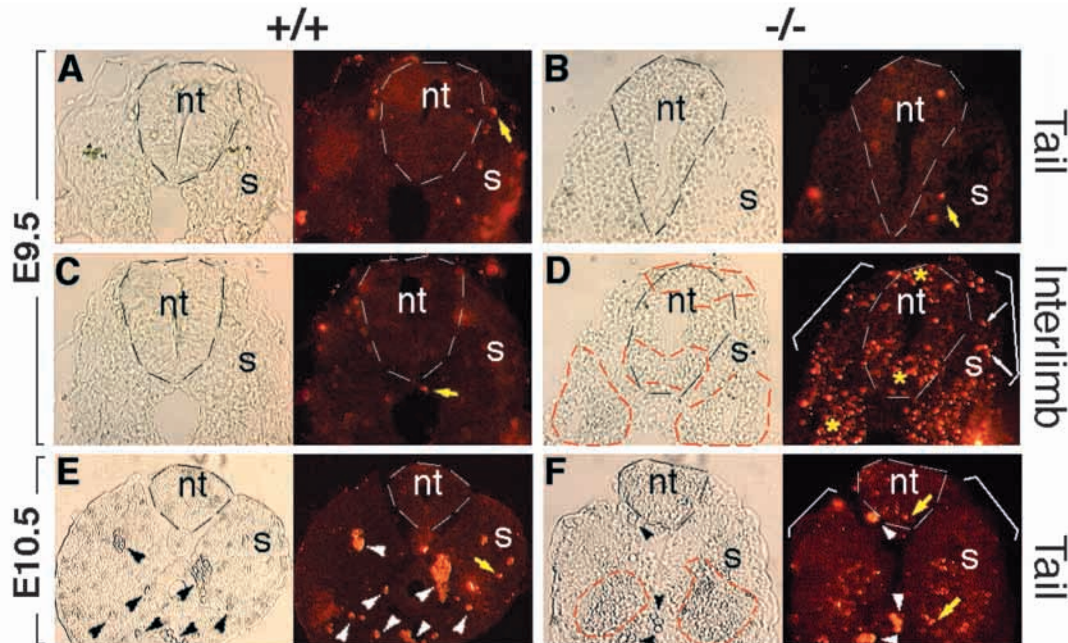

Fig. 4. Specific defects in cell survival in *Shh* null embryos. (A-F) TUNEL assay of E9.5 (A-D) and E10.5 (E,F) wild-type and *Shh* mutant embryos. (A,B) Transverse sections at the tail level of E9.5 embryos, at the time *Myf5* expression should be activated (see Fig. 2A,B), do not show an increase of apoptosis in *Shh* null embryos. (C,D) Transverse sections at the interlimb level of E9.5 embryos show specific increase in apoptosis in the ventral somite domain, the ventral neural tube, excluding the floor plate region, and the dorsal neural tube of *Shh* null embryos. In contrast, the dorsal somite domain does not show massive increase in cell death. (E,F) Transverse sections at the tail level of E10.5 embryos show cells that begin to die in the ventral neural tube and somite. Bright field is on the left and dark field on the right. Broken black and grey lines outline the neural tube (nt). Black and white arrowheads indicate autofluorescence of blood cells. Yellow arrows indicate examples of TUNEL-positive, apoptotic cells in somites (s) and neural tube (nt). Yellow asterisks (in dark field) and red dotted lines (in bright field) indicate extensive TUNEL-positive cells in ventral and dorsal neural tube, and in the ventral somite. White brackets indicate the dorsal epaxial somite domain where *Myf5* would be expressed and where there are few TUNEL-positive cells.

ventral domain of newly formed somites (Fig. 4F), consistent with the analysis of E9.5 embryos showing that apoptosis is mainly restricted to the cells of the ventral somite. As with E9.5 embryos, significant apoptosis is not observed in the dorsal somite of E10.5 *Shh* null embryos.

