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Pax3 acts cell autonomously in the neural tube and somites by controlling cell surface properties

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SUMMARY

***Pax3* is a member of the paired-box-containing transcription factors. It is expressed in the developing somites, dorsal spinal cord, mesencephalon and neural crest derivatives. Several loss-of-function mutations are correlated with the Splotch phenotype in mice and Waardenburg syndrome in humans. Malformations include a lack of muscle in the limb, a failure of neural tube closure and dysgenesis of numerous neural crest derivatives. In this study we have used embryonic stem (ES) cells to generate a *lacZ* knock-in into the *Pax3* locus. The *Pax3* knock-in Splotch allele (Sp^{2G}) was used to generate *Pax3*-deficient ES cells in order to investigate whether, in chimeric embryos, *Pax3* is acting cell autonomously in the somites and the neural tube. We found that while *Pax3* function is essential for the neuroepithelium and somites, a wild-type environment rescues mutant neural crest cells. In the two affected embryonic tissues, mutant and wild-type cells undergo segregation and do not intermingle.**

The contribution of mutant cells to the neural tube and the somites displayed temporal differences. All chimeric embryos showed a remarkable contribution of blue cells to the neural tube at all stages analyzed, indicating that the *Pax3*-deficient cells are not excluded from the neural epithelium while development proceeds. In contrast, this is not true for the paraxial mesoderm. The somite contribution of $Pax3^{-/-}$ ES cells becomes less frequent in older embryos as compared to controls with $Pax3^{+/-}$ ES cells. We propose that although *Pax3* function is related to cell surface properties, its role may differ in various tissues. In fact, apoptosis was found in *Pax3*-deficient cells of the lateral dermomyotome but not in the neural tube.

Key words: Mouse, *Pax3*, Cell-autonomous, Chimeras, Neural tube, Somites, Neural crest, Dermomyotome

INTRODUCTION

Pax3 belongs to the family of paired-box-containing transcription factors. Mutations in the *Pax3* gene lead to developmental defects in mouse and man. Thus, *Pax3*-deficient mice (Splotch) lack limb muscle, suffer from spina bifida and exencephaly, and exhibit defects in neural crest derivatives (Auerbach, 1954; Moase and Trasler, 1989; Franz and Khotary, 1993). In humans, *PAX3* mutations lead to Waardenburg syndrome, an autosomal dominant disorder that consists of numerous defects in neural crest-derived tissues (Tassebehji et al., 1992; Tassebehji et al., 1993). The observed phenotypes exhibit similar defects that suggest loss-of-function mutations. In fact, a mutant mouse generated recently by knocking *lacZ* into the *Pax3* locus by homologous recombination in embryonic stem (ES) cells, displays the same phenotype as Splotch mice (Mansouri and Gruss, 1998). Homozygous Splotch animals die at E14 of gestation and several defects are observed, such as spina bifida and exencephaly (Auerbach, 1954; Franz, 1989; Moase and Trasler, 1989).

Splotch mutants have been extensively characterized in order to elucidate *Pax3* function, especially in skeletal muscle (Bober et al., 1994; Goulding et al., 1994; Daston et al., 1996; Tajbakhsh et al., 1997). It has emerged that *Pax3* is an important regulator for the migration of myogenic precursors into the limb bud (Williams and Ordahl, 1994; Bober et al., 1994; Goulding et al., 1994; Daston et al., 1996; Mennerich et al., 1998). In addition, genetic and in vitro analysis revealed that *MyoD* (*Myod1* – Mouse Genome Informatics) activation is dependent on either *Pax3* or *MyF5*. Accordingly, two independent pathways have been postulated for the activation of myogenesis in the body (Tajbakhsh et al., 1997; Maroto et al., 1997).

Somite patterning is under the control of various signals provided by the dorsal neural tube, the notochord and the floor plate, the surface ectoderm, and the lateral plate mesoderm (Münsterberg et al., 1995; Pourquié et al., 1996; Hirsinger et al., 1997; Marcelle et al., 1997; Reshef et al., 1998; Tajbakhsh et al., 1998; Yamaguchi, 1997; Currie and Ingham, 1998). The multiple roles for *Pax3* in the paraxial mesoderm and the neural tube do not indicate which tissues require *Pax3* function. We therefore used $Splotch^{2H}$ mice (Sp^{2H}) and *lacZ*-expressing

Pax3 knockout mice (Sp^{2G}) to generate chimeras, composed of wild-type and $Pax3^{-/-}$ cells. Chimeric embryos with $Pax3^{-/-}$ cells could easily be identified by PCR through the presence of the Sp^{2H} allele (Epstein et al., 1991) and by X-gal staining. Embryos with a high proportion of *Pax3*-deficient cells display a similar phenotype to Splotch homozygous embryos, and have exencephaly and spina bifida. Moreover, these embryos die at embryonic day (E) 14. All the embryos that survived to term contained either the Sp^{2H} or the *lacZ* allele, indicating that high chimeric embryos containing homozygous cells do not survive to term. In order to trace more accurately the *Pax3*-deficient cells and to follow their behavior in a wild type environment we isolated three $Pax3^{-/-}$ ES cell lines from embryos derived from the knockout mice generated by the knock-in of the *lacZ* gene into the *Pax3* locus. Two homozygous and one heterozygous ES cell lines were used to generate chimeric embryos by aggregation with wild-type embryos. These chimeric embryos were analyzed at different stages of gestation (E9.0 to E13). The degree of ES cell contribution was monitored by the amount of pigmentation in the eye and by X-gal staining. Our analysis reveals that *Pax3*-deficient cells participate in forming all *Pax3*-expressing tissues. Chimeric embryos with high contribution of *Pax3*-deficient ES cells (>80%) often suffer, as do Splotch embryos, from spina bifida and exencephaly. At early stages of gestation (up to E10), mutant cells do not segregate from wild-type cells. However, later in development mutant cells are detected as isolated clones of blue cells, in the somites, spinal cord, brain and olfactory epithelium. Accordingly, $Pax3^{-/-}$ cells may have lost or modified some cell surface properties. This is in agreement with earlier observations proposing that Pax genes may be involved in cell-cell adhesion or cell-cell interactions (Chalepakis et al., 1994; Quinn et al., 1996; Stoykova et al., 1997; Collinson et al., 2000, Duncan et al., 2000). In addition, we found that in chimeric mice and in grafts of $Pax3^{-/-}$ ES cells into chick neural tube, *Pax3*-deficient neural crest cells are able to migrate. *Pax3* function in the neural crest may therefore be related to the maintenance of other properties required post migration. Alternatively, normal neighboring tissue may be necessary for neural crest migration and/or survival.

MATERIALS AND METHODS

Isolation of *Pax3*-deficient ES cells

A *Pax3* knockout mouse was generated by replacing the first exon and part of the first intron with the *lacZ* gene from *Escherichia coli* (data not shown; Mansouri and Gruss, 1998). This knockout mouse line was designated Sp^{2G}. The pattern of expression of the β-galactosidase protein from the mutated allele recapitulates the previously described expression of the *Pax3* gene (Fig. 1; Goulding et al., 1991). Highly chimeric males that had transmitted the mutated allele to the germline were mated to 129/Sv mice, and born animals carrying the *Pax3 lacZ* allele were used for the efficient derivation of ES cells. Females with vaginal plugs were opened at E3.5 of gestation (considering day of vaginal plug as E0.5). About 60 blastocysts were prepared as described (Hogan et al., 1994), and cultured for few days on mitomycin treated embryonic fibroblasts in the presence of 500 U/ml of LIF and 20% fetal calf serum (FCS). Sixteen ES cell lines were derived and three of them (*Pax3*/3, *Pax3*/9 and *Pax3*/15) were *Pax3*-deficient. Genotyping of the ES cells was performed by genomic Southern blot with an external probe used for screening the *Pax3* knockout mice (Mansouri and Gruss, 1998 and data not shown).

Generation of chimeric embryos

In preliminary experiments, we generated chimeric embryos by using Sp^{2H} and the knockout mice (Sp^{2G}). Using PCR, *lacZ* and Sp^{2H} alleles could be traced. Embryos with a genotype including both alleles indicated that homozygous cells are present in the chimeric embryo (Fig. 2). Embryos with heterozygous cells exhibit either the Sp^{2H} or the *lacZ* allele. Briefly, embryos at the morulae stage were prepared and the zona pellucida removed by passage through acidic Tyrode solution (Hogan et al., 1994) and aggregated according to the scheme shown in Fig. 2.

