

## Migration of cardiac neural crest cells in *Spotch* embryos

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### SUMMARY

*Pax3* encodes a transcription factor expressed during mid-gestation in the region of the dorsal neural tube that gives rise to migrating neural crest populations. In the absence of *Pax3*, both humans and mice develop with neural crest defects. Homozygous *Spotch* embryos that lack *Pax3* die by embryonic day 13.5 with cardiac defects that resemble those induced by neural crest ablation in chick models. This has led to the hypothesis that *Pax3* is required for cardiac neural crest migration. However, cardiac derivatives of *Pax3*-expressing precursor cells have not been previously defined, and *Pax3*-expressing cells within the heart have not been well demonstrated. Hence, the precise role of *Pax3* during cardiac development remains unclear. Here, we use a Cre-lox method to fate map *Pax3*-expressing neural crest precursors to the cardiac outflow tract. We show that although *Pax3* itself is extinguished prior to neural crest

populating the heart, derivatives of these precursors contribute to the aorticopulmonary septum. We further show that neural crest cells are found in the outflow tract of *Spotch* embryos, albeit in reduced numbers. This indicates that contrary to prior reports, *Pax3* is not required for cardiac neural crest migration. Using a neural tube explant culture assay, we demonstrate that neural crest cells from *Spotch* embryos show normal rates of proliferation but altered migratory characteristics. These studies suggest that *Pax3* is required for fine tuning the migratory behavior of the cardiac neural crest cells while it is not essential for neural crest migration.

Key words: *Pax3*, Cardiogenesis, connexin 43, Cre recombinase, Neural crest, Mouse

### INTRODUCTION

In birds and mammals the complex process of cardiac morphogenesis requires interactions between multiple cell types intrinsic and extrinsic to the primitive heart tube. A specific population of neural crest cells, defined by ablation studies in the chick, migrates to the rostral pole of the heart tube and mediates septation of the single great vessel emerging from the primitive heart (Kirby et al., 1983). This results in division of the truncus arteriosus into the aorta and the pulmonary artery and is associated with rotation of the outflow tract, resulting in juxtaposition of the aorta with the left ventricle. Studies in chick embryos indicate that disruption of neural crest migration can result in cardiac outflow tract defects. Thus, ablation of premigratory cardiac neural crest cells emerging between the mid-otic placode and the caudal boundary of somite 3 results in the failure of cardiac outflow tract septation (Kirby et al., 1983). Such studies indicate that errors in this process may underlie relatively common forms of congenital heart disease in humans which are often associated with other neural crest related abnormalities and syndromes (Driscoll, 1994; Jones, 1990).

Molecular pathways regulating cardiac neural crest function and interaction with cardiac tissues remain largely unknown.

Normally, neural crest cells make up only a small proportion of the final aorticopulmonary septum and outflow endocardial cushions, as demonstrated by quail-chick chimera studies in which quail neural crest cells are transplanted into chick embryos (Kirby et al., 1983; Waldo et al., 1999). These observations are confirmed by immunohistochemical analysis using HNK-1, an antibody specific for chick neural crest cells, and by diI labeling studies to fate map neural crest cells in the chick (Kirby et al., 1993). In humans and mice, however, there is no equivalent marker for tracking cardiac neural crest cells and thus the contribution of neural crest cells to cardiac formation has been inferred by analogy to chick and by mutational analysis (Epstein, 1996).

The *Spotch* mutant mouse has served as an important model for congenital heart disease involving the outflow tract of the heart, as homozygous mutant *Spotch* embryos display persistent truncus arteriosus in addition to other developmental defects (Auerbach, 1954; Franz, 1989). Several alleles of *Spotch* have been identified and all result from mutation or deletion of the *Pax3* gene (Epstein et al., 1991, 1993). *Pax3* encodes a transcription factor expressed during embryogenesis in the dorsal neural tube from which neural crest cells emerge (Goulding et al., 1991). Phenotypic resemblance to chick neural crest ablation models has suggested that *Spotch*

represents a mammalian animal model of failed neural crest migration. Some experimental studies have supported this supposition (Conway et al., 1997; Moase and Trasler, 1990), though the lack of appropriate murine crest-specific markers has made investigation of the fate of cardiac neural crest cells in *Spotch* (and wild-type) embryos difficult.

The cellular function of Pax3 in cardiac neural crest has remained unknown. Recent studies have suggested roles for Pax3 in neuronal differentiation (Koblar et al., 1999) and myelination of axons in the peripheral nervous system (Kioussi et al., 1995). By analogy to other cell types where Pax3 is expressed, it may also play a role in modulating neural crest migration, survival, proliferation and/or interaction with other tissues. For instance, in myogenic progenitors of hypaxial muscles, Pax3 is required for cell migration via pathways that include activation of the c-Met receptor (Bladt et al., 1995; Bober et al., 1994; Daston et al., 1996; Epstein et al., 1996; Goulding et al., 1994). Pax3 may also play important roles in myogenic differentiation (Maroto et al., 1997; Tajbakhsh et al., 1997), survival (Borycki et al., 1999) and proliferation (Barr et al., 1993; Galili et al., 1993; Shapiro et al., 1993).

In this report we have reinvestigated the question of whether Pax3 is required for neural crest migration to the heart. Using a neural crest-specific promoter element from the Pax3 gene to direct Cre-recombinase expression and appropriate Cre-reporter mice (Soriano, 1999), we have been able to track Pax3-expressing neural crest progenitors to the heart, even after Pax3 expression has abated. These results were independently confirmed using a neural crest specific transgene to fate map cardiac neural crest derivatives in the outflow tract of both wild-type and *Spotch* embryos. Such studies showed the presence of crest cells in the outflow tract of Pax3-deficient embryos. Further analysis of explanted cardiac neural crest cells from Pax3-deficient embryos revealed intact postotic neural crest cell migration, but at an altered rate. These studies suggest that Pax3 is not required for cardiac neural crest migration but may play a role in fine tuning the efficient targeting of crest cells to the outflow tract.