TUNEL analysis also detected extensive apoptosis in the ventral neural tube encompassing the domain of motorneuron formation, as well as in the dorsal roof plate of the neural tube and in cells lateral to the dorsal neural tube corresponding to sites of migrating neural crest cells (Fig. 4D). Therefore, the Shh signaling function for cell survival is highly patterned and affects well-defined domains of the dorsal and ventral neural tube and the ventral somite. Notably, Shh is not required for survival of the dorsal somite or the floor plate.

Significantly, Shh function for cell survival is transient during embryonic development. At E9.5, apoptosis is observed in these neural tube or somite domains in the interlimb region between the posterior hindlimb and the forelimb level. At E10.5, apoptosis is detected in more posterior regions of the embryo, in the neural tube at the level of the presomitic mesoderm and in the ventral somite beginning at the level of somite formation. Apoptosis extends at this stage up to the hindlimb level, where ventral somite cells decondense to initiate sclerotome formation, but is not detected in somites and neural tube at the interlimb level (data not shown). These findings indicate that early neural tube and somite lineages require the presence of Shh either directly for their survival or alternatively, for their determination, the absence of which leads to their death.

Myf5, and not *MyoD*, is the primary target of Shh signaling in presomitic mesoderm explants

As Shh is not required for cell proliferation or cell survival in dorsal epaxial somite cells at the stage that *Myf5* and *MyoD* are being activated, the possibility remains that Shh has an inductive function in epaxial myogenesis. To further investigate inductive functions of Shh, we developed an explant assay to investigate *Myf5* and *MyoD* expression. In this assay, presomitic mesoderm (PSM) from E9.5 embryos is explanted into culture with its associated dorsal ectoderm (SE), which is retained to ensure continued expression of *Pax3* (Fan and Tessier-Lavigne, 1994). We have also shown that the surface ectoderm is required for mesodermal cell survival, as assessed by TUNEL assay (data not shown) (Borycki et al., 1999), as well as for activation of *Gli* genes (Borycki et al., 1998), which mediate Shh signaling. To examine the response of presomitic mesoderm to Shh and other signals from surrounding tissues, we first examined *Myf5* and *MyoD* expression in PSM cocultured with signaling tissues, including surface ectoderm (SE), with or without neural tube and notochord (NT+NC) (that provide a natural source of Shh), or following exposure to recombinant N-Shh protein (Fig. 5). In this explant system, we assayed *Myf5* and *MyoD* expression by semi-quantitative RT-PCR and immunohistological analyses. We found that