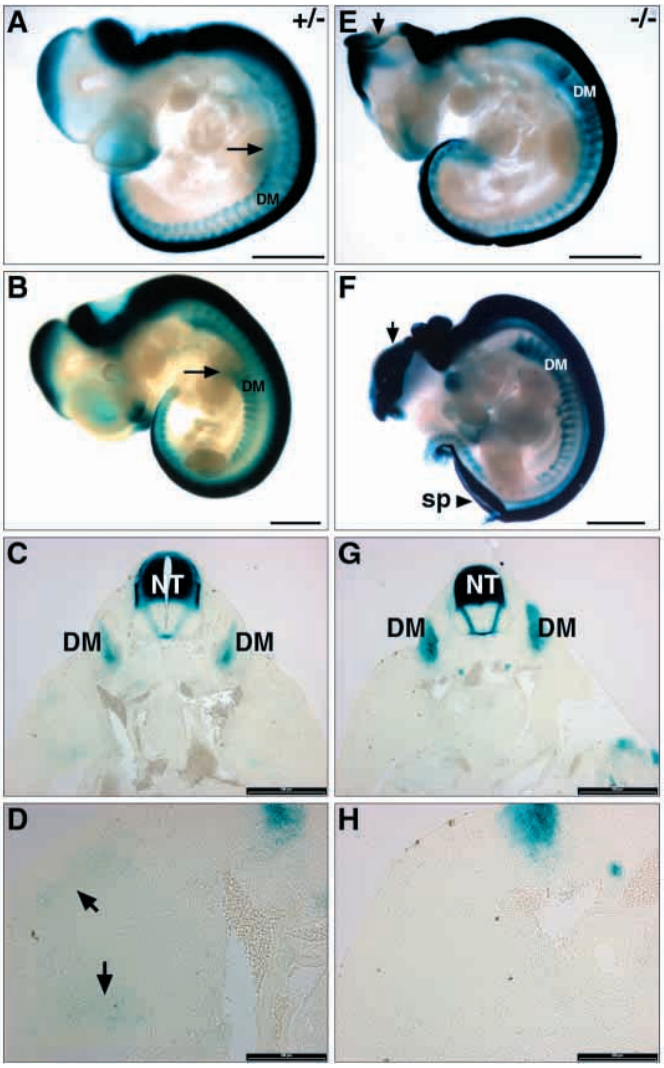

Fig. 1. The expression pattern of the *lacZ* gene from the mutated *Pax3* allele recapitulates the *Pax3* expression pattern. Whole mount X-gal staining at different developmental stages. Embryos in A and B are heterozygous, embryos shown in E and F are homozygous for the mutation (A,E: E10; B,F: E10.75). Exencephaly and spina bifida are indicated in black arrows in E and F. The lateral dermomyotome in E and F is disorganized and limb muscle is detected only in A and B, as indicated by black arrows. The lack of limb muscle is also documented by transverse sections at the limb level in G and H (higher magnification of G) and corresponding sections of heterozygous embryos in C and D (higher magnification of C) (E10.5). In D, myoblasts are indicated by black arrows. DM, dermomyotome; NT, neural tube; sp, spina bifida; +/–, heterozygous; –/–, homozygous. Scale bars: 1 mm in A,B,E,F; 500 μm in C,G; 200 μm in D,H.

Table 1. Chimeric embryos generated

	<30% chimerism			30-60% chimerism			>70% chimerism		
Method	E9.0-E9.5	E10.0-E10.5	E10.5-E12.5	E9.0-E9.5	E10.0-E10.5	E10.5-E12.5	E9.0-E9.5	E10.0-E10.5	E10.5-E12.5
$Sp^{2H}\times Sp^{2G}$	nd	4 1 3 0	3 1 2 0	nd	8 2 4 2	16 6 6 4	nd	29 8 10 11*	5 1 2 2‡
$ES^{-/-}$	4	4	5	15	14	8	20	23	7
$ES^{+/-}$	4	5	5	4	4	6	7	6	8

Aggregation chimeras were made by two different methods as indicated. Chimeras were analyzed mostly by X-gal staining at different stages of development. The number of chimeric embryos is listed with respect to stage of gestation, percentage of mutant cell contribution and method of aggregation.

nd, not done.

In the first row $Sp^{2H}\times Sp^{2G}$, the lower numbers indicate from the right to the left the number of $Sp^{2H}\leftrightarrow+/+$, of $Sp^{2G}\leftrightarrow+/+$ and of $Sp^{2H}/Sp^{2G}\leftrightarrow+/+$ chimeras, respectively.

*Nine of these embryos had spina bifida and/or exencephaly.

‡Indicates necrotic embryos with exencephaly.

Chimeric embryos were also generated by aggregating $Pax3^{+/-}$ and $Pax3^{-/-}$ ES cells with morulae from albino NMRI embryos (Table 1). The resulting degree of chimerism could easily be monitored by the amount of pigmentation in the eye. Embryos were prepared at different stages and whole-mount X-gal staining was performed to determine the degree of chimerism in *Pax3*-expressing tissues.

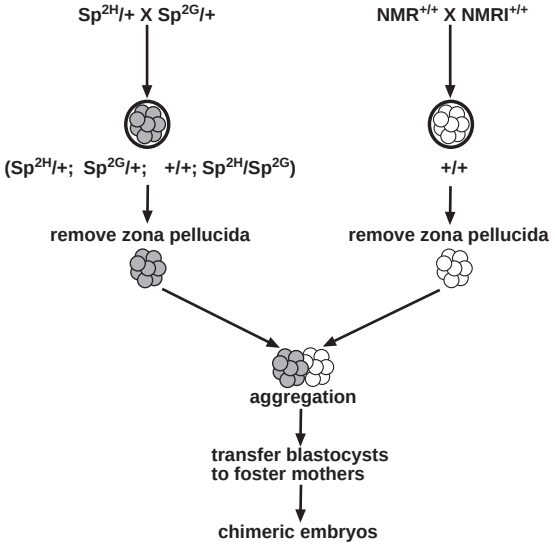

Fig. 2. The aggregation procedure using Sp^{2H} and Sp^{2G} embryos. The scheme for one of the aggregation procedures using Sp^{2H} and the allele generated by knocking-in the *lacZ* gene into the *Pax3* locus. Briefly, heterozygous animals (Sp^{2H}/+ and Sp^{2G}/+) were intercrossed and morulae were prepared at E2.5 of gestation. In parallel, embryos from NMRI (albino) wild-type intercrosses were also prepared. Embryos from both matings were denuded from the zona pellucida using acidic tyrode solution (Hogan et al., 1994) and subjected to aggregation as indicated. The embryos from Sp^{2H} and Sp^{2G} crosses are on a mixed 129/sV and C57Bl/6 background and presented in gray. The embryos were left to develop overnight to blastocysts and subsequently transferred to foster mothers. Chimeric embryos were prepared at the appropriate time. DNA was prepared from embryonic membranes and both alleles are detected by PCR. Embryos that are positive for both alleles are supposed to contain homozygous $Pax3^{-/-}$ cells. The embryos that are positive only for *lacZ* are considered to consist of wild-type and $Pax3^{+/-}$ cells and are used as controls. In older embryos, the degree of chimerism is also monitored by eye pigmentation.

Graft of murine ES cells into chicken embryos

For this specific experiment, the *Pax3*/6 heterozygous and *Pax3*/9 homozygous ES cells were grown on 0.1% (w:v) gelatine in the absence of feeder layers in standard medium containing 2000 U/ml mouse LIF (GIBCO-BRL). All the following procedures have been described previously (Beauvais-Jouneau et al., 1999). Briefly, ES cells were labelled with 10 μM of the vital fluorescent CFSE marker (C-1157, Molecular Probes) to localize them in the chicken embryo. Before grafting, ES cells were stained in DMEM containing 30 μg/ml Neutral Red (N-2889, Sigma) to visualize the cells during the manipulation. Chicken embryos were incubated until they had developed 20-25 somite pairs. Aggregates of 15-30 ES cells were pipetted onto the chicken embryo and gently inserted between a somite and the neural tube through a slit in the ectoderm. The graft was performed at the axial level where neural crest cells had just migrated off the dorsal surface of the neural tube (i.e., the fifth to last somite pairs). After grafting, the eggs were sealed with tape and incubated in humid atmosphere at 37°C for about 18 hours. Migrating ES cells were localized either by confocal scanning fluorescence or X-gal staining.

Histology and whole-mount X-gal staining

For whole-mount X-gal staining, embryos were briefly washed in cold phosphate buffered saline (PBS), fixed in a solution containing formaldehyde, glutaraldehyde and NP40, and stained overnight at 30°C for *lacZ* activity as described (Allen et al., 1988). Stained embryos were cleared in glycerol solution. For histological analysis X-gal-stained embryos were briefly fixed in 4% PFA, washed in PBS and saline, and dehydrated in ethanol and isopropanol before embedding in paraffin. Sections (10 μm) were used for the analysis. X-gal-stained sections were counterstained with nuclear Fast Red (Vector Laboratories). Additionally, vibratome sections (50 μm) were prepared after embedding X-gal-stained embryos in gelatin.

BrdU labeling and TUNEL staining

Cell proliferation analysis was studied by the incorporation of BrdU (Sigma) into embryos and labeled cells were detected by the immunohistochemical procedure on paraffin-embedded sections using anti-BrdU antibody (Bioscience, USA). Briefly, foster mothers were injected with 100 μg/g bodyweight bromodeoxyuridine (BrdU) at 12.00pm and 1 hour later the concepti were recovered. Embryos from E10 to E11.5 were processed for BrdU immunohistochemistry. They were fixed in 4% paraformaldehyde, infiltrated and embedded in paraffin. Serial sections (10 μm) were cut and processed for staining. Endogenous peroxidase activity was blocked with H_2O_2, the sections were treated with intervening washes in PBS with HCl, and then $Na_2B_4O_7$, pepsin, anti-BrdU antibody (Bio-Science Products), biotynlated anti-mouse/rabbit IgG (Vector Laboratories), avidinbionylated horseradish peroxidase complex (Vector Laboratories) and diaminobenzidine.

For TUNEL assay, E10 and E11.5 embryos were fixed in paraformaldehyde and processed as for paraffin-embedded sections. Cells undergoing apoptosis were analyzed by TdT-mediated dUTP-biotin nick end-labeling using in situ apoptosis detection kit (ApoTag, Oncor) according to the manufacturer's instructions.

