

Pax3 functions in cell survival and in *pax7* regulation

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SUMMARY

In developing vertebrate embryos, *Pax3* is expressed in the neural tube and in the paraxial mesoderm that gives rise to skeletal muscles. *Pax3* mutants develop muscular and neural tube defects; furthermore, *Pax3* is essential for the proper activation of the myogenic determination factor gene, *MyoD*, during early muscle development and *PAX3* chromosomal translocations result in muscle tumors, providing evidence that *Pax3* has diverse functions in myogenesis. To investigate the specific functions of *Pax3* in development, we have examined cell survival and gene expression in presomitic mesoderm, somites and neural tube of developing wild-type and *Pax3* mutant (*Splotch*) mouse embryos. Disruption of *Pax3* expression by antisense oligonucleotides significantly impairs *MyoD* activation by signals from neural tube/notochord and surface ectoderm in cultured presomitic mesoderm (PSM), and is accompanied by a marked increase in programmed cell

death. In *Pax3* mutant (*Splotch*) embryos, *MyoD* is activated normally in the hypaxial somite, but *MyoD*-expressing cells are disorganized and apoptosis is prevalent in newly formed somites, but not in the neural tube or mature somites. In neural tube and somite regions where cell survival is maintained, the closely related *Pax7* gene is upregulated, and its expression becomes expanded into the dorsal neural tube and somites, where *Pax3* would normally be expressed. These results establish that *Pax3* has complementary functions in *MyoD* activation and inhibition of apoptosis in the somitic mesoderm and in repression of *Pax7* during neural tube and somite development.

Key words: Apoptosis, Myogenesis, Gene regulation, Embryogenesis, Mouse

INTRODUCTION

During mouse embryogenesis, dorso-medial somitic mesoderm gives rise to the epaxial deep back muscles, and ventro-lateral somitic mesoderm gives rise to the hypaxial body wall and limb muscles (Buckingham, 1992). Multiple signaling processes are required to determine presomitic mesoderm to form these different muscle lineages. These signals initiate and maintain lineage-specific gene expression, regulate the spatial domain of gene expression, and control cell growth, survival and differentiation. Two bHLH transcription factors, *Myf5* and *MyoD*, are essential for the determination of the presomitic mesoderm to skeletal muscle lineages, because targeted mutations in *Myf5* and *MyoD* genes result in mice that entirely lack skeletal muscles and myogenic progenitor cells (Rudnicki et al., 1993). However, mice lacking either *MyoD* or *Myf5* form normal embryonic musculature, indicating that compensatory mechanisms can override initial defects (Braun et al., 1992; Rudnicki et al., 1993). The basis for this compensation is not yet well understood, but loss of *MyoD* function results in upregulation of *Myf5* in somitic cells (Rudnicki et al., 1992), and loss of *Myf5* function results in a delay in myotome formation, which recovers after the later onset of *MyoD* expression (Braun et al., 1994; Tajbakhsh et al., 1997). Insertion of *lacZ* into the *Myf5* locus reveals that somitic

mesodermal cells that lack *Myf5* function can adopt alternate dermatome or sclerotome somitic fates prior to *MyoD* activation, or can engage in myogenesis once *MyoD* is activated (Tajbakhsh et al., 1996), demonstrating that *Myf5* is an essential gene for the initiation of early myogenic determination. In agreement with this observation, *Myf5* is the earliest regulatory gene activated in epaxial and hypaxial muscle lineages in mouse embryos at E8.5, while *MyoD* expression is first observed at E9.75 (Ott et al., 1991; Sassoon et al., 1989; Tajbakhsh et al., 1996). Early expression of *Myf5* is confined to the dorso-medial epaxial domain of somites, and is then detected in the ventro-lateral hypaxial domain of somites (Ott et al., 1991; Tajbakhsh et al., 1996). *MyoD* expression follows that of *Myf5*, and is first detected in the ventro-lateral hypaxial domain of somites, and then in the dorso-medial epaxial domain (Ott et al., 1991; Sassoon et al., 1989; Tajbakhsh et al., 1996).

Another key regulatory gene in myogenesis is *Pax3*, which is a member of the Paired Box transcription factor family (Borycki and Emerson, 1997; Goulding et al., 1991; Tajbakhsh et al., 1997). *Pax3* function in appendicular muscle formation is well established; *Splotch* (*Sp*) mutant mice, in which a mutation disrupts *Pax3* function, do not form limb muscles (Auerbach, 1954; Franz et al., 1993; Goulding et al., 1994). In *Sp* mutants, hypaxial muscle progenitors do not

migrate into the limb bud (Bober et al., 1994; Goulding et al., 1994), likely because they do not express *c-Met*, a tyrosine kinase receptor implicated in cell migration (Bladt et al., 1995) and a transcriptional target of Pax3 (Epstein et al., 1996). Moreover, when transplanted into chick wing buds, *Sp* mutant hypaxial cells differentiate into muscle (Daston et al., 1996), showing that Pax3 is not essential for muscle differentiation. Pax3, however, has essential functions in myogenesis, as revealed by the discovery that compound *Pax3* and *Myf5* mutant embryos completely lack trunk muscles and do not express *MyoD* (Tajbakhsh et al., 1997). This phenotype mimics the absence of muscle observed in *Myf5/MyoD* double mutant embryos, indicating that Pax3 has a function upstream of *MyoD* during muscle determination in the embryo. One possibility is that Pax3 controls *MyoD* expression in somites, which is suggested by the finding that overexpression of *Pax3* in chick presomitic mesoderm induces *Myf5* and *MyoD* expression (Maroto et al., 1997). Furthermore, *Pax3* is expressed in somitic domains of future myogenesis, although transcripts are initially expressed throughout the somite and also are found in non-myogenic lineages such as the dorsal neural tube, the neural crest and the nasal epithelium (Goulding et al., 1991). An alternative possibility is that Pax3 has general or lineage-specific functions in somitic mesoderm that are necessary prior to myogenic differentiation and acting upstream of *MyoD* activation.

Another closely related member of the Paired Box transcription factor family, *Pax7*, is also expressed in the dorsal neural tube and in somites (Jostes et al., 1991). However, in contrast to *Pax3*, *Pax7* is not expressed in presomitic mesoderm but is gradually induced in late stages of somite maturation (Jostes et al., 1991). Following somite formation, *Pax3* transcripts are found throughout the somite, and then become restricted to the dermomyotome as somites mature (Goulding et al., 1991). *Pax3* expression then becomes concentrated in the ventro-lateral domain of the dermomyotome (Goulding et al., 1994; Tajbakhsh et al., 1997), whereas *Pax7* expression is concentrated to the central and dorso-medial regions of the dermomyotome (Jostes et al., 1991; Tajbakhsh et al., 1997). These overlapping but distinct patterns of expression of *Pax3* and *Pax7* in somites suggest that *Pax3* and *Pax7* may have partially overlapping functions in hypaxial and epaxial myogenic domains. Further evidence for shared functions in myogenesis comes from the observation that translocations of the human *PAX3* locus or *PAX7* locus to the *FKHR* locus are correlated with formation of rhabdomyosarcomas, muscle neoplasms (Davis et al., 1994; Galili et al., 1993).