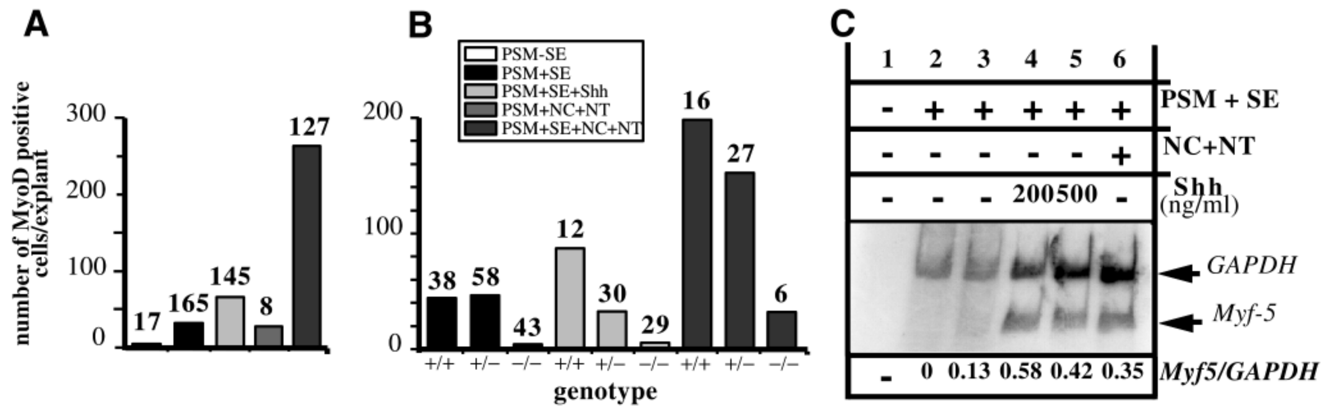

Fig. 5. Sonic hedgehog induces *MyoD* and *Myf5* expression in presomitic mesoderm explant cultures. (A) Activation of *MyoD* by surface ectoderm and axial tissue signals, and by N-Shh in vitro. Presomitic mesoderm (PSM) from E9.5 CD1 mice was cultured for 3 days, either as PSM alone, PSM with surface ectoderm (SE), PSM with SE+N-Shh, with neural tube/notochord (NC+NT) or PSM with SE+NC+NT. *MyoD* expression was assayed by immunohistochemistry. Numbers above bars represent the numbers of explants analyzed. (B) Activation of *MyoD* by SE, NC+NT and N-Shh is Myf5-dependent. *MyoD* activation is blocked in PSM+SE explants from E9.5 *Myf5* null embryos, and is reduced in PSM+SE from *Myf5* heterozygous mutant embryos, when challenged with N-Shh. (C) Shh induces *Myf5* expression in PSM+SE explant culture. PSM+SE from E9.5 CD1 embryos was cultured for 3 days, and *Myf5* expression was assayed by semi-quantitative RT-PCR. GAPDH primers were internal controls. Lane 1, negative control without RT product. Lane 2, PSM+SE prior to culture. Lane 3, PSM+SE after culture. Lanes 4, 5, PSM+SE in presence of N-Shh (200 and 500 ng/ml). Lane 6, PSM+SE+NC+NT. The ratio *Myf5/GAPDH* was calculated to normalize *Myf5* expression to the number of cells in the explant. Calculation shows a fourfold increase in *Myf5* expression following treatment with N-Shh, an increase similar to that obtained after culture in the presence of NC+NT.

MyoD expression is induced at low levels in PSM+SE explants. Explants cultured with N-Shh ($P<0.001$) or NC+NT ($P<0.0001$) express *MyoD* at significantly increased levels (Fig. 5A), demonstrating that NC+NT and N-Shh, and, to a lesser extent, SE provide inductive signals for *MyoD* activation. However, *MyoD* is not as highly activated by N-Shh as compared to NC+NT, suggesting that additional signals from NC+NT participate in *MyoD* activation, as previously reported (Cossu et al., 1996; Tajbakhsh et al., 1998).

To investigate whether *MyoD* is a direct PSM target of SE, NC+NT and N-Shh signaling, PSM from E9.5 *Myf5* null embryos was cultured in the presence of SE, NCNT and N-Shh. Significantly, we observed that *MyoD* induction is completely blocked in *Myf5*-null PSM associated with SE ($P<0.0001$) (Fig. 5B), indicating that Myf5 function is essential for the initial response of PSM to SE signals. However, myogenesis in *Myf5*-null PSM+SE explants has been reported after longer culture periods (Tajbakhsh et al., 1998), indicating that SE also can provide signals for Myf5-independent myogenesis, probably taking place in the hypaxial somite (Cossu et al., 1996). Similarly, *MyoD* activation in *Myf5*-null PSM+SE associated with NT+NC is severely reduced, but not entirely blocked ($P<0.007$) (Fig. 5B). This residual *MyoD* expression reflects signaling activity of an alternative Myf5-independent signaling pathway for *MyoD* activation. These findings are supported by in vivo studies of wild-type and *Myf5*-null embryos that reveal the existence of both Myf5-dependent and Myf5-independent pathways for activation of *MyoD* in both hypaxial and epaxial somite lineages (Tajbakhsh et al., 1997). It is notable that *MyoD* activation by N-Shh is completely impaired in PSM+SE explants from *Myf5* null homozygous embryos ($P=0.002$) (Fig. 5B), and greatly reduced in explants from heterozygous mutant *Myf5* embryos ($P=0.02$) (Fig. 5B). These findings establish that, during myogenesis, *MyoD* is downstream of *Myf5* in PSM induced by N-Shh. The disproportionate reduction in *MyoD* activation in PSM from the heterozygous *Myf5* null embryos also indicates that levels of *Myf5* expression are critical to its function as an upstream regulator of *MyoD*. This observation is consistent with earlier genetic evidence that myogenesis is defective in *MyoD* null embryos that are heterozygous for *Myf5* (Rudnicki et al., 1993).