RESULTS

Generation of chimeric embryos

Pax3-deficient embryos die at E14 of gestation so that the generation of chimeric embryos containing *Pax3*$^{-/-}$ cells requires the use of heterozygous crossings. We used Sp^{2H} and Sp^{2G} in order to genotype the chimeric embryos unambiguously. Sp^{2H} has a 34bp deletion in the *Pax3* gene (Epstein et al., 1991). Sp^{2G}, the knock-in allele of the bacterial *lacZ* gene into the *Pax3* locus recapitulates the expression of *Pax3* in the embryo (Fig. 1; Mansouri and Gruss, 1998). Both alleles could be monitored by PCR. In addition, X-gal staining was used to trace the expression of the Sp^{2G} locus corresponding to *Pax3*-deficient cells in chimeric embryos. Matings and aggregation of morulae were performed as shown in Fig. 2. We have generated a total of 65 chimeras, from which 19 were +/+ ↔ Sp^{2H}/Sp^{2G}; 19 were +/+ ↔ +/Sp^{2H}; 27 were +/+ ↔ +/Sp^{2G} and 29 were +/+ ↔ +/+. Chimeras were analyzed between E10 and E12.5 (Table 1). From the chimeras with a compound genotype (+/+ ↔ Sp^{2H}/Sp^{2G}), two embryos were necrotic (E12.5), 11 exhibited exencephaly, spina bifida or both defects, and six had no obvious malformation. The observed defects in the compound chimeras are consistent with those described previously in homozygous Splotch embryos (Auerbach, 1954).

In order to monitor *Pax3*$^{-/-}$ cells in chimeric embryos directly without the need to make compound embryos, we used the Sp^{2G} knockout mice to derive ES cells from blastocysts provided by crossing Sp^{2G}/+ males with Sp^{2G}/+ females. Three *Pax3*$^{-/-}$ and several *Pax*$^{+/-}$ ES cell lines were isolated. More than 100 chimeras were produced using *Pax3*$^{-/-}$ ES cells aggregated with NMRI morulae. With the *Pax3*$^{-/-}$ ES cells *Pax3*/9, several high chimeric (>80%) embryos were obtained, indicating that mutant Splotch cells are able to colonize all *Pax3*-expressing tissues such as the spinal cord, the mesencephalon and the dermomyotome, as revealed by X-gal staining (Fig. 3). In addition, a high number of these embryos displayed a Splotch phenotype with defects in the neural tube and dermomyotome (Figs 3, 4). Thus, chimeras generated with two independent methods indicate that *Pax3* function is required in the neural tube and the dermomyotome.

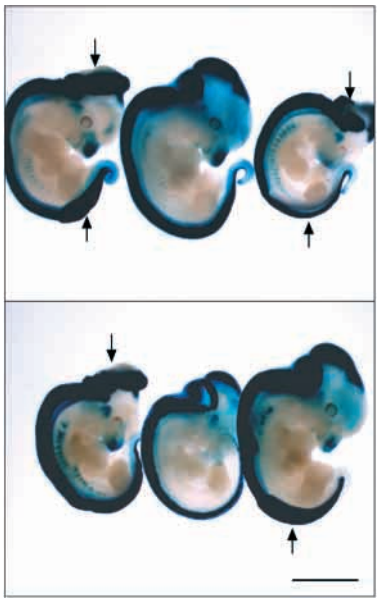

Fig. 3. Highly chimeric (>80%) embryos exhibit with high incidence a Splotch phenotype. Most of the shown embryos (E10.5-E11.5) are highly chimeric as revealed by the strong X-gal staining. Also strong eye pigmentation can be recognized in some embryos. The chimeras were generated by aggregating *Pax3*$^{-/-}$ ES cells to NMRI morulae. Arrows point to spina bifida or exencephaly (neural tube defects), as described in Splotch embryos. Scale bar: 1mm.

Segregation of mutant *Pax3* and wild-type cells in the somites, the neural tube and olfactory epithelium

As mentioned above, the degree of chimerism is documented by the percentage of blue cells (*lacZ* expression). In older embryos (E11) this is corroborated by the amount of pigmentation of the eye (Fig. 3; data not shown). In all chimeras, we found a tendency of mutant cells, documented by the *lacZ* expression pattern, to segregate from wild-type cells. In high chimeric (>80%) embryos, the somites, the neural tube and the olfactory epithelium exhibited only a few wild-type cells trapped in a mass of blue cells (Fig. 5 and data not shown).

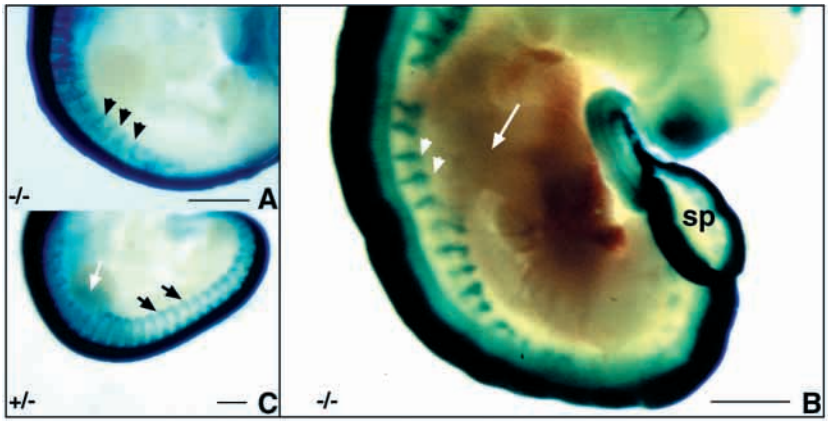

Fig. 4. Defects in the neural tube and dermomyotome of chimeric embryos are not rescued by wild-type cells. High chimeric embryos exhibit spina bifida and/or exencephaly. The remarkable contribution of mutant cells to the dermomyotome, documented by the X-gal staining, results in disorganized somites. In addition, the muscle precursor cells are not able to migrate into the limb (A,B) when compared with the embryo in C (chimera generated with *Pax3*$^{+/-}$ cells), where blue cells can be observed in the limb (white arrow). Black (A) and white (B) arrowheads show the disorganized dermomyotome. Black arrows in C indicate the regular and organized dermomyotome marked by *Pax3*$^{+/-}$ cells. Scale bars: 1 mm in A,C; 0.5 mm in B.

These embryos also have spina bifida and/or exencephaly, and are reminiscent of *Pax3* knockout homozygous embryos (Figs 1, 3). In moderate or low chimeric embryos, however, somites, neural tube and olfactory epithelium display a patchy *lacZ*-expression pattern, pointing to a failure of mixing capability between mutant (blue) and wild-type cells (white) (Fig. 5). This segregation behavior of mutant and wild-type *Pax3*-expressing cells is not detected before E10 (Fig. 5). In addition, it is interesting to note that in all chimeras with an open neural tube, no patchy expression of *lacZ* is observed in the spinal cord or mesencephalon, even in embryos older than E10. This indicates that mutant *Pax3* cells still contribute efficiently to the neural tube of the developing embryo.

In the lateral dermomyotome of chimeric embryos *Pax3*-deficient cells are not able to migrate into the limb

The limbs of Splotch embryos are devoid of muscle cells and *Pax3* was therefore suggested to be responsible for the migration of muscle precursors into the limb (Franz et al., 1993; Bober et al., 1994; Goulding et al., 1994; Daston et al., 1996; Mennerich et a., 1998). We analyzed our chimeras for the capability of *Pax3* mutant cells in the lateral dermomyotome to colonize the limb. In high chimeric embryos, the lateral dermomyotome is disorganized and has a 'fuzzy' structure. *Pax3*-deficient cells do not seem to migrate into the limb, where no blue cells can be detected when compared with chimeras generated from $Pax3^{+/-}$ ES cells (Fig. 4 and data not shown). Our analysis indicates that *Pax3* function is required for the structural organization of the dermomyotome and for the migration of muscle progenitors into the limb. In addition, *Pax3* mutant cells are not able to mix with wild-type cells (Fig. 5). Accordingly, *Pax3* mutant cells have lost or modified surface properties so that a rescue