## MATERIALS AND METHODS

### Transgenic and inbred mouse lines

*Spotch* mice were obtained from the Jackson Labs and maintained on a C57/B16 inbred background. Embryos and adult mice were genotyped by PCR analysis of DNA derived from extra-embryonic membranes and tail biopsy, respectively, as described by Tajbakhsh et al. (1997). *Cx43-lacZ* mice have been described by Lo et al. (1997). P3pro-Cre consisted of the 1.6 kb proximal Pax3 regulatory region upstream of Cre recombinase. The transgene was created by digestion of P3pro-Pax3 (Li et al., 1999) with *Bst*EII and *Not*I followed by ligation of a 1.4 kb PCR fragment encoding Cre recombinase and a bovine growth hormone polyadenylation site amplified with primers containing engineered *Bst*EII (5') or *Not*I (3') sites. P3pro-Cre was linearized with *Nar*I and *Not*I and gel purified to remove vector sequences, purified over an Elutip column and used for oocyte injection at the Institute for Human Gene Therapy Transgenic Core Facility at the University of Pennsylvania. Founders were screened for ability to pass the transgene to F<sub>1</sub> offspring, express Cre recombinase in an expected pattern as screened by in situ hybridization and express sufficient levels of Cre activity to result in *lacZ*-expressing cell populations after crossing with floxed *lacZ*-reporter mice (Soriano, 1999).

### $\beta$ -galactosidase detection and in situ hybridization

Embryos to be analyzed for *lacZ* expression were washed in PBS and fixed for 2 hours in 2% PFA in PBS, rinsed, and stained according to standard techniques. Whole-mount in situ hybridization was carried out essentially as described by Riddle et al. (1993) with modifications (Chen et al., 1999). Radioactive in situ hybridization was performed using <sup>35</sup>S-UTP and embryos fixed overnight in 4% PFA, dehydrated and embedded in paraffin and sectioned at 8  $\mu$ m. Prehybridization, hybridization and wash conditions were as described by Lutz et al. (1994). Slides were exposed to photographic emulsion for 5 days prior to developing and counterstaining with Hoechst 33258 nuclear dye. Embryos and sections were photographed digitally and images were processed using Adobe Photoshop.

### Immunohistochemistry

*Sp*<sup>-/-</sup> embryos, fixed in 4% paraformaldehyde, were processed in a graded series of ethanol, cleared in toluene and embedded in Polyfin (Polysciences Inc., Warrington, PA). The specimens were serially sectioned (5  $\mu$ m) and mounted on aminoalkylsilane-coated slides (Sigma Diagnostics, St. Louis, MO). For the detection of smooth muscle actin (SMA, Sigma, A2547) monoclonal antibody and the atrial Myosin Light Chain polyclonal antibody (aMLC; gift from Dr Kubalak) binding on mouse tissue sections, the indirect conjugated method was applied. Paraffin wax was removed in xylene, the sections were rehydrated through a series of ethanols, and then pretreated for 30 minutes with hydrogen peroxide (3% v/v in phosphate-buffered saline (PBS), pH 7.4) to reduce endogenous peroxidase activity, and preincubated with TENG-T (10 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.25% gelatin, 0.05% Tween-20, pH 8.0) for an additional 30 minutes to minimize nonspecific binding of antibodies. The SMA antibody was diluted in PBS/BSA/NRS (PBS with 1% bovine serum albumin (BSA), and 2.5% normal rabbit serum (NRS)), and the polyclonal antibody was diluted in PBS/BSA. After incubation of the pretreated sections with the primary antibodies overnight, antibody binding was detected with rabbit anti-mouse peroxidase (RAM-Po; Sigma, A-9044) and goat anti-rabbit peroxidase respectively. All incubations were followed by three washes with PBS for 5 minutes. The immunocomplex formed was visualized using a Metal Enhanced Diaminobenzidine (DAB) Substrate Kit (Pierce, Rockford, IL; product number 34065). Sections were mounted in Accu-Mount 60 (Stephens Scientific, Riverdale, NJ). Digital images were collected using a Polaroid Digital Microscope Camera mounted on an Olympus BX40 microscope.

### Immunofluorescence

*Sp*<sup>-/-</sup> embryos, stained as whole mounts for the presence of transgenic *lacZ* expression, were processed in a graded series of ethanol, cleared in iso-propanol, carefully oriented, embedded in Polyfin (Polysciences Inc., Warrington, PA), and serially sectioned. After removal of the paraffin wax and rehydration, individual sections on selected slides were pretreated with TENG-T for 30 minutes to minimize nonspecific binding of antibodies. Smooth muscle actin (SMA) was detected using anti-smooth muscle actin (Sigma, A2547). The binding of this monoclonal antibody was visualized with goat anti-mouse FITC. Fluorescent labeling was visualized using a Biorad MRC 1024 scanning laser confocal microscope.