To investigate Pax3 functions in skeletal myogenesis, we designed an in vitro presomitic mesoderm explant assay to examine early myogenesis in *Sp* mutant embryos. We show that presomitic mesoderm from *Sp* mutant embryos fails to activate *MyoD* in response to neural tube/notochord and surface ectoderm signals. Nevertheless, in vivo, *MyoD* activation occurs normally in the lateral hypaxial domain of somites of *Sp* mutant embryos, indicating that Pax3 is not essential for the initiation of *MyoD* transcription. Further analysis shows that apoptosis is greatly enhanced in presomitic mesoderm explants from *Sp* mutant embryos, as well as in *Pax3*-expressing cells in newly formed somites of *Sp* mutant embryos. However, apoptosis does not occur in dorsal neural tube or in more mature somites where *Pax7*

becomes ectopically expressed in the absence of Pax3 function. We further demonstrate that Pax3 represses *Pax7* expression in cultured muscle cells, providing an explanation for the ectopic expression of *Pax7* in neural tube and somites of *Sp* embryos. These data establish that Pax3 is essential for the survival of uncommitted somitic progenitor cells, as well as for the negative regulation of *Pax7* expression in somitic cells and neural tube.

MATERIALS AND METHODS

Presomitic mesoderm explant cultures

Splotch mice (Jackson Laboratory) were bred and embryos were collected by cesarean incision (E0.5 is designated as noon of the day of vaginal plug identification). Presomitic mesoderm with overlying ectoderm was dissected from E9.5 mouse embryos using electrolitically sharpened tungsten knives. When indicated, the axial notochord/neural tube complex was dissected together with the presomitic mesoderm. Explants were transversally cut into two anterior and posterior halves, transferred without prior dissociation of cells onto gelatin-coated 48-well plates, and cultured at 37°C in 0.5 ml DMEM/F-12 medium (Gibco/BRL) containing 15% FCS (Gibco/BRL), 1% penicillin/streptomycin (Gibco/BRL) and 2 ng/ml bFGF (Sigma), for 2 or 3 days in a humidified CO₂ incubator. Anterior and posterior explant halves were monitored for myogenesis and showed to behave in a similar manner. Addition of bFGF to the culture medium has previously been shown to increase cell survival (Fan and Tessier-Lavigne, 1994), but has also been shown to act on myogenic precursor cells (Stern et al., 1997). Here, we tested different concentrations of bFGF (1–10 ng/ml) on explant cultures for the absence of effect on MyoD activation but an increase in cell survival, and 2 ng/ml was found to be optimal. Oligonucleotides (5–8 µM final concentration) were added to culture medium 24 hours after the tissue was explanted and explants were cultured for an additional 16–48 hours.

Immunohistochemistry

Explant 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 the primary antibody (anti-mouse MyoD 5.8A, Novocastra) diluted 1:25, followed by a 30-minute incubation with the secondary antibody (biotin-conjugated anti-mouse IgG, Vector Labs) diluted 1:200. Peroxidase staining of positive cells was performed using the Vectastain Kit (Vector Labs) according to the manufacturer's instructions. MyoD-positive cells per explant were counted on an inverted LEITZ-DMIRB microscope.

Paraffin sections of mouse embryos were dewaxed and dehydrated through an ethanol series followed by antigen unmasking using Vector Labs Unmasking Solution as directed. Pax7 antibody (Ericson et al., 1996) was obtained from the Developmental Studies Hybridoma Bank and used at a dilution of 1:100. Biotinylated horse anti-mouse secondary antibody (Vector Labs) was used at a dilution of 1:200, followed by developing with Vector Labs ABC reagent.

Antisense oligonucleotides

Oligonucleotides were synthesized from phosphorothioate-modified nucleotides (Oligos Etc, Wilsonville, OR). Pax3 antisense (5'-GCGTGGTCATCCTGGGGGC-3') spanned the translation initiation site. Pax3 random (5'-GGTCATGGCCTGGGCTGCG-3') had the same nucleotide content as Pax3 antisense but the sequence was randomized. Pax3 control (5'-TGGATCCGACATGTCAGA-3') was an unrelated random sequence oligonucleotide.

Apoptosis assays

Apoptotic cells were detected in situ on paraffin sections or explant cultures fixed briefly in 2% paraformaldehyde in PBS (20 minutes) using a modified TUNEL technique (protocol kindly provided by E. Fernandez, University of Texas Southwestern, USA) by specific end-labeling of fragmented nuclear DNA with biotin-dCTP (Gibco/BRL). Slides were deparaffinized, hydrated, washed with PBS, treated with proteinase K (20 $\mu\text{g}/\text{ml}$ in PBS) for 10 minutes at room temperature, washed with PBS, permeabilized with 0.3% Triton X-100 in PBS for 10 minutes at room temperature, incubated in TdT buffer (30 mM Tris, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride) for 5 minutes at room temperature and incubated in TdT enzyme mix containing biotin-dCTP (TdT buffer containing 20 ng/ml biotin-dCTP and 300 units/ml TdT enzyme) at 37°C for 90 minutes in a humidified chamber. Slides were then washed in PBS, pH 8.2, and biotin-labeled fragmented DNA was detected by Texas Red-labeled streptavidin (Vector Labs) diluted 1:50 in PBS, pH 8.2 (30 minutes at room temperature), followed by final extensive washing in PBS and mounting in Vectashield (Vector Labs). DNase I-treated tissue was used as a positive control and equivalent slides without TdT enzyme were used as a negative control.

Apoptosis was also assessed by annexin-V staining of extracellular phosphatidylserine using fluorescein-conjugated annexin-V (Boehringer Mannheim) as directed. Annexin-V binds to phosphatidylserine, which is exposed on the extracellular surface of cells during the early phases of apoptosis (Vermes et al., 1995). Cells were simultaneously stained with propidium iodide to differentiate apoptotic cells from necrotic cells with a loss of membrane integrity.

In situ hybridization

^{35}S -labeled antisense riboprobes were synthesized by in vitro run-off transcription of linearized plasmids using SP6, T7 or T3 RNA polymerase and ^{35}S -labeled UTP. Prehybridization, hybridization and posthybridization procedures were as described (Lutz et al., 1994). Slides were then dipped in Kodak NTB2 emulsion and exposed for 8–10 days at 4°C, developed and fixed in Kodak Dektol developer and fixer, counterstained in Hoechst 33258 (Sigma) for 2 minutes, washed in water, dried and mounted in Canada Balsam. The *Pax3* probe has been described (Epstein et al., 1996). The *Pax7* probe was transcribed using T3 RNA polymerase from pKS-Pax7 (kindly provided by Dr Fred Barr) after linearization. This probe corresponds to the 872 bp sequence deposited in GenBank (accession number U20792). The *MyoD* probe (kindly provided by Dr M. Buckingham) was transcribed using T3 RNA polymerase following linearization with *Mlu*I. Whole-mount in situ hybridization was performed using DIG-labeled RNA probes (Borycki et al., 1998) according to the protocol previously described (Henrique et al., 1995), with post-antibody washes for 3 days.