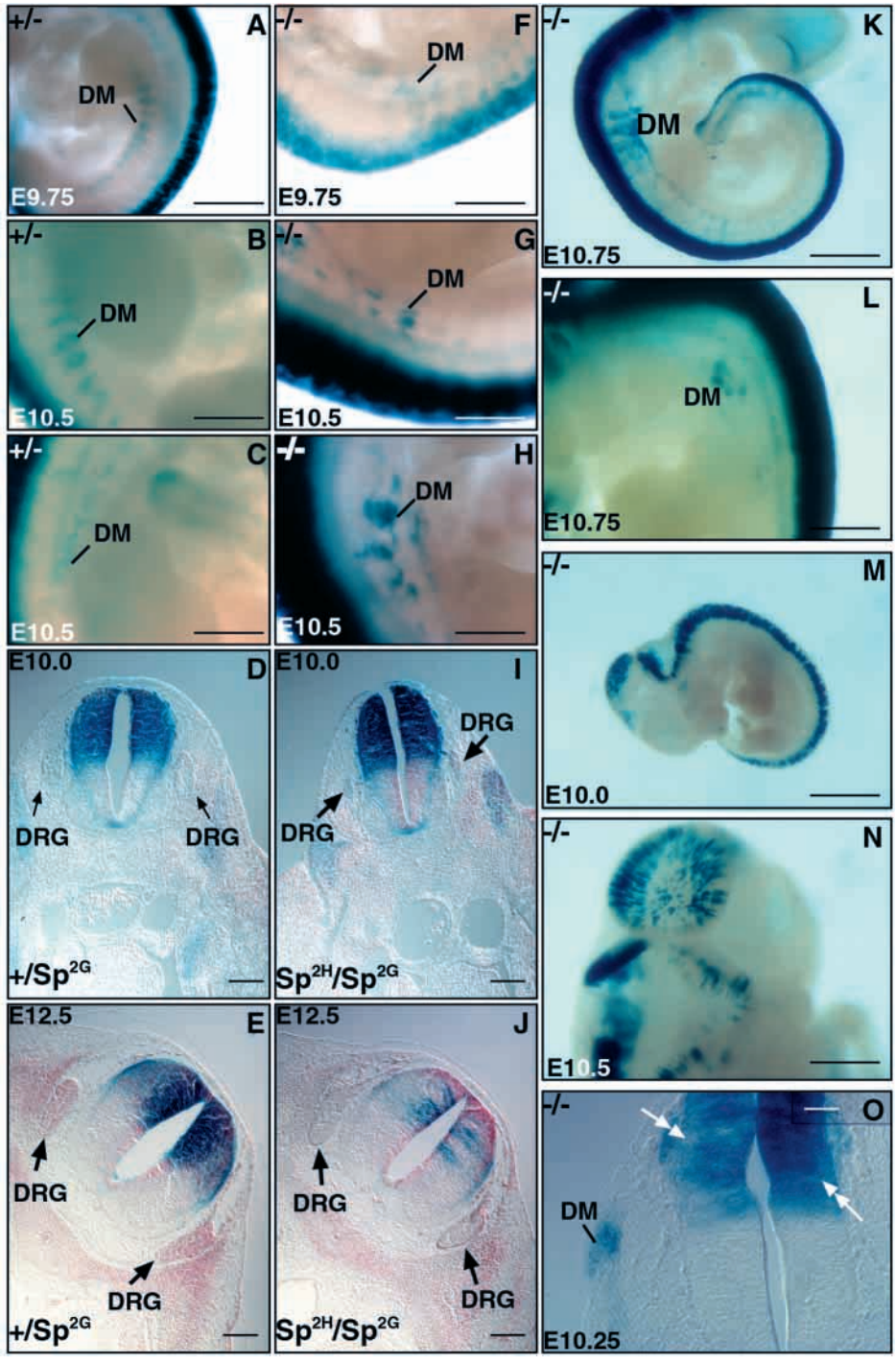

Fig. 5. Segregation of mutant $Pax3^{-/-}$ from wild-type cells in the neural tube and dermomyotome. Whole-mount X-gal staining of several chimeric embryos of different stages and transverse sections of chimeric embryos at the limb and trunk level. The embryos shown in A-C are derived from $ES^{+/-}$ cells and exhibit no segregation of wild-type and mutant cells in the dermomyotome; corresponding embryos from $ES^{-/-}$ cells, however, exhibit segregation in the dermomyotome after E10 (G,H,K,L,O) and in the neural tube in J,M-O. The few white cells (+/+) in the neural tube are indicated by double white arrows on a transverse section of E10.25 embryo (O). The chimeras shown in D,E,I,J were generated according to the scheme in Fig. 2 using Sp^{2H} and Sp^{2G} mice. From this aggregation, segregation of wild-type and mutant –/– cells is shown at E12.5 (J). Black arrows in D,E,I,J indicate the DRG. In I, the DRG are affected and smaller in size. In the older embryo (J), wild-type cells rescue them, shown in transverse section of a highly chimeric embryo of E12.5 (chimerism in this embryo was determined by eye pigmentation, data not shown). DM, dermomyotome; DRG, dorsal root ganglia; +/–, heterozygous; –/–, homozygous. Scale bars: 400 μm in A-C,F-H,L,N; 200 μm in D,E,I,J; 300 μm in K; 600 μm in M; 100 μm in O.

with wild-type cells does not occur. Thus, *Pax3* acts cell autonomously in the dermomyotome and the neural tube.

Pax3-deficient cells of the dermomyotome do not form a highly organized epithelium

Pax3 is already expressed in the presomitic mesoderm and is found in the newly formed somites. At later differentiation stages, it is confined to the dorsal somite compartment, the dermomyotome (Goulding et al., 1991; Mansouri et al., 1996a). The dermomyotome is an epithelial structure from which the myotome and the dermatome form. It differentiates through an epithelial-to-mesenchymal transition (Christ and Ordahl, 1995). The integrity of the epithelium is crucial for this process. The analysis of our chimeric embryos revealed that at E9.0 the development of the epithelium from *Pax3*$^{-/-}$ cells occurs normally (Fig. 6). At later stages, however, embryos with strong X-gal staining in the somites display severe defects in the structure of the dermomyotome. In these embryos only, the epithelium rather consists of a loosely packed cell mixture where some epithelial organization can be still recognized (Fig. 7D-I). Embryos with strong *lacZ* expression in the spinal cord but almost no blue staining in the somites have a well-formed dermomyotome (Fig. 7B). This indicates that during differentiation, *Pax3*$^{-/-}$ cells cannot maintain the epithelial architecture of the dermomyotome. Strikingly, the analysis using several markers (*Myf5*, *MyoD*, *myogenin*) revealed that only the expression of the gene for the fibroblast growth factor Fgf8 is abolished in the dermomyotome of Sp^{2H} or highly chimeric embryos (Fig. 8). *Fgf8* is normally detected in the rostral and caudal dermomyotomal lip (Fig. 8C); it has been shown to play an important role in cell proliferation and/or migration (Trumpp et al., 1999; Sun et al., 1999). All generated chimeras exhibit remarkable levels of *lacZ* expression in the neural tube at all stages of development analyzed so far. In contrast, this is not true for the somites. The contribution of *Pax3*$^{-/-}$ ES cells to the somites is detected very frequently from E9 to E10 when compared with E10.5 or older embryos. In contrast, control *Pax3*$^{+/-}$ ES cells contribute equally to the somites at all stages analyzed (data not shown). Therefore, proliferation and cell survival studies have been performed on chimeric embryos. BrdU labeling and TUNEL assay revealed that only cell survival is affected. In fact, apoptosis is detected in *Pax3*$^{-/-}$ cells of the lateral dermomyotome but not in the neural tube (Fig. 9A,G; data not shown).

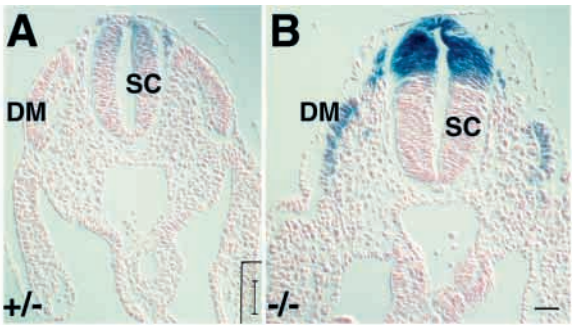

Fig. 6. Contribution of *Pax3*-deficient cells to the dermomyotome do not affect epithelial formation at E9.5. The high contribution of mutant cells to the dermomyotome at E9.5 for *Pax3*$^{+/-}$ and *Pax3*$^{-/-}$ ES cells is shown on frontal sections. The epithelial architecture looks normal indicating that *Pax3* is not required for the initiation process. This is in good correlation with earlier findings showing that truncation of the lateral dermomyotome occurs after E9.5 in Splotch embryos (Daston et al., 1996). (A) Section from a chimera generated with *Pax3*$^{+/-}$ cells. (B) Section from a chimera generated with *Pax3*$^{-/-}$ cells. DM, dermomyotome; SC, spinal cord. Scale bar: 100 μm.

Neural tube closure is affected in chimeric embryos but *Pax3*-deficient neural crest cells are able to migrate

Several highly chimeric embryos suffer from spina bifida and/or exencephaly, indicating that *Pax3* function is required in the neural tube. Our analysis demonstrates that *Pax3* mutant cells contribute efficiently to the neural tube. Although *Pax3*-