### Neural tube explant cultures and neural crest migration and proliferation studies

To obtain embryos for neural crest outgrowth studies, mice were mated and the day the vaginal plug was found was considered E0.5. Embryos were collected at E8.5, and the yolk sac from each embryo was harvested for genotyping by PCR analysis. The method used to establish the neural crest outgrowth cultures has been described previously (Huang et al., 1998). Briefly, each embryo was treated with 0.5 mg/ml of collagenase/dispase (Sigma Co., St. Louis, MO.), then bisected longitudinally to expose the hindbrain neural folds. Each half

was further transected to isolate the postotic region of the hindbrain, after which the dorsal ridge of the hindbrain neural fold was surgically removed from the surrounding tissues and cultured on fibronectin-coated 35 mm Petri dishes containing Dulbecco's modified Eagle's medium (DMEM) with high glucose and 10% fetal bovine serum. Digital images of the outgrowth cultures were captured at 24 and 48 hours by videomicroscopy, and then analyzed using the NIH Image software. We measured the area of the outgrowth and subtracted from it the area of the dense central neural tube mass. To control for differences in migration area resulting from random variations in the shape of the explant (as neural crest cells emerge only from the edges of the explant), the outgrowth area ( $\text{mm}^2$ ) was divided by the perimeter (mm) of the explant to arrive at the migration index. For monitoring cell proliferation, 48-hour explant cultures were incubated with BrdU (10  $\mu\text{M}$ ) for 2 hours, then fixed, and processed for immunodetection using an anti-BrdU antibody in conjunction with the True Blue™ peroxidase substrate (Kierkegaard and Perry Laboratories, Gaithersburg, MD). To determine the total cell number in the outgrowth after BrdU immunostaining, the cultures were further stained with hematoxylin. The proliferation rate was then calculated as the percentage of BrdU-labeled cells over the total number of cells in the outgrowth. Labeled cells were counted with the aid of NIH Image. For time lapse videomicroscopy, images of the explants were captured every 10 minutes over a 20-hour interval using IPLab software. For these studies, the explant cultures were maintained at 37°C on a heated microscope stage in phosphate-buffered L-15 medium containing 10% fetal bovine serum. The captured images were then converted to a Quicktime movie for viewing and motion analysis. The motion analysis was carried out using the Dynamic Image Analysis Software (Solltech, Inc.).

## RESULTS

### *Pax3* is not expressed by neural crest cells in the heart

In *Spotch* mice, a mutation in *Pax3* at the splice acceptor site for exon four results in aberrant RNA splicing and a non-functional transcript (Epstein et al., 1993). Nevertheless, mutant RNA transcripts can be detected by in situ hybridization and cells that would normally be expressing *Pax3* can therefore be detected. *Pax3* is expressed by neural crest progenitors within the dorsal neural tube and mutations in *Pax3* result in neural crest defects. A prior report has suggested that *Pax3* is expressed by cardiac neural crest cells after migrating from the neural tube and within the outflow tract of the heart (Conway et al., 1997). We attempted to detect the expression of the non-functional *Pax3* transcript in *Spotch* embryos to determine the fate of cardiac neural crest cells in the absence of functional *Pax3*. In parallel, we re-examined the normal expression pattern of *Pax3* transcripts in cardiac neural crest and in the hearts of wild-type embryos. As shown in Fig. 1A (arrow), a stream of *Pax3*-expressing cells can be seen emerging from the dorsal region of the embryo at E10.5 and migrating ventrally towards the developing heart. This expression pattern is missing in *Spotch* littermates (Fig. 1B) as previously reported (Conway et al., 1997). However, a detailed examination of such whole-mount in situ hybridized embryos revealed that this stream of cells does not represent neural crest cells destined to populate the heart. Rather, these are hypoglossal myogenic progenitors populating the hypoglossal cords that will form the muscles of the neck (Mackenzie et al., 1998) (Fig. 1C). A detailed analysis by whole-mount in situ hybridization of

embryos between E9.5 and E12.5 failed to detect *Pax3*-expressing cells within the heart, either in wild-type or *Spotch* embryos (Fig. 1 and data not shown).

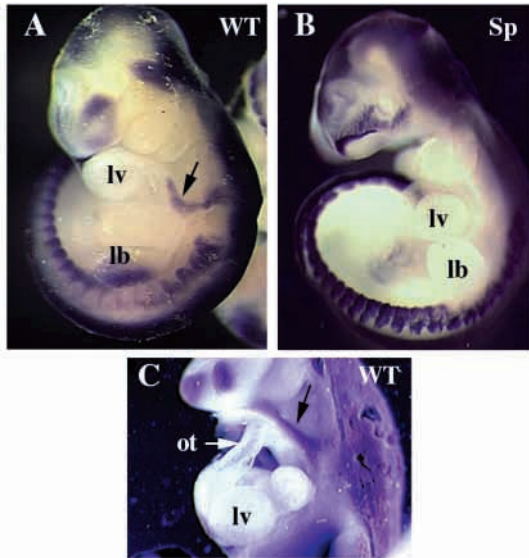
These findings were confirmed by radioactive in situ hybridization studies. Analysis of consecutive sections through the cardiac and aortic arch regions of E9.5 to E12.5 wild-type embryos failed to reveal any specific *Pax3* signal in the heart, outflow tract or great vessels (data not shown). Likewise, immunohistochemistry using a recently characterized *Pax3* antibody (Li et al., 1999) failed to detect *Pax3* protein expression in this region, though *Pax3*-positive cells in the neural tube, dorsal root and sympathetic ganglia and the limb buds were present (Fig. 3A and data not shown). Hence, we conclude that *Pax3* is not expressed at detectable levels in the embryonic heart at these time points and that *Pax3* expression significantly or completely abates prior to cardiac neural crest cell arrival in the outflow tract of the heart. These results raise the question of whether *Pax3* expression is indeed associated with the cardiac neural crest cell progenitors. They also indicate the need for an alternate strategy for detecting cardiac neural crest cells in *Spotch* and wild-type embryos. Therefore, we have taken advantage of a Cre-lox approach in combination with the *Pax3* promoter/enhancer to determine if *Pax3* is expressed in cardiac neural crest progenitors.