Based on the finding that *MyoD* activation by Shh requires Myf5, we examined whether Shh induces *Myf5* expression in PSM+SE explants, as assayed by semi-quantitative RT-PCR. *Myf5* expression in PSM+SE explants is undetectable at t_0 prior to culture (Fig. 5C, lane 2) and is expressed at only very low levels following 3 days of culture in the presence of SE (*Myf5/GAPDH*=0.13) (Fig. 5C, lane 3). In contrast, *Myf5* expression is highly induced in PSM+SE explants cultured in the presence of N-Shh (*Myf5/GAPDH*=0.58) (Fig. 5C, lanes 4 and 5). The lower ratio of *Myf5/GAPDH* (0.35) in PSM+SE explants cultured in the presence of NC+NT as compared to explants cultured in the presence of N-Shh is probably an underestimation of *Myf5* activation as NC+NT cells do not express *Myf5* (Fig. 5C, lane 6). Together, our results provide evidence that *Myf5*, and not *MyoD*, is the primary target in PSM of inductive signals from the SE, NC+NT and N-Shh.

DISCUSSION

In this study, we have investigated gene expression in somites of *Shh* null mouse embryos to examine the inductive, proliferative and cell survival functions of *Shh* in the regulation of myogenesis. Previous evidence for a role of *Shh* in somitogenesis has not discriminated between inductive and trophic functions of *Shh*. A genetic approach, such as that

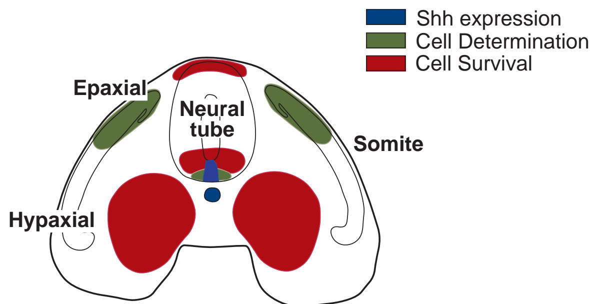

Fig. 6. Summary describing specific defects in cell determination and cell survival in the somites and neural tube of *Shh* null embryos. Shh, secreted by the notochord and the floor plate of the neural tube (blue) is required for the determination of the floor plate cells and the epaxial muscle progenitor cells (green). In addition, lack of Shh results in increased apoptosis in the sclerotomal progenitor cell in the ventral somite, and the motorneuron and neural crest progenitors in the neural tube (red).

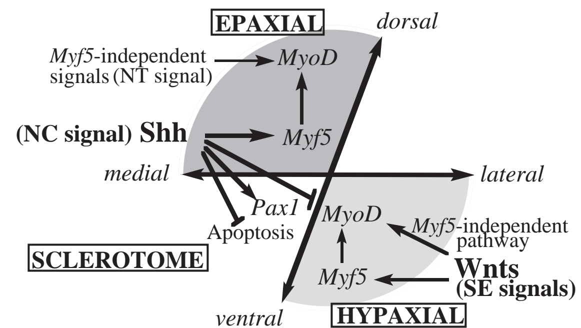

Fig. 7. Model for epaxial and hypaxial muscle lineage determination showing that two different signaling pathways control the determination of the epaxial and hypaxial lineages in somites. Hypaxial muscle cell progenitors are determined in the ventral lateral compartment of somites (light gray) and epaxial muscle cell progenitors are determined in the dorso-medial compartment of somites (dark gray). Our present work demonstrates the essential role of Sonic hedgehog in the determination of the epaxial muscle lineage during embryogenesis, and its function in restricting expansion of hypaxial gene expression into the ventral medial compartment. Epaxial muscle lineage determination occurs via a *Myf5*-dependent pathway, although a *Myf5*-independent route may also occur in absence of *Myf5*. Surface ectoderm signals, possibly Wnts (Tajbakhsh et al., 1998), are likely to control the determination of the hypaxial muscle lineage through a *Myf5*-dependent, but also a *Myf5*-independent, pathway.