Transfection and western blotting

C2C12 myoblasts (ATCC) were transfected with pcDNA3-Pax3-HA, which encodes full-length murine Pax3, with an amino-terminal epitope tag consisting of three copies of the hemagglutinin peptide (YPYDVPDYAG) (Epstein et al., 1995) and polyclonal stable transfectants were selected with 500 $\mu\text{g}/\text{ml}$ G418. Western blotting was performed with the mouse monoclonal anti-HA antibody 12CA5 (Boehringer Mannheim) as recommended and described previously (Epstein et al., 1995), and developed with the ECL kit (Amersham Life Sciences).

Northern blotting

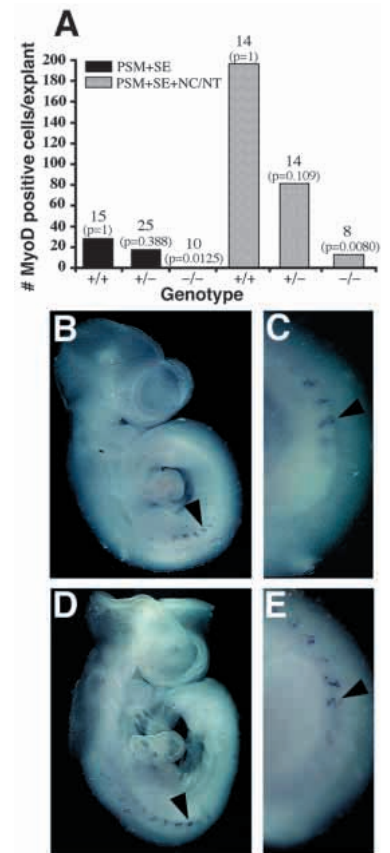
RNA was prepared from fresh tissue using Trizol™ Reagent (Gibco/BRL) and poly(A)RNA was purified using Quiagen Oligotex mRNA purification kit. 1 μg mRNA was loaded per lane, transferred to GeneScreen II (Dupont NEN) and probed with random hexamer ^{32}P -labeled probes.

RESULTS

MyoD expression is impaired in presomitic mesoderm in response to signals from axial tissues or surface ectoderm

To investigate the role of Pax3 in the regulation of myogenic determination genes in somitic mesoderm, we cultured presomitic mesoderm (PSM) explants from E9.5 wild-type, heterozygous and homozygous *Spotch* (*Sp*) embryos in association with surface ectoderm (SE), which provides signals for hypaxial muscle lineage determination, or in association with axial tissues (neural tube and notochord), which provide signals for epaxial muscle lineage determination (Cossu et al., 1996). After 72 hours, MyoD-expressing cells in explants were quantified by immunohistochemistry (Fig. 1A). PSM+SE from wild-type mice had an average of 28 MyoD-positive cells per explant, and PSM cultured with SE and axial tissues had greater than 190 MyoD-positive cells per explant (Fig. 1A). PSM explants from heterozygous *Sp* mutant embryos had similar numbers of MyoD-positive cells when co-cultured with SE ($P=0.388$) or with SE and axial tissues ($P=0.109$). In contrast, no MyoD-expressing cells were detected in PSM explants derived from homozygous *Sp* mutant embryos cultured in the presence of SE ($P=0.01$), and MyoD expression

Fig. 1. Impaired MyoD activation in vitro but not in vivo in *Pax3* mutant mice. (A) Presomitic mesoderm explants (PSM) from wild-type (+/+), heterozygous (+/-), and homozygous (-/-) *Pax3* mutant E9.5 mouse embryos were cultured with surface ectoderm (SE) (black bars) or with surface ectoderm and axial tissues (notochord and neural tube, NC/NT) (grey bars) for 72 hours. MyoD expression was assessed by immunohistochemistry. MyoD-stained cells were counted, and the average number of MyoD-positive cells per explant for each condition was plotted (y-axis). The number of explants examined and the *P* value determined by *t*-test analysis compared to wild type (in parenthesis) are indicated above the bars for each condition. Whole-mount in situ hybridization for *MyoD* transcripts in wild-type (B,C) and *Pax3* mutant (D,E) E10 embryos shows *MyoD* activation in interlimb somites (arrowheads) in both wild-type and mutant embryos. The onset of *MyoD* activation is not altered, but somites are disorganized in *Pax3* mutant embryos (arrowheads in C and E). C and E are high magnifications of the embryos shown in B and D, respectively.



was markedly impaired in PSM cultured with SE and axial tissues (15 MyoD-positive cells per explant; $P=0.008$ compared to wild type) (Fig. 1A). These data confirm earlier work showing that Pax3 is required for MyoD activation by signals from the surface ectoderm in the hypaxial domain of somites (Tajbakhsh et al., 1997), and demonstrate that Pax3 is also required for activation of MyoD by signals from axial tissues in the epaxial domain.

To test whether Pax3 is essential for MyoD activation *in vivo*, we examined the onset of MyoD expression in *Sp* embryos by performing *in situ* hybridization on E9.75 wild-type and Pax3 mutant embryos (Fig. 1B-E). We found that MyoD is activated normally at E9.75 in the ventro-lateral hypaxial domain of somites of wild-type and Pax3 mutant littermates (Fig. 1B,D). MyoD-expressing cells are disorganized in Pax3 mutant embryos (Fig. 1E), likely reflecting the disruption of the ventral lateral lip of the dermomyotome, as described previously (Franz et al., 1993). We also have observed that PSM explants from Pax3 mutant mice cultured in the presence of surface ectoderm grow poorly and have poor survival, compared to PSM explants from wild-type littermates. These findings suggest that PSM from Pax3-deficient mice is defective in cell survival, which may lead to loss of MyoD-expressing cells.