### *Pax3*-expressing progenitors are fated to populate the cardiac outflow tract

We have created transgenic mice expressing Cre recombinase driven by the proximal *Pax3* regulatory elements (Li et al., 2000). These regulatory elements when used to direct *Pax3* expression, are sufficient to rescue cardiac development in *Spotch* embryos (Li et al., 1999). Hence they will likely target gene expression in the precise cell types in which *Pax3* is required for cardiac development, presumably the cardiac neural crest progenitors. Examination of Cre recombinase expression in the *Pax3*-Cre transgenic mice by in situ hybridization confirmed a pattern of expression which recapitulated a subset of the endogenous *Pax3* expression domains that included prominent expression in the dorsal neural tube (data not shown). This pattern of expression is identical to that previously observed for this regulatory element directing *lacZ* expression (Natoli et al., 1997; Li et al., 1999).

When these Cre-expressing transgenic mice were crossed with a floxed-*lacZ* reporter mouse line (Soriano, 1999), *lacZ* expression was induced in neural crest cells that are derived from progenitors that normally express *Pax3* (Fig. 2). For these studies, embryos at various stages of development were harvested to examine the distribution of  $\beta$ -gal-expressing cells. As the floxed *lacZ* locus once activated, will continue to provide  $\beta$ -gal expression even after Cre expression has ceased, the pattern of  $\beta$ -gal expression is in fact an accurate fate map of *Pax3*-expressing neural crest cells.

At E10.5,  $\beta$ -gal-expressing cells were seen invading the cardiac outflow tract, forming two parallel columns lining the truncus arteriosus (see arrow in Fig. 2A). This distribution is consistent with that previously described for cardiac crest cells (Kirby et al., 1983; Waldo et al., 1999). These results confirm that *Pax3*-expressing cells are fated to populate the outflow tract, even though *Pax3* expression itself has abated by the time crest cells have migrated into the heart. By E12.5, the truncus arteriosus has septated into two vessels, the aorta and



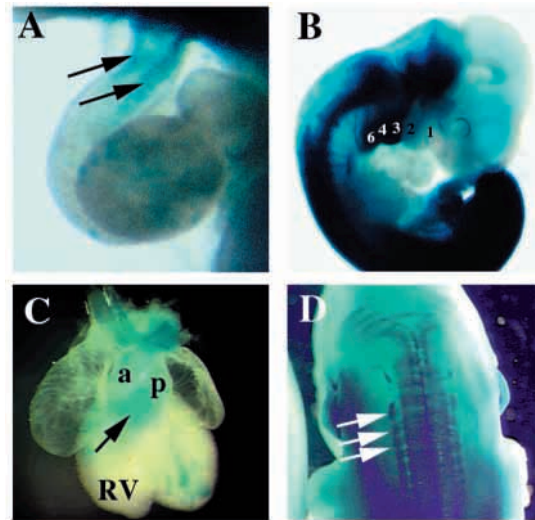
**Fig. 1.** *Pax3* is expressed by migrating hypoglossal muscle precursors but not in the heart. (A) Whole-mount in situ hybridization of E10.5 wild-type (WT) embryo reveals *Pax3* expression in dorsal neural tube, somites, limb bud (lb) and in migrating myoblasts (arrow). (B) In *Sp*<sup>-/-</sup> embryos (Sp) the non-functional *Pax3* transcript is detected in dorsal neural tube and somites, but not in limb bud (lb) or in migrating hypoglossal myoblasts. (C) Close-up view of wild-type embryo, similar to that shown in A, oriented to display the hypoglossal chord (arrow) which is expressing *Pax3*. Note the absence of *Pax3* expression in the outflow tract of the heart (ot). lv, left ventricle.

pulmonary arteries. Labeled cells derived from *Pax3*-expressing precursors are seen within the aorticopulmonary septum (arrow, Fig. 2C) and within the walls of the great vessels. In addition, labeled cells populate the dorsal root ganglia (arrows, Fig. 2D) which are also neural crest derived.

A striking difference was noted in the abundance of  $\beta$ -gal-expressing cells populating the third, fourth and sixth pharyngeal arches as compared to the first two pharyngeal arches which contained very few labeled cells (Fig. 2B). Note that *Pax3* itself is not expressed at significant levels by cells of the pharyngeal arches at this time (Fig. 1A). While we cannot rule out that our Cre-expressing transgene is missing regulatory elements required for expression by *Pax3*-positive precursors of the first two arches, these results suggest that *Pax3*-expressing neural crest cells specifically populate arches 3, 4 and 6. This finding is of significance given that only crest cells associated with these three caudal arches participate in cardiac development (Phillips et al., 1987).

#### **A *Cx43-lacZ* transgene and the labeling of neural crest cells in *Spotch* mutants**

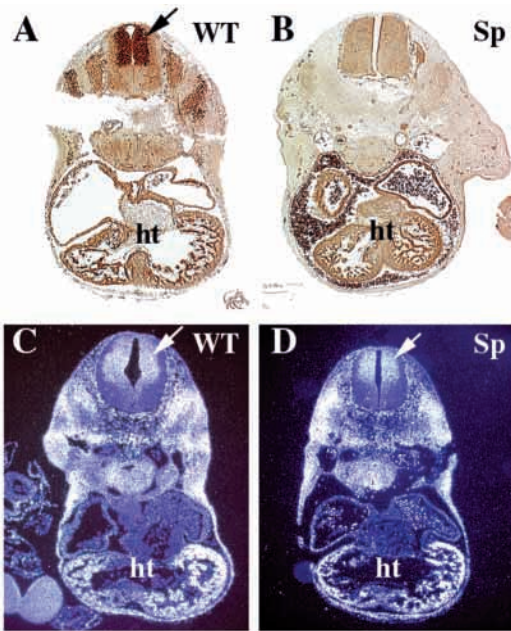
*Pax3*pro-Cre transgenic mice were useful for identifying cardiac neural crest cells in wild-type embryos, as demonstrated above. However, the usefulness of this system for tracking cardiac neural crest in *Spotch* embryos is unclear since any potential alterations of  $\beta$ -galactosidase expression could be attributed to altered *Pax3* promoter activity in the absence of *Pax3*. Therefore, to determine if cardiac neural crest cells in *Spotch* embryos migrate to the cardiac outflow tract,