presented here, reveals the essential functions of *Shh* in somite patterning and in the determination of specific tissues in the embryo. Such an analysis of *Shh* null embryos was undertaken in this study specifically to define the essential functions of *Shh* in somitogenesis. Our results show that *Shh* has both inductive and trophic functions during somitogenesis (summarized in Fig. 6). Our studies specifically identify *Shh* as a key upstream regulatory gene which encodes a signal that controls epaxial myogenic determination and medio-lateral somite patterning. We also show that *Shh* regulates cell survival in the somite sclerotome progenitors of the ventral somite as well as in the motorneuron progenitors in the ventral neural tube and neural crest progenitors in the dorsal neural tube.

An inductive function for *Shh* in epaxial muscle determination

Analysis of myogenic regulatory gene expression in the *Shh* null embryo has established that Shh has an essential, inductive function in the epaxial somite as an activator of *Myf5*, a key upstream regulatory gene that controls epaxial muscle determination in mouse embryos (Fig. 7) (Tajbakhsh et al., 1996b). Our findings support earlier studies that identified a role of *Shh* in *MyoD* and *Myf5* activation in somites of avian embryos (Johnson et al., 1994; Munsterberg et al., 1995; Dietrich et al., 1997; Borycki et al., 1998) and in slow muscle formation in Zebrafish embryos (Weinberg et al., 1996; Blagden et al., 1997).

Analysis of *Myf5* and *MyoD* expression in the *Shh* null embryos reveals that *Shh* is not essential for the activation of these genes in the hypaxial dermomyotome, in the limb or in the head (Fig. 7). These findings, therefore, indicate that other Shh-independent signals function to induce myogenesis at these other sites in the embryo. In vitro explant studies have shown that different Wnts produced by surface ectoderm and dorsal neural tube can differentially induce *Myf5* and *MyoD* (Tajbakhsh et al., 1998), and therefore are candidates for Shh-independent signals that regulate epaxial and hypaxial myogenesis. To date, genetic analysis has not demonstrated a requirement for any single Wnt in muscle lineage determination. However, multiple Wnts are produced by the surface ectoderm and dorsal neural tube and may have redundant functions in myogenic determination. Such a redundant function during somitogenesis has already been demonstrated in *Wnt1/Wnt3a* double knockout mice (Ikeya and Takada, 1998). Interestingly, in these embryos, the dorso-medial lip of the dermomyotome forms initially but is not maintained. By E10.5, in a similar fashion as in *Shh* null somites, *Wnt1/Wnt3a* null somites lack the dorso-medial lip of the dermomyotome. However, in contrast to *Shh* null embryos, *Myf5* is activated, although to a lower level, in the dorso-medial domain of somites in *Wnt1/Wnt3a* null embryos (Ikeya and Takada, 1998). One possible explanation for these observations is that *Wnt* signaling in the neural tube is affected in *Shh* null mice, leading to structural defects of the dermomyotome as in *Wnt1/Wnt3a* null embryos. Further experiments will be required to address this possibility. Interestingly, in *Shh* null embryos, *Pax3* expression in the epaxial dermomyotome of interlimb somites is affected, suggesting a possible role of *Shh* in *Pax3* expression. In chick embryos, ablation of the notochord also results in ventralization of *Pax3* transcripts and the concomitant loss of dorsal *Pax3* expression (Goulding et al., 1993, 1994). However, in vitro explant assays show that Shh alone does not induce/maintain *Pax3* expression in the presomitic mesoderm, but in combination with Wnt1, Shh is capable of inducing/maintaining *Pax3* expression (Maroto et al., 1997). Moreover, Wnt1 has been directly implicated in the formation/maintenance of the dorso-medial lip of the dermomyotome in chick embryos (Marcelle et al., 1997). Therefore, loss of epaxial *Pax3* expression and disruption of the dorso-medial lip of the dermomyotome in *Shh* null embryos may result from the disruption of dorsal neural tube Wnt signaling.