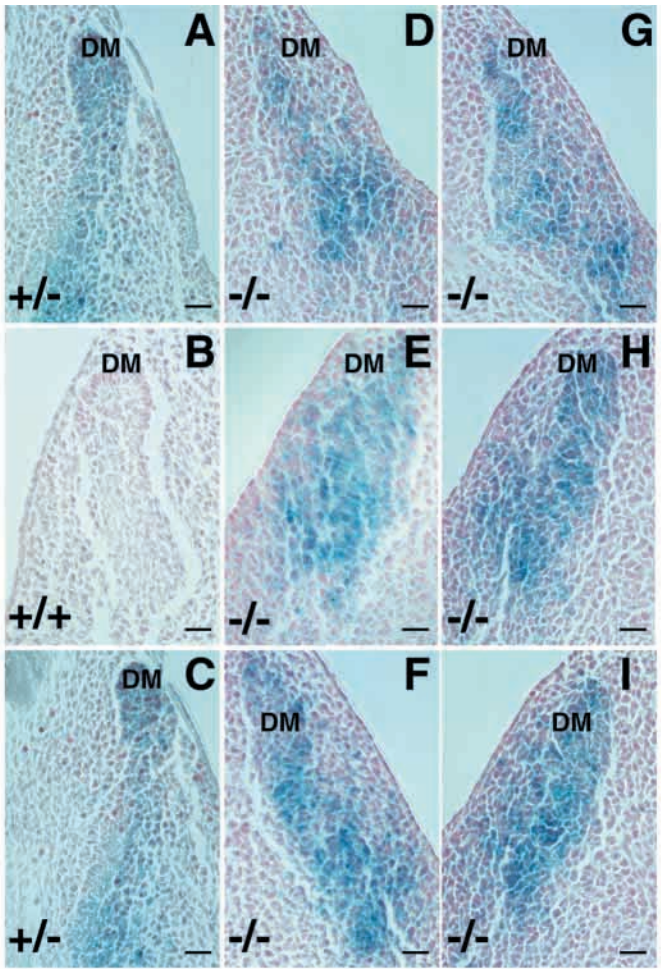

Fig. 7. The high contribution of mutant *Pax3* cells to the dermomyotome results in a failure to maintain an organized epithelium. Transverse sections showing the dermomyotome of chimeric embryos at E11 at the limb level. (A-C) The dermomyotome appears highly organized. (A,C) the blue cells refer to chimeras generated from *Pax3*$^{+/-}$ ES cells. (B) Section from a chimera derived from *Pax3*$^{-/-}$ ES cells: there is no contribution of blue cells to the dermomyotome; however, the neural tube contains mutant cells (data not shown). In D-I, several transverse sections at the limb level are shown from chimeric E11 embryos with high contribution to the somite. They document the disorganized architecture of the epithelium made from blue *Pax3*$^{-/-}$ cells. Four embryos were analyzed. DM, dermomyotome. Scale bar: 100 μm.

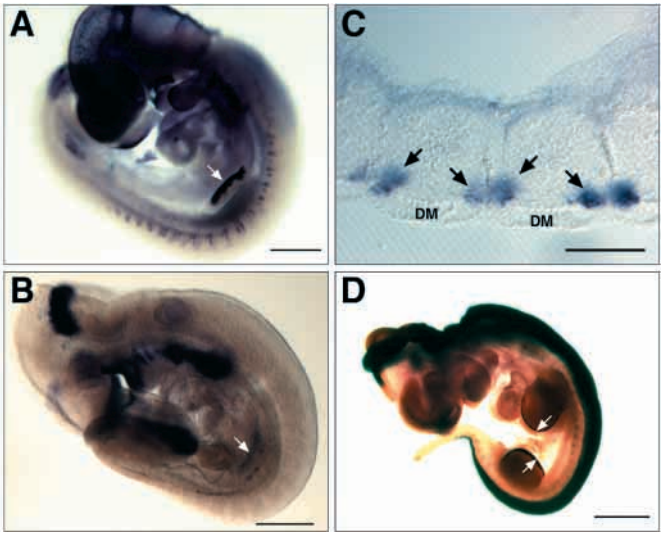

Fig. 8. *Fgf8* is not detected in the dermomyotome of Splotch embryos. Whole-mount in situ hybridization showing the expression pattern of *Fgf8* in control and Splotch embryos. *Fgf8*, which is normally expressed in the dermomyotome, is not detectable in the somite of Splotch and high chimeric (>80% of $Pax3^{-/-}$ cells) embryos at all stages analyzed (E9.0-E10.5). The expression in the hindbrain and limb bud is not affected in Splotch embryos. (A) Control embryo (E9.5); (B) $Splotch^{2H}$ embryo (E9.0); (C) Sagittal section of embryo in A showing the expression of *Fgf8* in the caudal and rostral lips of the dermomyotome as indicated by black arrowheads, rostral is to the left; (D) chimeric embryo (generated with $Pax3^{-/-}$ ES cells) stained for β-galactosidase and hybridized with Fgf8 probe; hybridization in the limb bud is indicated by arrows; in the somites only the X-gal staining is detectable. DM, dermomyotome. Scale bars: 500 μm in A,B,D; 100 μm in C.

deficient cells do not intermingle with wild-type cells, they are not excluded from the neural tube during later development. As mentioned above, cell survival is not affected.

Splotch mice and individuals with Waardenburg syndrome suffer from defects of neural crest derivatives. Hence, *Pax3* is an important regulator of neural crest cell migration. We therefore wanted to know how $Pax3^{-/-}$ neural crest cells behave in a chimeric environment with wild type cells. The analysis of several chimeras at E9 and E11 of development revealed that the neural crest cells of the rostral neural tube (hindbrain) seemed to migrate more efficiently than those located caudally. In addition, more caudal neural crest cells were able to migrate and blue $Pax3^{-/-}$ cells could be detected in dorsal root ganglia (DRG) and spinal ganglia (SG) (Fig. 10A-D; data not shown). Furthermore, DRG of highly chimeric embryos were affected and smaller in size at early stages of development, but were later rescued by wild-type cells. In fact, older embryos (E11.5) always exhibited normally formed SG and DRG (Fig. 5I,J; data not shown). A normal contribution of blue cells to the trigeminal ganglion was also observed (data not shown). Accordingly, in contrast to other tissues, *Pax3* does not act cell autonomously in neural crest cells. The contribution of mutant neural crest cells to the formation of melanocytes were therefore analyzed by grafting $Pax3^{+/-}$ and $Pax3^{-/-}$ ES cells into chick embryos.

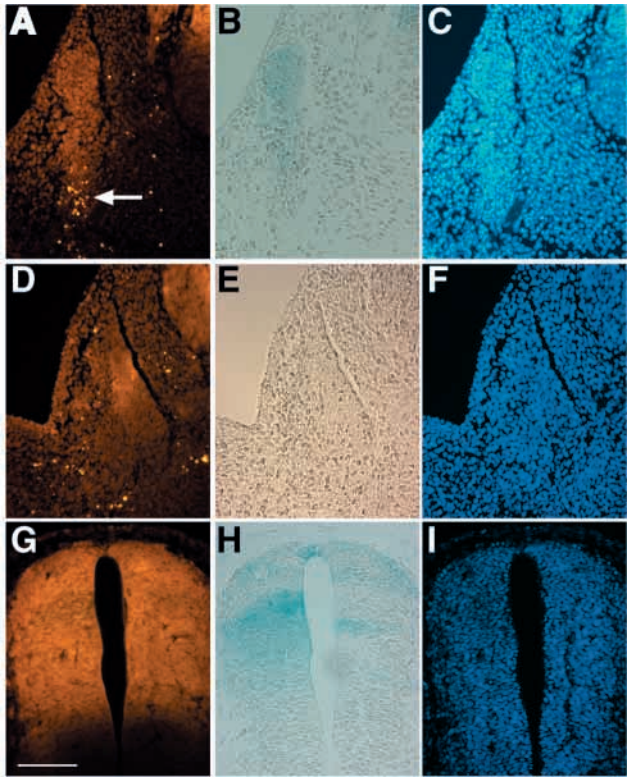

Fig. 9. Apoptosis is detected in *Pax3*-deficient cells of the lateral dermomyotome but not in the neural tube. Cell survival is analyzed in the somites and in the neural tube of chimeric embryos using the TUNEL assay. (A,D,G) Apoptosis staining using TUNEL assay; (B,E,H) corresponding adjacent sections showing X-gal staining; and (C,F,I) corresponding adjacent sections showing DAPI staining. (A-C) Chimera with blue $Pax3^{-/-}$ cells in the dermomyotome showing apoptosis only in the lateral dermomyotome (white arrow). In wild-type embryos (D-F) no apoptosis is detected. (G-I) TUNEL assay in the spinal cord of a chimeric embryo as indicated by the X-gal staining (H). No apoptosis is detectable in $Pax3^{-/-}$ cells (blue cells) of the neural tube. Three embryos analyzed for somite and four for spinal cord. Scale bar: 100 μm.

Grafted *Pax3*-deficient ES cells migrate on the dorsolateral and on the dorsoventral neural crest cell pathway

Heterozygous *Pax3*/6 and homozygous *Pax3*/9 ES cells were labelled with a vital fluorescent marker (CFSE) and grafted into early trunk neural crest cell migratory pathways. The migratory behavior of the cells was assessed by fixing the host embryos 18 hours after receiving the graft. ES cells were detected in the embryos either by confocal scanning fluorescence microscopy or by histochemistry after staining for β-galactosidase. In the presence of *Pax3*, wild-type and heterozygous ES cells were able to migrate in both pathways simultaneously (data not shown and Beauvais-Jouneau et al., 1999). In the absence of *Pax3*, ES cells were also able to migrate in both the dorsoventral and dorsolateral pathways (Fig. 10E,F). Moderate β-galactosidase activity was revealed by whole-mount X-gal staining of *Pax3* homozygous and heterozygous ES cells when migrating in chicken embryo (Fig. 10F; not shown). Paraffin sections revealed that cells migrating dorsolaterally and dorsoventrally were expressing *lacZ* (data not shown).