Apoptosis in Pax3-deficient PSM explants

To examine cell death in PSM explants, we performed TUNEL analysis, which detects DNA fragmentation in apoptotic nuclei, and annexin-V immunohistochemistry, which detects extracellular phosphatidylserine in apoptotic cells (Vermes et al., 1995). PSM explants from wild-type embryos co-cultured with surface ectoderm and axial structures produce large numbers of MyoD-positive cells (Figs 1A, 2A) and have few TUNEL-positive nuclei (Fig. 2C). In contrast, PSM explants from Pax3 mutant embryos have few MyoD-expressing cells (Figs 1A, 2B) and have large numbers of apoptotic nuclei (Fig. 2D). These findings suggest that the defect in MyoD activation by surface ectoderm and neural tube/notochord in Pax3 mutant mice is associated with programmed cell death of somitic cells. To examine whether apoptosis observed in PSM cultures from Pax3 mutant embryos is directly caused by loss of Pax3, rather than due to the loss of a neural tube survival factor under the control of Pax3, we performed TUNEL assays on PSM explants treated with Pax3 antisense oligonucleotides. In control western blot experiments, we established that antisense Pax3 oligonucleotides, but not control or random oligonucleotides, impaired Pax3 protein expression in cultured cells (see Materials and methods; Fig. 3A). Random, control and Pax3 antisense oligonucleotides were applied on PSM explants for 72 hours, and MyoD expression was analyzed by immunohistochemistry. We found that PSM explants cultured in the presence of antisense Pax3 oligonucleotides had eightfold fewer MyoD-positive cells per explant as compared to explants cultured without oligonucleotides or with random oligonucleotides (data not shown). Furthermore, treatment of explants with antisense Pax3 oligonucleotides (Fig. 3D), but not with control or random oligonucleotides (Fig. 3B,C), triggered a dramatic increase in the number of apoptotic cells, indicating that Pax3 is required in a cell-autonomous manner for PSM cell viability. In agreement, we observed many apoptotic cells in Pax3 mutant PSM explants as compared to

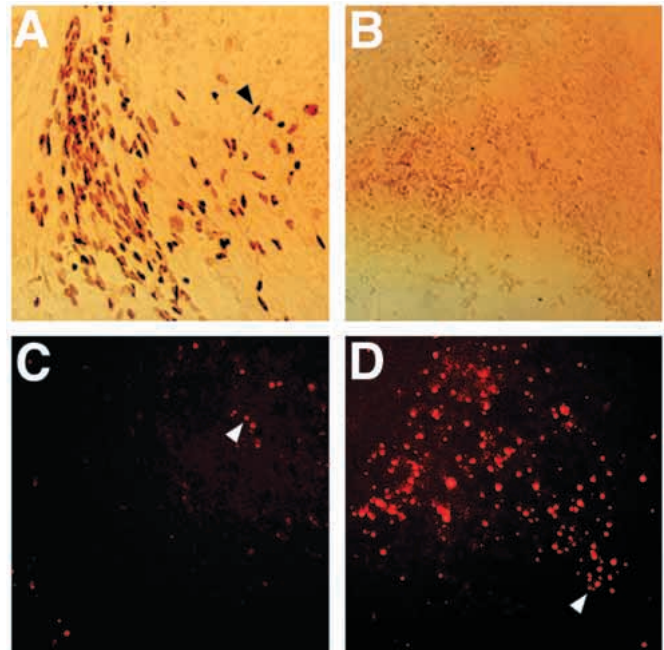


Fig. 2. Apoptosis and deficient myogenesis in PSM explants from Pax3 mutant mice. Presomitic mesoderm (PSM) with associated surface ectoderm and axial structures (notochord and neural tube) was explanted from wild-type (A,C) or Pax3 mutant (B,D) E9.5 embryos and cultured for 72 hours. Explants were then stained for MyoD protein expression (A, black arrowhead) (A,B) and for apoptosis by TUNEL assay (white arrowheads) (C,D). Bright field and fluorescent images of identical fields are shown (A and C, B and D). Numerous MyoD-positive cells are seen in wild-type explants (A) with few apoptotic cells (C), while few MyoD-positive cells are seen in Pax3 mutant explants (B) and many apoptotic cells are evident (D). Axial tissue and surface ectoderm cells could be identified morphologically in cultures and did not show significant apoptosis.

wild-type PSM, as assayed by staining with fluorescein-conjugated annexin-V (Fig. 3E,F) and by TUNEL assays (data not shown). Apoptosis was also observed in wild-type PSM explants cultured without surface ectoderm (data not shown), which is required for Pax3 expression (Fan and Tessier-Lavigne, 1994). This provides additional evidence that Pax3 function in PSM cells is required for their survival. Interestingly, we noticed that less apoptosis occurs in PSM cocultured with both surface ectoderm and axial tissues than in PSM cocultured with surface ectoderm alone, indicating that axial tissues (neural tube and notochord) provide additional cell survival factors.

Apoptotic cells in somites of Pax3-deficient embryos

The apoptosis observed in PSM explants from Pax3 mutant embryos led us to compare apoptosis in the somites of wild-type and Pax3 mutant embryos. Transverse sections from E9.5-11.5 wild-type and Pax3 mutant embryos were analyzed by *in situ* hybridization for Pax3 expression, and by TUNEL assay for apoptosis (Fig. 4). In E11.5 wild-type embryos, Pax3 transcripts are detected in the dorsal neural tube, dorsal root ganglia and dermomyotome (Fig. 4A), and TUNEL-positive cells are scattered throughout the embryo (Fig. 4C). In E11.5

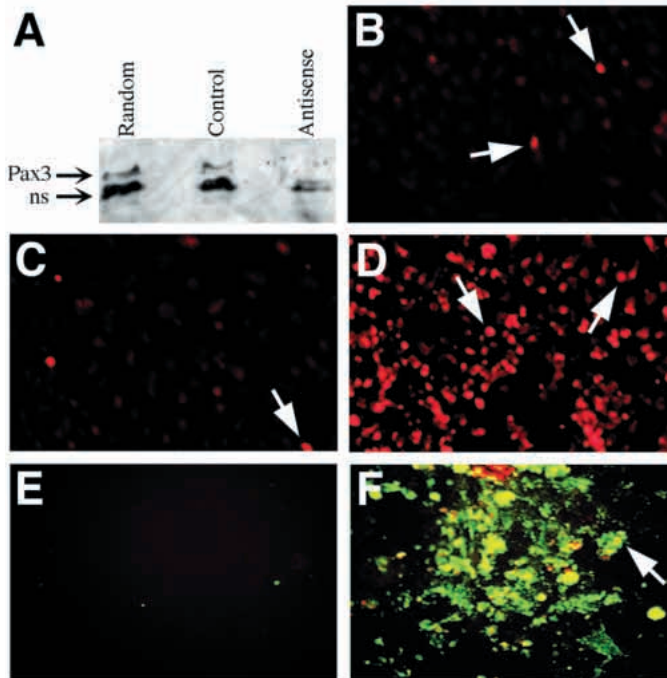


Fig. 3. Apoptosis in *Pax3*-deficient PSM explants is cell autonomous. Western blot (A) of C2C12 myoblasts stably transfected with an epitope-tagged form of *Pax3* and cultured for 72 hours with random, control or antisense oligonucleotides (8 μ M) demonstrates specific inhibition of *Pax3* protein expression by antisense oligonucleotides. The *Pax3*-specific band (approx. 49 kDa) is indicated running just above a non-specific doublet (ns). (B–D) PSM with surface ectoderm from wild-type E9.5 embryos was cultured for 24 hours followed by addition of control or *Pax3* random or antisense oligonucleotides (8 μ M) and further culture for 48 hours. Explants were then fixed and stained for apoptotic cells using a TUNEL assay. Numerous apoptotic nuclei (white arrows) were identified in cultures containing antisense *Pax3* oligonucleotides (D), while fewer apoptotic cells were seen in cultures containing control (B) or random (C) oligonucleotides. PSM with surface ectoderm was cultured from wild-type (E) or *Pax3* mutant (F) E9.5 embryos and cultured for 72 hours followed by staining with fluorescein-conjugated annexin V to detect apoptotic cells (green). Counterstaining with propidium iodide (red) shows fragmented nuclei in *Pax3* mutant explants.