**Fig. 2.** *Pax3*-expressing neural crest cells are fated to populate the cardiac outflow tract, pharyngeal arches 3, 4 and 6, and the dorsal root ganglia. (A) E10.5 embryo resulting from a cross of a transgenic P3pro-Cre mouse with a Cre-reporter mouse (Soriano, 1999) and stained for  $\beta$ -galactosidase expression reveals labeled cells invading the outflow tract of the heart (arrows). (B) E10.5 embryo, similar to that shown in A, reveals labeled cells in the neural tube and populating pharyngeal arches 3, 4 and 6. Considerably fewer labeled cells were seen in arches 1 and 2. Pharyngeal arches are numbered. (C) Explanted heart from E12.5 P3pro-Cre X Cre-reporter embryo reveals labeled cells in the aorticopulmonary septum (arrow) and in the walls of the aorta (a) and pulmonary artery (p). RV, right ventricle. (D) Dorsal view of E12.5 embryo reveals labeled cells in neural tube and in dorsal root ganglia (arrows).

we used a transgene composed of a *connexin 43* (*Cx43*) promoter driving *lacZ* (Lo et al., 1997). This transgene provides  $\beta$ -gal expression in mouse neural crest cells, including the cardiac crest cells (Lo et al., 1997; Waldo et al., 1999). It contains 6.5 kb of the proximal upstream genomic regulatory regions of *Cx43*. Although the specific regulatory regions responsible for tissue specific expression have not been identified, the pattern of  $\beta$ -galactosidase expression in the developing heart displayed by *Cx43-lacZ* embryos (Waldo et al., 1999) is strikingly reminiscent of the pattern of  $\beta$ -galactosidase expression that represents *Pax3* fate mapped cardiac neural crest cells (compare Fig. 2A with 4A, 2C with 6A). Therefore, we chose to use this transgene to track neural crest cells in *Spotch* embryos. However, first we had to determine whether absence of *Pax3* activity in *Spotch* embryos might perturb *Cx43* promoter activity. This was examined by analyzing *Cx43* expression by in situ hybridization of E10.5 wild-type and *Spotch* embryos. As shown in Fig. 3A and C, *Pax3* and *Cx43* display similar expression patterns in wild-type embryos. Both are expressed in the dorsal neural tube (arrows) and in neural crest derivatives including dorsal root and sympathetic ganglia. In *Spotch* embryos, *Cx43* expression is grossly unchanged (Fig. 3D) while *Pax3* protein is absent (Fig. 3B), demonstrating that *Pax3* is not required for tissue-specific *Cx43* expression. These results suggested that the *Cx43-lacZ* transgene can be used as a marker to track neural crest cells in *Spotch* embryos.

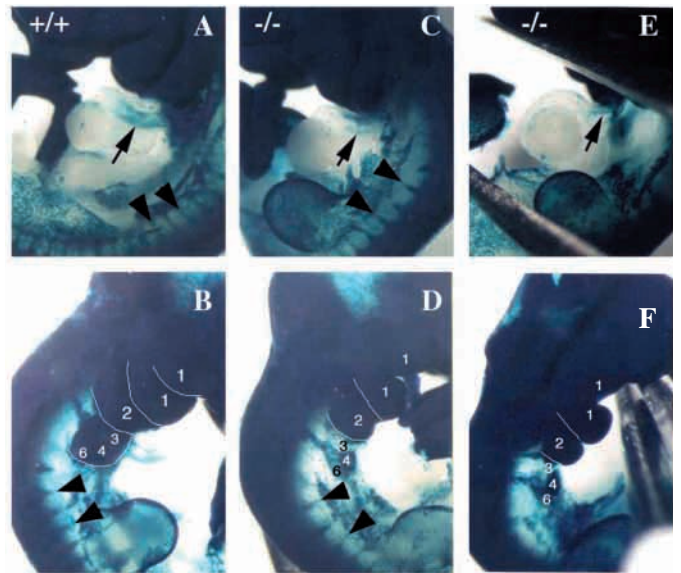




**Fig. 3.** *Cx43* expression is not grossly affected by the absence of Pax3. (A,B) Immunohistochemistry to detect Pax3 protein (dark brown) in wild-type (WT, A) and *Sp*<sup>-/-</sup> (Sp, B) E10.5 embryos reveals Pax3 protein in the dorsal neural tube of wild-type embryos (arrow, A). No Pax3 protein is detected in *Sp*<sup>-/-</sup> embryos. (False positive signal in the pericardial space of the embryos shown in B is due to red blood cell artifact). (C,D) In situ hybridization to detect *Cx43* expression in wild-type (WT, C) and *Sp*<sup>-/-</sup> (Sp, D) embryos indicates *Cx43* expression in the dorsal neural tube (arrows) and the heart (ht) that is not grossly affected by the presence (C) or absence (D) of Pax3.