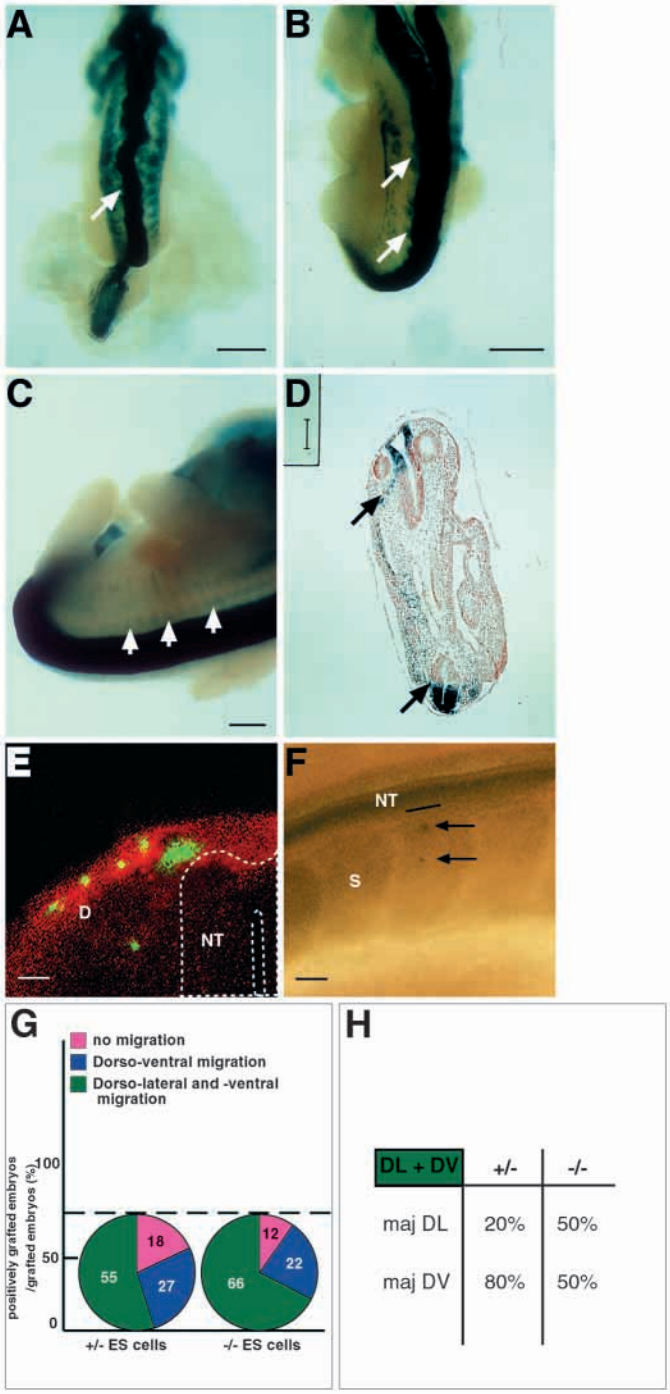

Fig. 10. *Pax3*-deficient neural crest cells are able to migrate in chimeric environment of mouse and chick. (A-D) Several embryos show the migration of *Pax3*-deficient neural crest cells in an environment mixed with wild-type cells. All the presented chimeras are made using aggregation of *Pax3*-deficient ES cells. (A,B) The migration of the neural crest cell is indicated by white arrows in two different chimeric embryos at E9.0 and E9.75, and stained in whole mount for β-galactosidase. At E11.5, DRG are detected in embryo C, as revealed by whole-mount X-gal staining; white arrowheads point to some of the ganglia between forelimb and hindlimb. In D, a frontal section from an E9.0 embryo documents the migration of neural crest cells at the hindbrain and at the more caudal neural tube level (arrows). In E,F, grafted *Pax3*-deficient ES cells migrate on the DL and on the DV neural crest cell pathway of the chicken embryo. (E) Transverse section of a chicken embryo (in red) fixed 18 hours after the graft of *Pax3*/9 homozygous ES cells (in green). These embryos were analyzed by optical scanning using a confocal scanning fluorescence microscope. ES cells were previously labeled with a vital green fluorescent marker (CFSE). After fixation, embryos were immunostained with an anti-laminin antibody that recognised the basal lamina of the embryo (red). (F) DL view of the trunk of an X-gal-stained embryo fixed 18 hours after grafting of *Pax3*/9 homozygous ES cells. The cells located on the DV pathway of neural crest cells are not visible. The black bar on the side of the neural tube indicates the grafting site. (G) Statistical analysis of embryos grafted with *Pax3*/6 heterozygous ES cells (*n*=24) or *Pax3*/9 homozygous ES cells (*n*=26). The diameter of the pie charts represents the percentage of positive grafted embryos. The embryos were classified as follows: embryos with no migrating cells (pink areas), embryos containing cells migrating solely on the DV pathway (blue areas), embryos containing cells migrating on the DL and DV pathways (green areas). (H) Analysis of embryos containing cells migrating on the DL and DV pathways. For each embryo, the cells were migrating preferentially either on the DV pathway (maj DL) or on the DV pathway (maj DV). D, dermomyotome; NT, neural tube; S, somite. Scale bars: 300 μm in A-C; 100 μm in D; 15 μm in E; 200 μm in F.

In the presence or absence of *Pax3*, the percentage of positively grafted embryos over the grafted embryos was similar. The proportions of embryos in which ES cells were not migrating or migrating exclusively dorsoventrally were similar in heterozygous and in homozygous ES cells (Fig. 10G). In embryos in which ES cells were migrating dorsolaterally and dorsoventrally, the number of cells was determined on each pathway. The embryos were classified as a function of the cells migrating in majority on the dorsolateral (DL) or the dorsoventral (DV) pathway (Fig. 10H). In this respect, in the absence of *Pax3*, the proportions of DL and DV embryos were equal to 50%. In the presence of wild-type or heterozygous *Pax3*, the proportion of DL embryos was equal to 80%. These results suggest that ES cells that lack *Pax3* have a stronger tendancy to migrate dorsolaterally. Finally, the average distance of migration is similar for wild type, heterozygous and homozygous *Pax3* ES cells.

DISCUSSION

Loss-of-function mutations of the paired-box-containing gene *Pax3* lead to developmental defects documented by the Splotch phenotype in mice and the Waardenburg syndrome in humans. These abnormalities include a failure of neural tube closure, dysgenesis of neural crest-derived tissues and the incapability of myogenic precursors of the lateral dermomyotome to migrate into the limb (Mansouri et al., 1996a). In this study we used different Splotch alleles and also *Pax3*$^{-/-}$ embryo-derived stem cells to generate chimeric embryos consisting of wild-type and *Pax3*$^{-/-}$ cells. *Pax3*-deficient cells were monitored by the expression of the *lacZ* gene inserted into the *Pax3* locus by homologous recombination. Thus, the amount of *Pax3*$^{-/-}$ cells in the affected tissues can easily be traced in the individual embryo. The chimeric embryos provide a system in which *Pax3*-deficient cells are allowed to mix and interact with surrounding wild-type cells, enabling them to be rescued in the affected tissues. A failure to rescue mutant cells would suggest

that *Pax3* acts cell autonomously in that specific tissue (Rossant and Spence, 1998; West, 1999).

Pax3 acts cell autonomously in the neural tube and somites

Somite patterning is under the control of different signals provided by the neural tube and other tissues such as the surface ectoderm, and the axial and the lateral mesoderm (Münsterberg et al., 1995; Pourquié et al., 1996; Hirsinger et al., 1997; Marcelle et al., 1997; Reshef et al., 1998; Tajbakhsh et al., 1998). The multiple roles of *Pax3* in the paraxial mesoderm and the neural tube do not disclose which tissue requires *Pax3* function.

In chimeric embryos, where only the neural tube exhibits a high proportion of mutant cells, the dermomyotome develops normally. Thus, *Pax3* function in the neural tube is not related to signals exerted on the somites. However, the contribution of mutant cells to the dermomyotome leads to disorganized somites, as has been reported for Splotch embryos. In addition, *Pax3* mutant cells in the lateral dermomyotome are not able to migrate into the limb. (Franz et al., 1993; Bober et al., 1994, Goulding et al., 1994; Daston et al., 1996; Mennerich et al., 1998). Accordingly, *Pax3* acts cell autonomously in the lateral dermomyotome. Furthermore, mutant cells in the neural tube are not able to interact with wild-type cells, resulting in an open neuroepithelium and suggesting a cell autonomous role for *Pax3* in this tissue.

The analysis of chimeric embryos further revealed that in the neural tube, somites and olfactory epithelium, mutant and wild-type cells do not intermingle. $Pax3^{-/-}$ and wild-type cells fail to mix and clear segregation is readily detectable in the affected tissues in whole-mount X-gal-stained embryos. The affected tissues consist exclusively of patches of blue cells (mutant) or white cells (wild type) revealing a distinct boundary. However, this abnormal participation of mutant cells is not observed before E10 of gestation. In addition, the neural tube alone displays segregation of heterozygous mutant and wild-type cells in some chimeras generated by the aggregation of $Pax3^{+/-}$ ES cells with wild-type embryos. This is in close correlation with the often observed failure of neural tube closure at the posterior neuropore (spina bifida) in heterozygous Splotch embryos (Mansouri et al., 1996a). The failure of mutant cells to mix and interact normally with surrounding wild-type cells suggests that *Pax3* may control cell surface properties. Similar observations have been made in chimeras generated with mutant *Pax6* Sey cells (Quinn et al., 1996; Collinson et al., 2000). Differences in cell-cell adhesion documented by the expression of various cadherins may confer segregation behavior and thus define cell identity (Takeichi, 1991). In fact, it has been suggested that some *Pax* genes act on cell surface molecules (R-, N-cadherin), members of the immunoglobulin superfamily (N-CAM, L1) or integrins (Stoykova et al., 1997; Brand-Saberi et al., 1996; Moase and Trassler, 1991; Mansouri and Gruss, 1998; Chalepakis et al., 1994; St-Onge et al., 1997; Quinn et al., 1996; Collinson et al., 2000; Kozmik et al., 1992; Duncan et al., 2000). Alternatively, molecules involved in modulating cell surface properties may also act downstream of *Pax* genes. Such a protein may be the c-met tyrosine kinase, the receptor for HGF/SF (hepatocyte growth factor/scatter factor), which has been proposed to act downstream of *Pax3* and initiate the migration of myoblasts from the lateral dermomyotome (Bladt et al., 1995; Daston et al., 1996; Epstein et al., 1996; Yang et al., 1996). Accordingly, the tendency of *Pax*-deficient cells to segregate from wild-type cells in the affected tissues indicates a common mechanism, reflecting a similar role for *Pax* genes in various organs.