Our studies show that, in addition to Shh, surface ectoderm

is required for induction of *Myf5* and *MyoD* in presomitic mesoderm explants, providing evidence that Shh alone is not a sufficient signal for epaxial myogenesis. As Shh and Wnts have synergistic acitivities in somite myogenesis (Munsterberg et al., 1995; Tajbakhsh et al., 1998), Wnts also are candidate signaling molecules that work cooperatively with Shh in epaxial myogenesis. Our explant studies also show that *Myf5* is essential for the activation of *MyoD* in presomitic mesoderm explants in the presence of surface ectoderm. However, others have shown that *Myf5* null explants will undergo muscle differentiation after more prolonged culture (Tajbakhsh et al., 1998), indicating that surface ectoderm can also provide signals for Myf5-independent hypaxial myogenesis. Similarly, we show that *MyoD* activation is reduced, but not entirely blocked in *Myf5*-null explants of presomitic mesoderm associated with both surface ectoderm and neural tube and notochord, indicating that an alternative *Myf5*-independent signaling pathway also exists to promote epaxial *MyoD* activation. These conclusions are supported by earlier findings that *MyoD* is activated in *Myf5* null embryos in both the epaxial and hypaxial domains (Braun et al., 1994; Tajbakhsh et al., 1997), after a substantial delay (Tajbakhsh et al., 1997). Therefore, *MyoD* is normally activated in epaxial and hypaxial domains by *Myf5*, but alternative backup signaling pathways also exist to promote *MyoD* activation in the absence of *Myf5* function.

In addition to its function in the induction of *Myf5* and epaxial myogenesis, *Shh* has an essential function in mediolateral somite patterning. This conclusion is based on our finding that *Myf5*, *Pax3* and *Sim1* expression in the hypaxial domain becomes expanded into the ventro-medial somite in *Shh* null embryos. The expansion of hypaxial gene expression, which is evident at the interlimb level, indicates that Shh functions to maintain the proper patterning of epaxial and hypaxial myogenic domains in the somite (Fig. 7), probably by antagonizing the lateral signaling pathways that control hypaxial gene expression (Pourquie et al., 1996; Amthor et al., 1999). These findings are summarized in a signaling pathway model for epaxial and hypaxial determination, shown in Fig. 7.

Trophic functions of *Shh* in somites and neural tube

BrdU and TUNEL assays were used to show that *Shh* is not essential for proliferation or survival of epaxial somite cells, strongly supporting the above evidence that *Shh* has an inductive role in epaxial myogenesis. Ectopic *Shh* expression in limbs (Duprez et al., 1998) or deregulated Shh signaling in *Patched* null embryos (Goodrich et al., 1997; Hahn et al., 1998) results in a proliferative response of limb and neural cells. Conversely, loss of *Shh* function does not result in lineage-specific proliferative defects in neural tube or somites, although overall cell proliferation in E9.5 *Shh* null embryos is decreased. This observation implies that any proliferative function of *Shh* during somitogenesis and neurogenesis is ubiquitous and minor, but that overexpression of Shh signal transduction, such as occurs in the *Patched* mutant embryos, leads to aberrant regulatory processes.

In contrast, *Shh* is required specifically to maintain cell survival in the ventral somite as well as in the ventral and dorsal neural tube. The requirement of *Shh* for cell survival in these regions is restricted to a narrow window of developmental time. In the ventral somite, apoptosis occurs following somite formation at E9.5, during the time of *Pax1* activation and initiation of sclerotome differentiation. As previous work has shown that *Pax1* expression is initiated in the ventral somite in E9.5 *Shh* null embryos, but is lost by E10.5 (Chiang et al., 1996), this indicates that apoptosis is downstream of *Pax1* activation, accounting for the loss of sclerotome progenitors by cell death. It remains to be demonstrated whether *Shh* directly regulates sclerotomal progenitor cell survival or whether *Shh* is required for the maintenance of *Pax1* expression, in the absence of which cells die. It is notable that Shh can induce and maintain *Pax1* expression in explants of presomitic mesoderm (Fan and Tessier-Lavigne, 1994; Fan et al., 1995), suggesting that Shh functions in *Pax1* activation. *Pax1* being activated in somites of the *Shh* null embryo, this implies that redundant signals probably control *Pax1* activation, but not *Pax1* maintenance, in *Shh* null embryos.