Pax3 mutant embryos, nonfunctional *Pax3* transcripts are detected in the dorsal domain of the open neural tube and at the hindlimb level, in clusters of somitic cells (Fig. 4B). This observation correlates with previous work (Daston et al., 1996) showing that elongated somites are absent in *Pax3* mutant mice. Significantly, TUNEL assays on adjacent sections revealed clusters of apoptotic cells that colocalize with *Pax3*-expressing cells (Fig. 4D), establishing that *Pax3*-deficient dermomyotomal cells undergo apoptosis. These clusters of apoptotic cells were seen at multiple levels along the rostral-caudal axis of *Pax3* mutant embryos at E9.5 (Fig. 4F) but not in wild-type embryos (Fig. 4E). In somites of older E11.5 embryos, apoptotic cells were detected in significant numbers only at the hindlimb level and more posteriorly (Fig. 4D), suggesting that a compensatory mechanism may prevent extensive cell death, at later stages at the level of interlimb somites where *MyoD* is activated.

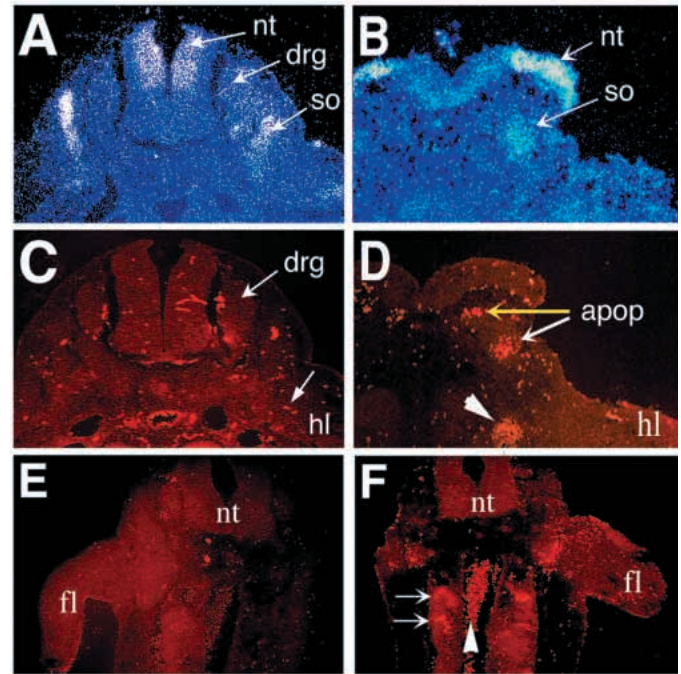
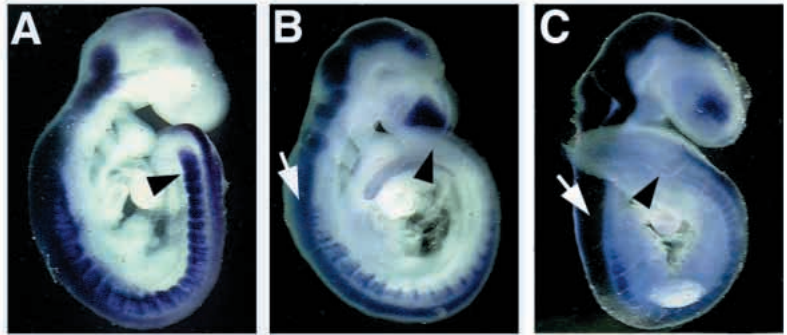


Fig. 4. Increased apoptosis in early somites of *Pax3* mutant embryos. In situ analysis of *Pax3* expression in wild-type (A) E11.5 embryos using transverse sections at the level of the hindlimb (hl) reveals strong expression in the dorsal neural tube (nt), and weaker expression in the dorsal root ganglia (drg) and in the somite (so). In *Pax3* mutant embryos (B) the neural tube does not close in the lumbosacral region (spina bifida), the dorsal root ganglia are small or absent, and the somites are poorly formed, remaining as spherical structures. The mutant (non-functional) *Pax3* transcript is detected in *Pax3* mutant embryos (B) in the dorsal neural tube (nt) and in the somite (so). TUNEL analysis of adjacent sections reveals scattered apoptotic cells in wild-type embryos (C). In *Pax3* mutant embryos, clusters of apoptotic cells (apop) colocalizing with the *Pax3* expression domain are evident in somites (white arrow, D). In addition, clusters of apoptotic cells are seen adjacent to the neural tube (yellow arrow, D) in the region where a dorsal root ganglion is normally located. TUNEL analysis of coronal sections through the forelimb (fl) at E9.5 reveals scattered apoptotic cells in wild-type embryos (E) and clusters of apoptotic cells in the region of the somites in *Pax3*-mutant embryos (arrows, F). Increased numbers of apoptotic cells in the proximal forelimb are also seen. (Autofluorescence of red blood cells within vascular structures gives a false positive signal in D and F, indicated by arrowheads).

We observed additional clusters of TUNEL-positive cells in the hindlimb region of *Pax3* mutant E11.5 embryos, located just lateral to the neural tube (yellow arrow, Fig. 4D). These apoptotic cells are located in the region occupied by the dorsal root ganglia that express *Pax3* in wild-type embryos (drg, Fig. 4C), suggesting that these dying cells are derived from the neural crest that is missing in the hindlimb region of *Pax3* mutant embryos (Auerbach, 1954). This observation indicates that *Pax3*-expressing somitic cells as well as *Pax3*-expressing neural crest cells undergo apoptosis in the embryo. However, at these developmental stages, we did not observe increased apoptosis in the dorsal neural tube of *Pax3* mutant embryos, where *Pax3* is expressed at high levels (Fig. 4B,D).