In addition, the contributions of mutant cells to the neural tube and the somites displayed temporal differences. All chimeric embryos showed a remarkable contribution of blue cells to the neural tube at all stages analyzed, indicating that the *Pax3*-deficient cells are not excluded from the neural epithelium while development proceeds. In contrast, this is not true for the paraxial mesoderm. Somite contribution of $Pax3^{-/-}$ ES cells becomes less frequent in older embryos, when compared with controls with $Pax3^{+/-}$ ES cells. This suggests that although in the neural tube and somites cell surface properties are related to *Pax3* function, *Pax3* may play different roles in both tissues. In the somites, *Pax3* may be necessary for cell proliferation and/or survival as suggested previously (Bernasconi et al, 1996; Amthor et al., 1999; Borycki et al., 1999). Although natural cell death was described in the somites (Cotrina et al., 2000), we think that in the lateral dermomyotome of Sp^{2H} embryos, observed apoptosis is significant. Our results support the idea that *Pax3* function in the dermomyotome is related to cell survival (Borycki et al., 1999). Furthermore, our findings suggest that *Pax3* is also required for maintenance of the integrity of the dermomyotome, where it may drive a proper epithelial-to-mesenchymal transition process that is necessary for the formation of the myotome (Christ and Ordahl, 1995). This is in close correlation with earlier observations in Splotch embryos, where after E9.5 the lateral dermomyotome becomes truncated, leading to a loss of epithelial morphology (Daston et al., 1996). The lack of *Fgf8* expression in the dermomyotome of Splotch embryos provides further evidence for the role of *Pax3* in the cytoarchitecture of this structure. In the absence of *Fgf8* cell survival is affected in the first branchial arch. During gastrulation, the lack of *Fgf8* causes a failure of cell migration (Trumpp et al., 1999; Sun et al., 1999). *Fgf8* may therefore be one of the factors that mediates cell proliferation and/or migration in the dermomyotome. In addition, the expression of *Fgf8* in the rostral and caudal dermomyotomal lips points to a role in the epithelial-to-mesenchymal transition during the differentiation of the dermomyotome. In the absence of the Fgf8 receptor, Fgfr1, it was proposed that the primary defect is a deficiency in the ability of cells to make the transition from an epithelial-to-mesenchymal morphology (Ciruna et al., 1997). Altogether, these results suggest an important role for *Pax3* in the morphogenesis of the epithelium of the lateral dermomyotome. *Fgf8* acts downstream of *Pax3* to achieve this function. *Pax7* possibly restores the integrity of the medial dermomyotome, as in *Pax3/Pax7* double mutants the whole epithelium is truncated (A. M. and P. G., unpublished).

In the neural tube, *Pax3* has been also suggested to be necessary for the migration of neural crest cells (Moase and Trasler, 1990). However, grafting of dorsal neural tube tissue from Splotch mice into chick host embryo results in normal neural crest migration (Serbedzija and McMahon, 1997). Our studies clearly provide further evidence that *Pax3*-deficient neural crest cells are able to migrate from the neural tube and that wild-type cells always rescue neural crest derivatives (DRG and SG). The role of *Pax3* in neural crest cells may be

related to the maintenance of other properties required post migration, such as proliferation and/or survival. Similar findings have recently been described for cardiac neural crest cells (Epstein et al., 2000). Alternatively, neural crest migration may require interaction with neighboring tissues (Serbedzija and McMahon, 1997; LaBonne and Bronner-Fraser, 1999). As stated above, it is conceivable that these neighboring cues are also related to cell surface molecules, such as extracellular matrix proteins (Duncan et al., 2000). Strikingly, overexpression of the extracellular matrix protein versican has been described and associated with defective neural crest migration in Splotch embryos (Henderson et al., 1997). Our results however, do not correlate with earlier studies that suggest a cell autonomous role for *Pax3* in neural crest cells (Li et al., 1999). Our analysis of ES cell grafts into the chick neural tube confirms the experiments reported previously (Serbedzija and McMahon, 1997) and support the idea that *Pax3* does not act cell autonomously in neural crest migration. In addition, these results indicate that *Pax3* is not necessary for ES cells to migrate in chicken embryo via the DV and DL pathways. A slight difference in the behavior of the cells that lack *Pax3* should be noticed. By an as yet unexplained mechanism, the absence of *Pax3* seems to favor slightly the DL migration.

We cannot exclude the possibility that *Pax3* function in neural crest is related to intrinsic properties, which are required post migration. The difference in the migration potential of mutant neural crest cells between rostral and caudal neural tube of Splotch embryos is most likely related to a redundant function of *Pax7* in the hindbrain (Mansouri et al., 1996b; Serbedzija and McMahon, 1997).

In summary, the chimeric analysis using *Pax3*-deficient ES cells revealed a cell-autonomous function of *Pax3* in the somites and neural tube. A common denominator of *Pax3* function may be the modulation of cell surface properties, although distinct roles are enacted in various tissues.

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REFERENCES

Allen, N. D., Cran, D. G., Barton, S. C., Hettle, S., Reik, W. and Surani, M. A. (1988). Transgenes as probes for active chromosomal domains in mouse development. *Nature* **333**, 852-855.

Auerbach, R. (1954). Analysis of the developmental effects of a lethal mutation in the house mouse. *J. Exp. Zool.* **127**, 305-329.

Amthor, H., Christ, B. and Patel, K. (1999). A molecular mechanism enabling continuous embryonic muscle growth – balance between proliferation and differentiation. *Development* **126**, 1041-1053.

Beauvais-Jouneau, A., Pla, P., Bernex, F., Dufour, S., Salamero, J., Fassler, R., Panthier, J. J., Thiery, J. P. and Larue, L. (1999). A novel model to study the dorsolateral migration of melanoblasts. *Mech. Dev.* **89,** 3-14.

Bernasconi, A., Remppis, A., Fredericks, W. J., Rauscher, F. J. and Schäfer, B. W. (1996). Induction of apoptosis in rhabdomyosarcoma cells through down-regulation of PAX proteins. *Proc. Natl. Acad. Sci. USA* **93**, 13164-13169.

Bladt, F., Riethmacher, D., Isenmann, S., Aguzzi, A. and Birchmeier, C. (1995). Essential role for the *c-met* receptor in the migration of myogenic precursor cells in the limb bud. *Nature* **376**, 768-771.

Bober, E., Franz, T., Arnold, H.-H., Gruss, P. and Tremblay, P. (1994). *Pax-3* is required for the development of limb muscles: a possible role for the migration of dermomyotomal muscle progenitor cells. *Development* **120**, 603-612.

Borycki, A.-G., Li, J., Jin, F., Emerson, C. P. J. and Epstein, J. A. (1999). *Pax3* functions in cell survival and in Pax7 regulation. *Development* **126**, 1665-1674.

Brand-Saberi, B., Gamel, A. J., Krenn, V., Müller, T. S., Wilting, J. and Christ, B. (1996). N-cadherin is involved in myoblast migration and muscle differentiation in the avian limb bud. *Dev. Biol.* **178**, 160-173.

Chalepakis, G., Wijnholds, J., Giese, P., Schachner, M. and Gruss, P. (1994). Characterization of Pax-6 and Hoxa-1 binding to the promoter region of the neural cell adhesion molecule L1. *DNA Cell Biol.* **13**, 891-900.

Christ, B. and Ordahl, C. P. (1995). Early stages of chick somite development. *Anat. Embryol.* **191**, 381-396.

Ciruna, B. N., Schwarz, L., Harpal, K., Yamaguchi, T.P. and Rossant, J. (1997) Chimeric analysis of fibroblast growth factor receptor-1 (Fgfr-1) function: A role in morphogenetic movement through the primitive streak. *Development* **124**, 2829-2841.

Collinson, J. M., Hill, R. E. and West, J. D. (2000). Different roles for Pax6 in the optic vesicle and facial epithelium mediate early morphogenesis of the murine eye. *Development* **127**, 945-956.

Cotrina, M. L., González-Hoyuela, M., Barbas, J. A. and Rodríguez-Tébar A. (2000). Programmed Cell Death in the Developing Somites Is Promoted by Nerve Growth Factor via Its p75NTR Receptor. *Dev. Biol.* **228**, 326-336.

Currie, P. D. and Ingham, P. W. (1998). The generation and interpretation of positional information within the vertebrate myotome. *Mech. Dev.* **73**, 3-21.

Daston, G., Lamar, E. and M., G. (1996). *Pax-3* is necessary for migration but not differentiation of limb muscle precursors in the mouse. *Development* **122**, 1017-1027.