### Abnormal dorsal root ganglia and pharyngeal arches in *Spotch* embryos

We crossed *Cx43-lacZ* transgenic *Spotch* mice with *Spotch* heterozygotes and litters were examined at E10.5. In wild-type embryos, *Cx43-lacZ*-labeled neural crest cells emerge from the neural tube along the entire rostral-caudal axis of the embryo at evenly spaced intervals (Fig. 4B, arrowheads). In *Spotch* embryos, labeled cells are also seen emerging from the neural tube though the intensity of staining is reduced (Fig. 4D, arrowheads). By E12.5, these  $\beta$ -gal-expressing cells are observed in all of the dorsal root ganglia in wild-type embryos (Fig. 5A), while only a few irregularly spaced and small clusters of labeled cells were evident in *Spotch* embryos (Fig. 5B). We confirmed that the labeled cells were neuronal in nature by performing immunohistochemistry using a neural-specific neurofilament monoclonal antibody (not shown). In *Spotch* embryos, neurofilament staining confirmed a paucity of ganglionic structures and those that were detected were small. The *Cx43-lacZ* transgene also labeled cranial ganglia in wild-type embryos (Fig. 5C). In addition to abnormalities previously reported for cranial ganglia in *Spotch* (Tremblay et al., 1995), we noted a marked reduction in the facial ganglia and nerves (compare arrows, Fig. 5C,D). The *Cx43-lacZ* transgene also labeled all pharyngeal arches in wild-type embryos (Fig. 4B, arches are numbered). We found a significant decrease in the size of pharyngeal arches 3, 4 and



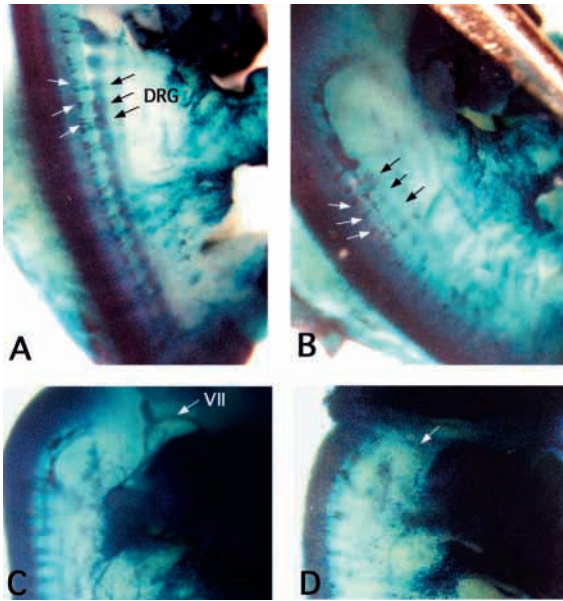
**Fig. 4.** Detection of neural crest cells in the cardiac outflow tract and pharyngeal arches using a *Cx43-lacZ* transgene. Wild-type (+/+, A,B) and *Sp*<sup>-/-</sup> (-/-, C-F) E10.5 embryos carrying the *Cx43-lacZ* transgene were stained to detect  $\beta$ -galactosidase expression. In all cases, labeled cells were seen invading the cardiac outflow tract (arrows, A,C,E) though the extent of migration and the intensity of signal was variable in *Sp*<sup>-/-</sup> embryos (compare C to E). Cells expressing the *Cx43-lacZ* transgene were also detected in all pharyngeal arches (numbered 1-4 and 6 in B,D,F). Pharyngeal arches 3, 4 and 6 were small and malformed in *Sp*<sup>-/-</sup> embryos (compare D and F to B). Note that regularly spaced streams of labeled cells emerge from both wild-type and mutant neural tubes (arrowheads, A-D).

6 in *Spotch* embryos (Fig. 4D,F), consistent with the observation that *Pax3*-expressing neural crest cells were specifically fated to populate these arches as demonstrated in Fig. 2B. Significantly, these caudal arches contain crest cells that are destined to migrate to the heart.

### Cardiac neural crest cells populate the outflow tract of *Spotch* embryos

Examination of E10.5 embryos revealed  $\beta$ -gal-positive crest cells in the outflow tract of both the wild-type and *Spotch* mutant embryos. Fig. 4A shows two  $\beta$ -gal-positive neural crest cell streams migrating into the cardiac outflow tract of wild-type E10.5 embryos (arrow). This same pattern was also observed in the outflow tract of *Spotch* embryos (Fig. 4C,E), but generally the labeled crest cells show less distal progression into the outflow tract as compared to the crest cell streams found in wild-type embryos of the same developmental stage (compare Fig. 4C,E with Fig. 4A). Nevertheless, it is of significance to note that in all cases, some labeled cells were identified in the cardiac outflow tract of the *Spotch* mutant embryos.

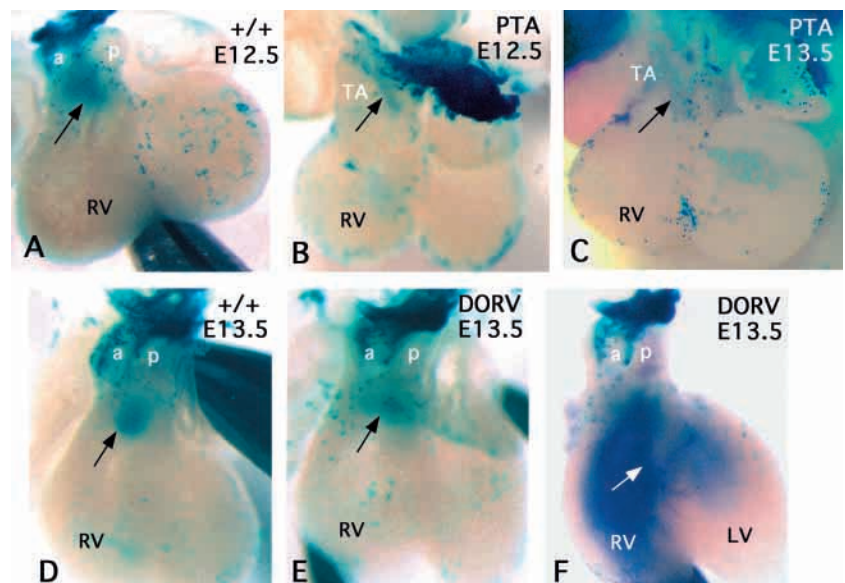
Further examination of wild-type and *Spotch* *Cx43-lacZ* hearts at E12.5 and E13.5 confirmed that neural crest cells are capable of long range migration in the absence of Pax3. Between E12.5 and E13.5, the outflow tract of the heart completes septation to give rise to two distinct vessels, the aorta and the pulmonary artery (Fig. 6A,D). At these stages of development, an abundance of  $\beta$ -gal-positive cells are found proximally at the base of the outflow tract (arrows, Fig. 6A,D).