Fig. 5. *Pax7* is not expressed in PSM of E9.5 wild-type or *Splotch* embryos. Whole-mount in situ hybridizations of E9.5 wild-type (A,B) and *Pax3*-mutant (C) embryos using *Pax3* (A) and *Pax7* (B,C) DIG-labeled RNA probes are shown. In wild-type embryos, in addition to neural tube expression, *Pax3* is expressed in mature and newly formed (caudal) somites and in PSM (A, black arrowhead), while *Pax7* is not expressed in PSM (B, black arrowhead). In *Pax3* mutant embryos, *Pax7* is strongly upregulated in the neural tube (compare white arrows in B and C), but *Pax7* is not prematurely expressed in the PSM (black arrowhead, C). Note that the neural tube is open in the caudal region of this mutant embryo.



Misexpression of *Pax7* transcripts in the neural tube and rostral somites of *Pax3* mutant embryos

To investigate the differential responses of dorsal neural tube and somites in *Pax3* mutant embryos, we examined the expression of *Pax7*, a gene that has sequence homology and developmental expression patterns similar to *Pax3* (Jostes et al., 1991). In E10.5 wild-type embryos, *Pax7* is expressed in the medial region of the neural tube along the dorsal/ventral axis (Figs 5B, 6A,C), but is excluded from the dorsal extreme of the neural tube (compare Figs 4A and 6A,C). *Pax7* also is transiently expressed in the dermomyotome of somites after *Pax3* expression has initiated (Jostes et al., 1991) (Fig. 5C). However, unlike *Pax3*, *Pax7* is not detected in PSM and newly formed somites, as assayed by whole-mount in situ hybridization (Fig. 5A,B).

In E9.5 *Pax3* mutant embryos, *Pax7* expression is strongly upregulated in the dorsal neural tube (Fig. 5C), and by E10.5, *Pax7* mRNA transcripts were detected in the most dorsal regions of the neural tube including the lumbosacral area, where loss of *Pax3* results in failure of neural tube closure (Fig. 6D). At E10.5, the dorsal expansion of *Pax7* expression occurs in the neural tube along the entire rostral-caudal axis (Figs 5C, 6B,D), as well as in the lamina terminalis of the brain, where normally *Pax3* is expressed and *Pax7* is at very low levels (Fig. 6A,B, red arrow). The dorsal expansion of *Pax7* into the *Pax3* domain of *Splotch* embryos indicates that *Pax3* normally functions to repress *Pax7*.

To investigate whether *Pax7* also is mis-expressed in rostral somites of *Pax3* mutant embryos, we examined *Pax7* expression in somites of E9.5, E10.5 and E11.5 wild-type and *Pax3* mutant embryos (Figs 5, 7). At E9.5, *Pax7* expression is activated in anterior, but not in posterior somites of *Pax3*-mutant embryos (Fig. 5B,C), indicating that the onset of *Pax7* expression is not disrupted in *Pax3* mutant embryos. However, in the interlimb somites, *Pax7* transcripts are misexpressed in both the posterior and the anterior compartments of somites in E9.5 *Pax3* mutant embryos (Fig. 7D,E), reflecting the pattern of *Pax3* expression in wild-type embryos (Fig. 7G,H). In contrast, in wild-type embryos *Pax7* transcripts are abundant mainly in the posterior compartment of somites (Fig. 7A,B). In addition, *Pax7* expression expands into the dorsomedial lip of the dermomyotome in E9.5 *Pax3* mutant embryos (compare Fig. 7C,F), where *Pax3* is normally expressed at this developmental stage (Fig. 7I). In the medial portion of the somite, *Pax3* and *Pax7* expression domains overlap. By E10.5, expression of *Pax7* protein in *Pax3* mutant embryos expands dorsally into the dorsomedial lip of the dermomyotome (Fig.

8A,B) where *Pax3* is normally expressed at this time (Tajbakhsh et al., 1997). By E11.5, *Pax7* is abundantly expressed in the medial domain of the dermomyotome and expression is increasing in the dorsomedial lip in wild-type embryos (Fig. 8C) (Jostes et al., 1991). In *Pax3* mutant embryos, *Pax7* expands into the ventral dermomyotome (Fig. 8C,D, open arrows), where *Pax3* expression would normally predominate. We also noted that *Pax7* is misexpressed in the remnants of dorsal root ganglia in *Pax3* mutant embryos (Fig. 8B,D arrowheads), but not in wild-type embryos (Fig. 8A,C). These data further support the conclusion that *Pax3* is a negative regulator of *Pax7*.

Pax3 represses endogenous *Pax7* expression in C2C12 myoblasts

To test directly whether *Pax3* can repress *Pax7* expression, we stably transfected C2C12 cells, which normally express *Pax7* but not *Pax3* (Fig. 9B), with a *Pax3* expression construct. Northern blot analysis of polyadenylated RNA from C2C12 myoblasts and two stably transfected C2C12 cell lines revealed

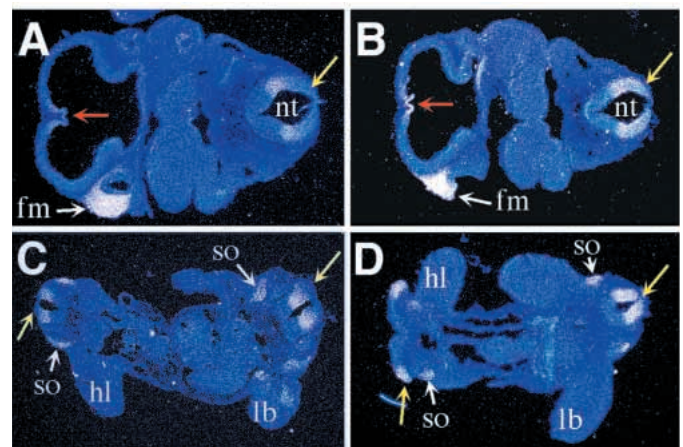
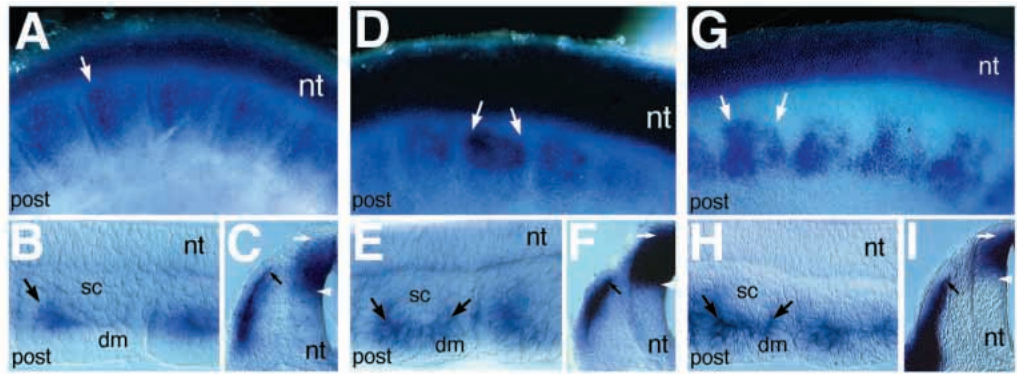


Fig. 6. *Pax7* expression expands in the neural tube of *Pax3* mutant embryos. Radioactive in situ analysis of *Pax7* expression using transverse sections of E10.5 wild-type embryos (A,C) reveals expression in the medial portion of the neural tube (nt), in the facial mesenchyme (fm), the somite (so) and the forelimb bud (lb). In *Pax3* mutant embryos (B,D), *Pax7* expression in the neural tube is expanded dorsally (yellow arrows) and appears in the lamina terminalis (red arrow in A and B) where *Pax3* is normally expressed. Expression of *Pax7* is absent in the limb bud (lb) of *Pax3* mutant embryos (D). Sections at the level of the brain (A,B), and the hindlimb (hl) (C,D) are shown.