Duncan, M. K., Kozmik, Z., Cveklova, K., Piatigorsky, J. and Cvekl, A. (2000). Overexpression of PAX6(5a) in lens fiber cells results in cataract and upregulation of a5b1 integrin expression. *Development* **13,** 3173-3185.

Epstein, D. J., Vekemans, M. and Gros, P. (1991). splotch *(Sp^{2H})*, a mutation affecting development of the mouse neural tube, shows a deletion within the paired homeodomain of *Pax-3*. *Cell* **67**, 767-774.

Epstein, J. A., Shapiro, D. N., Cheng, J., Lam, P. Y. and Maas, R. L. (1996). Pax3 modulates expression of the *c-met* receptor during limb muscle development. *Proc. Natl. Acad. Sci. USA* **93**, 4213-4218.

Epstein, J. A., Li, J., Lang, D., Chen, F., Braun, C. B., Jin, F., Min Lu, M., Thomas, M., Liu, E.-C. J., Wessels, A. and Lo, C. W. (2000). Migration of cardiac neiral crest cells in *Splotch* embryos. *Development* **127**, 1869-1878.

Franz, T. (1989). Persistent truncus arteriosus in the splotch mutant mouse. *Anat. Embryol.* **180**, 457-464.

Franz, T. and Kothary, R. (1993). Characterization of the neural crest defect in Splotch (Sp^{1H}) mutant mice using a lacZ transgene. Dev. *Brain Res.* **72**, 99-105.

Franz, T., Kothary, R., Surani, M. A. H. and Grim, M. (1993). The Splotch mutation interferes with muscle development in the limbs. *Anat. Embryol.* **187**, 153-160.

Goulding, M. D., Chalepkis, G., Deutsch, U., Erselius, J. R. and Gruss, P. (1991). Pax-3, a novel murine DNA binding protein expressed during early neurogenesis. *EMBO J.* **10**, 1135-1147.

Goulding, M., Lumsden, A. and Paquette, A. J. (1994). Regulation of *Pax-3* expression in the dermomyotome and its role in muscle development. *Development* **120**, 957-971.

Henderson, D. J., Ybot-Gonzalez, P. and Copp, A. J. (1997). Over-expression of the chondroitin sulphate proteoglycan *versican* is associated with defective neural crest migration in the Pax3 mutant mouse (*Splotch*). *Mech. Dev.* **69**, 39-51.

Hirsinger, E., Duprez, D., Jouve, C., Malapert, P., Cooke, J. and Pourquié (1997). Noggin acts downstream of Wnt and sonic Hedgehog to antagonize BMP4 in avian somite patterning. *Development* **124**, 4605-4614.

Hogan, B. L. M., Constantini, F. and Lacy, E. (1994). *Manipulating The Mouse Embryo: A Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press.

Kozmik, Z., Wang, S., Dörfler, P., Adams, B. and Busslinger, M. (1992). The promoter of the CD19 gene is a target for the B-cell-specific transcription factor BSAP. *Mol. Cell. Biol.* **12**, 2662-2672.

LaBonne, C. and Bronner-Fraser, M. (1999). Molecular mechanisms of neural crest formation. *Annu. Rev. Cell Dev. Biol.* **15**, 81-112.

Li, J., Liu, K. C., Jin, F., Lu, M. M. and Epstein, J. A. (1999). Transgenic rescue of congenital heart disease and spina bifida in Splotch mice. *Development* **126,** 2495-2503.

Mansouri, A., Hallonet, M. and Gruss, P. (1996a). Pax genes and their role in cell differentiation and development. *Curr. Opin. Cell Biol.* **8**, 851-857.

Mansouri, A., Stoykova, A., Torres, M. and Gruss, P. (1996b). Dysgenesis of cephalic neural crest derivatives in *Pax 7*$^{-/-}$ mutant mice. *Development* **122**, 831-838.

Mansouri, A. and Gruss, P. (1998). *Pax3* and Pax7 are expressed in commissural neurons and restrict ventral neuronal identity in the spinal cord. *Mech. Dev.* **78**, 171-178.

Marcelle, C., Stark, M. R. and Bronner-Fraser, M. (1997). Coordinate actions of BMPs, Wnts, Shh and Noggin mediate patterning of the dorsal somite. *Development* **124**, 3955-3963.

Maroto, M., Reshef, R., Münsterberg, A., Koester, S., Goulding, M. and Lassar, A. (1997). Ectopic Pax-3 activates MyoD and MyF5 expression in embryonic mesoderm and neural tissue. *Cell* **89**, 139-184.

Mennerich, D., Schaefer, K. and Braun, T. (1998). Pax3 is necessary but not sufficient for lbx1 expression in myogenic precursor cells of the limb. *Mech. Dev.* **73**, 147-158.

Moase, C. E. and Trasler, D. G. (1989). Spinal ganglia reduction in the Splotch-delayed mouse neural tube defect mutant. *Teratology* **40**, 67-75.

Moase, C. E. and Trasler, D. G. (1990). Delayed neural crest emigration from Sp and Sp^{d} mouse neural tube explants. *Teratology* **42**, 171-182.

Moase, C. E. and Trasler, D. G. (1991). N-CAM alterations in splotch neural tube defect mouse embryos. *Development* **113**, 1049-1058.

Münsterberg, A. E., Kitajewski, J., Bumcrot, D. A., McMahon, A. P. and Lassner, A. B. (1995). Combinatorial signaling by sonic hedgehog and Wnt family members induces myogenic bHLH gene expression in the somite. *Genes Dev.* **9**, 2911-2922.

Pourquié, O., Fan, C. M., Coltey, M., Hirsinger, E., Watanabe, Y., Breant, C., Francis-West, P., Tessier-Lavigne, M. and Le Douarin, N. M. (1996). Lateral and axial signals involved in avian somite patterning: a role for BMP4. *Cell* **84**, 461-471.

Quinn, J. C., West, J. D. and Hill, R. E. (1996). Multiple function for *Pax6* in mouse eye and nasal development. *Genes Dev.* **10**, 435-446.

Reshef, R., Maroto, M. and Lassar, B. L. (1998). Regulation of dorsal somitic cell fates: BMPs and Noggin control the timing and pattern of myogenic regulator expression. *Genes Dev.* **12**, 290-303.

Rossant, J. and Spence, A. (1998). Chimeras and mosaics in the mouse. *Trends Genet.* **14**, 358-363.

Serbedzija, G. N. and McMahon, A. P. (1997). Analysis of neural crest cell migration in Splotch mice using a neural crest-specific LacZ reporter. *Dev. Biol.* **185**, 139-147.

St Onge, L., Sosa-Pineda, B., Chowdhury, K., Mansouri, A. and Gruss, P. (1997). Pax6 is required for differentiation of glucagon-producing α-cells in mouse pancreas. *Nature* **387**, 406-409.

Stoykova, A., Götz, M., Gruss, P. and Price, J. (1997). Pax6-dependent regulation of adhesive patterning, R-cadherin expression and boundary formation in developing forbrain. *Development* **124**, 3765-3777.

Sun, X., Meyers, E. N., Lewandoski, M. and Martin, G. R. (1999). Targeted disruption of FGF8 causes failure of cell migration in the gastrulating mouse embryo. *Genes Dev.* **13**, 1834-1846.

Tajbakhsh, S., Rocancourt, D., Cossu, G. and Buckingham, M. (1997). Redefining the genetic hierarchies controlling skeletal myogenesis: Pax-3 and MyF-5 act upstream of MyoD. *Cell* **89**, 127-138.

Tajbakhsh, S., Borello, U., Vivarelli, E., Kelly, R., Papkoff, J., Duprez, D., Buckingham, M. and Cossu, G. (1998). Differential activation of MyF5 and MyoD by different Wnts in explants of mouse paraxial mesoderm and the later activation of myogenesis in the absence of Myf5. *Development* **125**, 4155-4162.

Takeichi, M. (1991). Cadherin cell adhesion receptors as a morphogenetic regulator. *Science* **251**, 1451-1455.

Tassabehji, M., Read, A. P., Newton, V. E., Harris, R., Balling, R., Gruss, P. and Strachan, T. (1992). Waardenburg's syndrome patients have mutations in the human homologue of the *Pax-3* paired box gene. *Nature* **355**, 635-636.

Tassabehji, M., Read, A. P., Newton, V. E., Patton, M., Gruss, P., Harris, R. and Strachan, T. (1993). Mutations in the *PAX3* gene causing Waardenburg syndrome type 1 and type 2. *Nat. Genet.* **3**, 26-30.

Trumpp, A., Depew, M. J., Rubenstein, J. L. R., Bishop, J. M. and Martin, S. G. R. (1999). Cre-mediated gene inactivation demonstrates that FGF8 is required for cell survival and patterning of the first branchial arch. *Genes Dev.* **13**, 3136-3148.

Williams, B. A. and Ordahl, C. P. (1994). Pax-3 expression in segmental mesoderm marks early stages in myogenic cell specification. *Development* **120**, 785-796.

West, J. D. (1999). Insights into development and genetics from mouse chimeras. *Curr. Topics Dev. Biol.* **44**, 21-66.

Yamaguchi, T. P. (1997). New insights into segmentation and patterning during vertebrate somitogenesis. *Curr. Opin. Genet. Dev.* **7**, 513-518.

Yang, X.-M., Vogan, K., Gros, P. and Park, M. (1996). Expression of the *met* receptor tyrosine kinase in muscle progenitor cells in somites and limbs is absent in *Splotch* mice. *Development* **122**, 2163-2171.