**Fig. 5.** Neural crest cells emerge from the neural tube of *Sploch* embryos but fail to form normal dorsal root ganglia. (A–D) E12.5 *Cx43-lacZ* transgenic embryos are stained to detect  $\beta$ -galactosidase expression. Labeled cells are seen after emerging from the neural tube and labeling the dorsal roots of wild-type (A) and *Sp*<sup>-/-</sup> (B) embryos (white arrows). Well formed dorsal root ganglia (DRG) are labeled in wild-type embryos (black arrows, A). Disorganized and small dorsal root ganglia are labeled in *Sp*<sup>-/-</sup> embryos (arrows, B). Cranial ganglia, including the facial nerves (VII, C), are labeled in wild-type embryos (C) but are severely deficient in *Sploch* embryos (white arrow in D).

Histologic analyses have previously shown that these correspond to  $\beta$ -gal-positive crest cells situated in the outflow septum (Waldo et al., 1999). In *Sploch* mutant embryos exhibiting absent or abnormal septation of the outflow tract,  $\beta$ -gal-positive cells can still be seen in the heart (Fig. 6B,C,E), albeit with reduced abundance in mutants with the most severe cardiac abnormalities (see below).

**Fig. 6.** Cardiac neural crest cells populate the outflow tract of wild-type and *Sp*<sup>-/-</sup> hearts. (A–E) *Cx43-lacZ* embryos were stained to detect  $\beta$ -galactosidase expression and the hearts were excised. Labeled cells were seen populating the aorticopulmonary septum of E12.5 wild-type hearts (arrow, A) and labeled cells were also seen in the outflow tract of E12.5 *Sp*<sup>-/-</sup> hearts with PTA (arrow, C). The orientation of the two prongs of labeled cells invading the outflow tract is abnormal in the *Sp*<sup>-/-</sup> hearts. Labeled cells were also seen in the region of the A–P septum of E13.5 wild-type hearts (arrow, D) and *Sp*<sup>-/-</sup> hearts with persistent truncus arteriosus (PTA, arrow, B) or with double outlet right ventricle (DORV, arrow, E). (E) The heart shown in E had a ventricular septal defect associated with DORV that was apparent after injecting the right ventricle (RV) with blue dye and observing it cross the interventricular septum (arrow, F). a, aorta; p, pulmonary artery; TA, truncus arteriosus; LV, left ventricle.

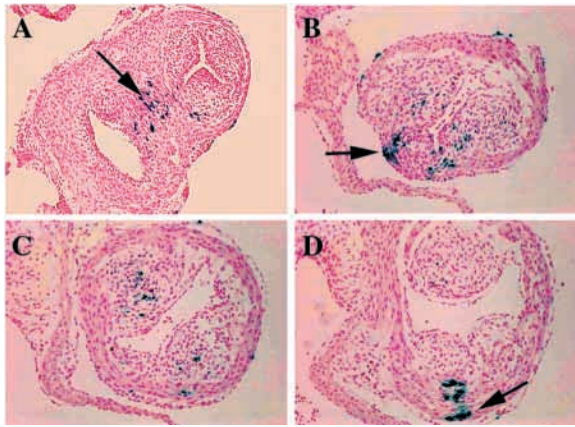


In *Sploch* mutants exhibiting the absence of outflow septation, that is with persistent truncus arteriosus (PTA),  $\beta$ -gal-positive cells are significantly reduced in abundance in the outflow tract (Fig. 6B,C; compare to Fig. 6A,D). In contrast, in *Sploch* mutant embryos in which partial septation has taken place, i.e. in embryos exhibiting a double outlet right ventricle (DORV),  $\beta$ -gal-positive cells are found at levels approaching that of wild-type embryos, but with a distinctly different distribution (Fig. 6E, compare to 6D). This may reflect the abnormal positioning of the outflow septum in these *Sploch* mutants (Fig. 6E) which is associated with a failure of rotation that normally juxtaposes the aorta with the left ventricle. Injection of dye into such DORV hearts (E13.5) indicates the presence of a ventricular septal defect, an abnormality frequently seen in conjunction with cardiac defects arising from cardiac neural crest perturbation (Fig. 6F).

Wild-type and *Sploch Cx43-lacZ* transgenic hearts were sectioned after staining for  $\beta$ -galactosidase activity to determine the precise distribution of neural crest cells within the outflow tract. Fig. 7A (arrow) shows the normal location of neural crest cells clustered between the aorta and pulmonary artery (see also Waldo et al., 1999). In Fig. 7B–D, serial sections through the outflow tract of a *Sploch* transgenic embryo with PTA reveal an atypical pattern of neural crest cell distribution. Some  $\beta$ -galactosidase positive cells were located within the myocardial layer instead of within the mesenchymal regions of the endocardial cushions (arrows, Fig. 7B,D). These results suggest disturbances in the targeting of neural crest cells in the outflow tract of *Sploch* mutants exhibiting PTA.