Fig. 7. *Pax7* expression expands in mature somites of E9.5 *Pax3* mutant embryos. Sagittal (B,E,H) and transverse sections (C,F,I) of E9.5 embryos after whole-mount in situ hybridization (A,D,G) to detect *Pax7* (A-F) and *Pax3* (G-I) expression are shown from wild type (A-C, G-I) and *Pax3* mutant (D-F) embryos. Sagittal sections reveal that *Pax7* expression expands anteriorly in *Pax3* mutant somites (arrows in D,E) as compared to wild-type somites (arrows in A,B). *Pax7* is also expressed in the dorsomedial lip (DML) of the somite in *Pax3* mutant embryos (black arrow, F), whereas it is excluded from the DML in wild-type somites (black arrow, C). The expression pattern of *Pax7* in *Pax3* mutant somites and neural tube resembles the expression pattern of *Pax3* in wild-type embryos (G-I). Note the dorsal expansion of *Pax7* in the neural tube (nt) of *Pax3* mutant embryos compared to wild-type (white arrows in C and F). sc, sclerotome; dm, dermomyotome; post, posterior.



that *Pax7* expression is downregulated strongly in both *Pax3*-transfected cell lines (Fig. 9A), establishing that *Pax3* can repress *Pax7* expression in cultured C2C12 myoblasts. This result is consistent with our in vivo expression data suggesting a reciprocal relationship between *Pax3* and *Pax7* in muscle precursor cells.

DISCUSSION

In this report, we provide in vivo and in vitro evidence that *Pax3* has previously unknown functions in apoptosis and *Pax7* regulation during embryogenesis. We first show that *Pax3* is required for the survival of somitic mesodermal cells,

providing a mechanism by which *Pax3* may act genetically upstream of *MyoD* in somitic mesoderm. In addition, we show that *Pax3* represses the expression of the closely related *Pax7* gene in both neural tube and somites. The misexpression of *Pax7* in *Pax3* mutant embryos provides a likely compensatory mechanism for cell survival and for cell fate determination in neural tube and in mature somites.

Pax3 is required for survival of somitic mesoderm

Multiple functions have been attributed to *Pax3* in the control of muscle cell progenitor migration into the limb bud (Bober et al., 1994; Goulding et al., 1994), in cell transformation (Galili et al., 1993) and finally in the control of *MyoD* transcription (Maroto et al., 1997). However, these previously known functions of *Pax3* do not account for the observation that the epithelial organization of the somite and the dermomyotome is disrupted in *Pax3* mutant embryos (Franz et al., 1993), a phenomenon that is observed in newly formed somites. We now show that *Pax3* mutant somitic cells undergo extensive apoptosis, as assayed both in vivo and in vitro. Therefore, apoptosis contributes to the disorganization of *Pax3*

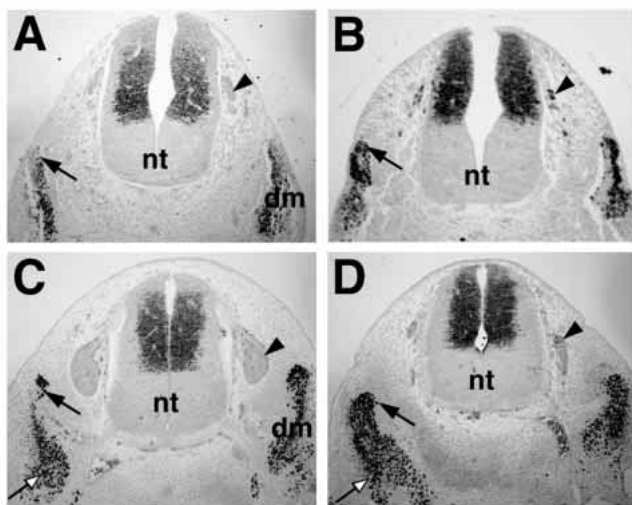
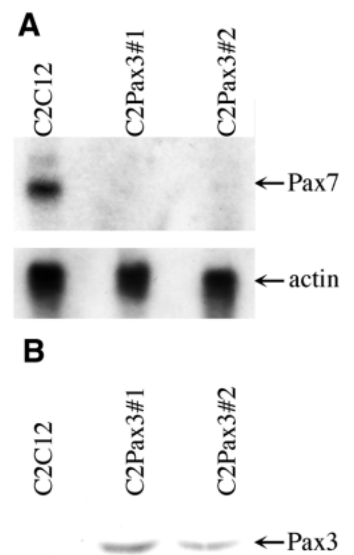


Fig. 8. *Pax7* protein expression expands in somites of *Spotch* embryos. Immunohistochemistry using an anti-*Pax7* antibody on transverse sections of E10.5 (A,B) and E11.5 (C,D) wild-type (A,C) and *Pax3*-mutant (B,D) embryos. In E10.5 *Pax3* mutant embryos, *Pax7* protein expression expands dorsally in the dorsomedial lip of the somites (black arrow, B). By E11.5 (D), *Pax7* protein expression also expands in the ventral lateral domain of the dermomyotome (open arrow in D). *Pax7* misexpression is also observed in the residual, small dorsal root ganglia of *Pax3* mutant embryos (black arrowheads B,D). dm, dermomyotome; nt, neural tube.

Fig. 9. *Pax3* represses endogenous *Pax7* expression in C2C12 myoblasts. (A) Northern blot analysis of *Pax7* mRNA expression in C2C12 myoblasts and two different clonal C2C12 lines stably expressing *Pax3* (C2Pax3#1, C2Pax3#2) is shown. *Pax7* mRNA expression is markedly downregulated in the clones expressing *Pax3*. 1 μ g of polyadenylated RNA is loaded per lane. *Actin* expression is shown below to indicate loading of lanes. (B) Western blot using a *Pax3* antibody reveals *Pax3* protein expression by the two C2C12 clones shown in A but not by untransfected C2C12 cells.



mutant somites and to the failure of somitic cells to express *MyoD* in response to signals from axial tissues and surface ectoderm in vitro.