Our findings that *Shh* null embryos do not show an increase in cell death in the dorsal somite contrast with previous reports that notochord degeneration or notochord ablation leads to massive cell death in the dorsal domain of somites (Asakura and Tapscott, 1998; Teillet et al., 1998). This observation indicates that the notochord either produces additional cell survival factors that suppress apoptosis in the dorsal somite, or that it induces the neural tube to express such survival factors. In this regard, it is of interest that the apoptotic activity in both the somite and neural tube of the *Shh* null embryo is very transient, which accounts for the absence of gross tissue disruptions in these mutant embryos.

As the formation of motorneurons is blocked in the *Shh* mutant embryo and *Pax3* and *Pax6* become misexpressed throughout all of the ventral neural tube (Chiang et al., 1996), it is interesting to note that apoptosis is restricted to the ventral motorneuron domain, but does not occur in adjacent prospective floor plate cells. This indicates that floor plate cells are distinct from the motorneuron progenitor cells in their survival requirements for *Shh*, perhaps reflecting the independent lineage origins of the floor plate and neural tube (Le Douarin et al., 1998) or reflecting distinct signaling pathways that transduce Shh signals. In favor of this latter hypothesis, *Gli2* null embryos show differential defects in floor plate and motorneuron development (Ding et al., 1998; Matise et al., 1998). The question remains as to whether apoptosis in the ventral neural tube occurs prior to the initiation of motorneuron determination, in which case *Shh* would have a direct role on motorneuron progenitor cell survival. Shh has inductive activity for motorneuron differentiation in explant assays (Roelink et al., 1994; Ericson et al., 1996). Therefore, the apoptosis in ventral neural tube cells of *Shh* null embryos may be a direct response to the lack of Shh signaling or may be an indirect response of those neural cells that fail to receive inductive signals for motorneuron differentiation. Our data show that apoptosis precedes motorneuron differentiation. Therefore, apoptosis occurs either as a consequence of lack of determination cues or as a consequence of lack of cell survival cues. Careful comparative timing analysis of motorneuron determination and *Shh* requirement for cell survival will distinguish between these possibilities. Alternatively, apoptosis in ventral neural tube cells may reflect a failure of adjacent ventral somite cells to produce requisite survival factors for ventral neural tube cells. Indeed, in many instances, somite development has been shown to influence neural tube

development (Fontaine-Perus et al., 1989; Pituello et al., 1999). However, it is noteworthy that in *Shh* null embryos, cell death in the ventral neural tube is detected prior to cell death and/or loss of gene expression in the ventral somite. Interestingly, apoptosis is also observed in the dorsal-most neural tube cells, probably neural crest, in the *Shh* null embryo, which are located beyond the dorsal extent of Shh signaling. Apoptosis in these distant neural crest cells must therefore be controlled by a cascade of cell survival signals which are first induced in the more ventral regions of the neural tube that are under the control of Shh signaling. Possible repercussions of this effect on the dorsal neural tube and subsequently on the adjacent epaxial somite remain to be evaluated. However, our explant experiments clearly indicate that *Shh* plays a direct role in the activation of *Myf5* in the paraxial mesoderm, in the absence of neural tube.

Shh signal transduction is mediated through the three *Gli* transcription factors, *Gli1*, *Gli2* and *Gli3*, which are patterned in their expression in both the somite and the neural tube (Borycki et al., 1998; Ding et al., 1998; i Altaba, 1998; Matise et al., 1998). *Gli* transcription factors are expressed in the epaxial dermomyotome (Borycki et al., 1998), consistent with the possibility that Shh regulates *Myf5* directly, although this remains to be established. The trophic functions of Shh in suppression of apoptosis in the ventral neural tube and ventral somite may be mediated directly by Glis or by other survival factors under the control of Shh and Gli signal transduction. Certainly, the regulated apoptosis of neural crest cells in the dorsal neural tube must be under the control of survival factors regulated by ventral Shh signals. The identification of the specific target genes regulated by Shh signaling is now required to understand the mechanisms by which Shh transduces its inductive and trophic functions in the embryo.

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