Pax3 has upstream functions in muscle cell determination, as revealed by the complete loss of *MyoD* expression in trunk muscles of *Pax3* and *Myf-5* double mutant embryos (Tajbakhsh et al., 1997). Retrovirus-mediated ectopic expression of Pax3 in chick presomitic mesoderm explants can activate the expression of *MyoD* (Maroto et al., 1997), indicating that Pax3 may have a direct role in *MyoD* transcription. In *Myf5* mutant embryos, *MyoD* activation is delayed developmentally in both epaxial and hypaxial somite muscle lineages, suggesting that *MyoD* activation is controlled by an early acting, Myf5-dependent mechanism, and a later-acting Myf5-independent mechanism. Early acting *MyoD* activation may be indirectly influenced by the role of Pax3 in somite cell survival. Alternatively, Pax3 may act upstream and be required for survival of the lateral dermomyotome cells prior to *MyoD* expression in *Pax3/Myf5* double mutant embryos (Tajbakhsh et al., 1997). In support of this latter hypothesis, the most drastic disruptions in myogenesis in *Pax3/Myf5* double mutant embryos occur in cell lineages derived from the lateral dermomyotome (Tajbakhsh et al., 1997). Furthermore, disruption of *Pax3* expression by removal of surface ectoderm (Fan and Tessier-Lavigne, 1994) increases cell death in the lateral, but not the medial compartment of somites in chick embryos (Schmidt et al., 1998). In this respect, ablation of the neural tube/notochord complex in chick embryos and the *Danforth's short tail (Sd)* mutation causing notochord degeneration in mouse embryos led to increased apoptosis of epaxial somite cells (Asakura and Tapscott, 1998; Teillet et al., 1998). These data indicate that lateral and medial domains of the dermomyotome are subject to different environmental influences that control cell survival. Our data indicate that Pax3 is required for the survival of the lateral dermomyotome domain.

Our data do not distinguish whether Pax3 directly or indirectly represses genes in the apoptotic pathway during the development of the paraxial mesoderm. There is some evidence that Pax genes directly regulate programmed cell death. A conserved Pax binding site is located in the first untranslated exon of the human *p53* gene (Stuart et al., 1995), which can promote apoptosis (Levine, 1997), and Pax2, Pax5, and Pax8 can transcriptionally inhibit *p53* expression (Stuart et al., 1995). Pax8 binds the *bcl-2* promoter and activates in vitro the transcription of the *bcl-2* gene (Hewitt et al., 1997), which suppresses apoptosis (Allsopp et al., 1993; Sentman et al., 1991). Further experiments are required to address whether Pax3 prevents apoptosis by controlling *p53* or *bcl-2* gene expression during embryonic development. However, a generalized function for Pax gene products in promoting cell survival is supported by the observation that *Pax1/Pax9* compound homozygous mutant embryos have increased cell death in the sclerotome (H. Peters and R. Balling, personal communication). Finally, antisense-mediated inhibition of Pax3 in rhabdomyosarcoma cells results in apoptosis (Bernasconi et al., 1996), suggesting that one mechanism contributing to malignancy in these rhabdomyosarcomas may be PAX3/FKHR inhibition of an apoptotic pathway. Further understanding of the target genes of Pax3 is required to fully elucidate its role in apoptosis.

Pax3 negatively regulates Pax7 expression in neural tube and somites

Pax7 and *Pax3* proteins are closely related, and both genes likely arose by gene duplication from a common ancestor (Noll, 1993). We now show that, in absence of Pax3 function, *Pax7* is misexpressed in the dorsal neural tube and in specific domains of somites where *Pax3* would normally be expressed. Furthermore, we establish experimentally that Pax3 can repress *Pax7* expression in C2C12 cultured myoblast cells. These findings indicate that Pax3 negatively regulates *Pax7* expression in specific domains of somites and neural tube. Such cross-gene repression is also observed for two other closely related Pax genes, *Pax1* and *Pax9*, which are expressed in the ventral sclerotome domain of somites, based on the observation that *Pax9* expression is upregulated in homozygous *Pax1* mutant mice (H. Peters and R. Balling, personal communication). We did not observe upregulation of the non-functional *Pax3* transcripts in *Pax3* mutant embryos, indicating that Pax3 does not regulate its own expression, as is the case for *Pax6* expression (Grindley et al., 1995). The purpose of such a regulatory network among homologous gene family members is probably to regulate the overall summation of Pax3 and Pax7 proteins in embryonic tissues. *Pax3* and *Pax7*, however, also have overlapping domains of expression in somites and in neural tube (Goulding et al., 1991; Jostes et al., 1991), suggesting that, in specific regions of the embryo, Pax3 protein levels are insufficient to downregulate *Pax7* expression. If this is the case, we would predict that overexpression of *Pax3* in the neural tube would result in a gradual downregulation of *Pax7* expression. Such cross-regulatory mechanisms amongst closely related factors provide a sophisticated layer of functional redundancy that protects the embryo from individual gene mutation or inactivation. There is evidence for similar regulatory networks amongst other transcription factor gene families, such as GATA genes and bHLH genes, whose functions are critical for the embryo (Kuo et al., 1997; Rudnicki et al., 1993; Weiss et al., 1994). Consistent with this idea, the paired and homeobox domains of Pax3 and Pax7 are 91% and 95% identical, respectively (Goulding et al., 1991; Jostes et al., 1991). Structural homology and the expression data presented here suggest that Pax7 may be able to compensate, at least partially, for loss of Pax3, as has been suggested for GATA factors and bHLH myogenic factors (Kuo et al., 1997; Rudnicki et al., 1993; Weiss et al., 1994). Furthermore, we have also observed that *Pax7* misexpression occurs in regions of the somite in *Pax3*-deficient mice that do not undergo apoptosis. Conversely, in presomitic mesoderm and newly formed somites, where *Pax7* expression has not initiated, apoptosis is prevalent. Similarly, *Pax3* mutant embryos have neural tube closure defects, and we hypothesize that Pax3 function is required for neural tube closure at a stage when *Pax7* expression is not activated in the neural tube (Jostes et al., 1991). In support of our hypothesis of the overlapping redundant functions of Pax3 and Pax7, *Pax3/Pax7* double homozygous embryos die earlier than *Pax3* mutant embryos and have extensive neural tube abnormalities, including spina bifida and exencephaly (Mansouri and Gruss, 1998). In addition, the dermomyotome of the *Pax3/Pax7* double mutant embryos appears to be severely affected at E10.5, as assessed by the loss of *En1* expression and the absence of somite expression of the LacZ

reporter gene inserted into the *Pax3* locus (Mansouri and Gruss, 1998).

In summary, Pax3 plays a role in somitic cell survival and this function may be especially critical prior to activation of myogenic determination genes. While Pax3 acts genetically upstream of *MyoD*, its cellular function may be critical to the undifferentiated myogenic precursor rather than as a mediator of myogenic differentiation. The possibility of functional compensation by the related *Pax7* gene is supported by our finding that Pax3 represses *Pax7* in specific neural and somitic tissue domains.

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