The *Sploch* heart with PTA shown in Fig. 6C was sectioned and examined histologically confirming a complete absence of outflow tract septation. Shown in Fig. 8A–D are sections of  $\beta$ -gal-positive cells seen in the outflow endocardial ridges of this mutant *Sploch* heart (arrows, Fig. 8B). Neural crest origin of these *lacZ*-positive cells was indicated by immunohistochemical analysis of smooth muscle actin (SMA) expression, a marker found in cardiac neural crest cells but not in mesenchymal cells of the endocardial cushions (Waller III, R., A. Phelps, R. R. Markwald, C. W. L., R. P. Thompson and A. W., unpublished data). *LacZ*-positive cells expressed SMA



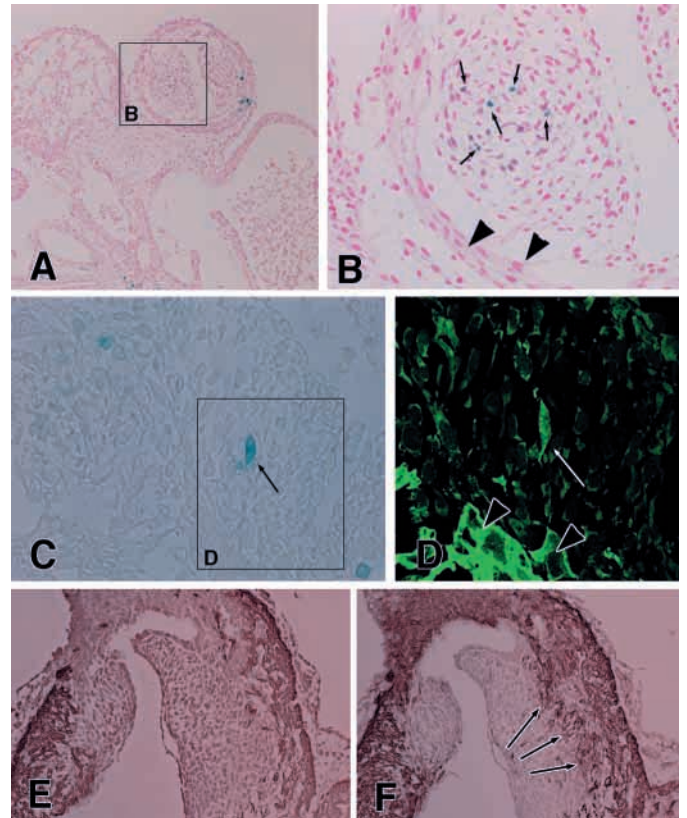


**Fig. 7.** *Cx43-lacZ*-labeled cells are ectopically located in some *Sp<sup>-/-</sup>* embryos with PTA. Transverse sections through wild-type (A) and *Sp<sup>-/-</sup>* (B-D) transgenic hearts reveals labeled neural crest cells clustered between the aorta and pulmonary artery of wild-type embryos (arrow, A). Serial sections through the persistent truncus arteriosus of a severely affected *Spotch* embryo reveals labeled cells within the mesenchymal portions of the endocardial cushions, but also in unusual locations near the periphery of the great vessel (arrows, B,D).

(arrows, Fig. 8C,D). We also examined the expression of atrial myosin light chain (aMLC, Fig. 8E) compared to SMA (Fig. 8F) in adjacent sections of *Spotch* hearts. aMLC is expressed by myocardial cells while SMA is expressed by myocardial cells and neural crest cells. Examination of serial sections through a *Spotch* mutant heart showed some SMA positive cells populating the compact mesenchyme of the endocardial cushions (arrows, Fig. 8F). Significantly, these SMA-positive cells do not express aMLC (Fig. 8E). These results also confirm the presence of neural crest derivatives in the outflow tract of *Spotch* mutant embryos.

#### Analysis of the migratory behavior of Pax3-deficient neural crest cells in explant cultures

Since neural crest cells from *Spotch* embryos appeared to emerge from the neural tube at approximately the correct stage of embryonic development, we sought to analyze the migratory behavior of cardiac neural crest cells using an explant culture system. For these studies, E8.5 neural tube segments caudal to the mid-otic placode and rostral to somite 3 were dissected from wild type, *Sp<sup>+/-</sup>* and *Sp<sup>-/-</sup>* littermates and cultured for 24 or 48 hours to monitor neural crest outgrowth. By measuring the area of the outgrowth, we obtained an estimate of the overall rate of neural crest migration, referred to as the migration index (MI in Table 1, see methods for details). This analysis showed no deficiency in the ability of neural crest cells to emerge and migrate from the *Sp<sup>-/-</sup>* neural tube explants. In fact, we observed a marked elevation in the apparent rate of neural crest migration in *Sp<sup>-/-</sup>* embryos as compared to that of *Sp<sup>+/-</sup>* or wild-type embryos (Table 1). This is indicated by a significant increase in the migration index of the *Sp<sup>-/-</sup>* neural crest outgrowths (Table 1). This was observed both at 24 hours and 48 hours of culture. Analysis of BrdU incorporation demonstrates this increase in the migration index is not likely accounted for by changes in the rate of cell proliferation (Table 1). To further examine whether the rate of neural crest cell



**Fig. 8.** *Cx43-lacZ*-labeled cells in *Sp<sup>-/-</sup>* outflow tracts express smooth muscle actin (SMA) but not  $\alpha$ -myosin light chain ( $\alpha$ MLC) consistent with neural crest cell identity. (A,B) Nuclear fast red counterstained section of a *Cx43-lacZ* transgenic *Sp<sup>-/-</sup>* embryo that had severe persistent truncus arteriosus reveals  $\beta$ -galactosidase-expressing cells in the endocardial cushions of the outflow tract at E13.5. The boxed area in A is shown at higher magnification in B and labeled cells are indicated with arrows. (C,D) Immunohistochemistry to detect SMA indicates that  $\beta$ -galactosidase-expressing cells (arrow, C) also express elevated levels of SMA (white arrow, D). The same cell is indicated by the arrows in C (brightfield) and D (fluorescence). SMA is expressed by myocardial cells and by neural crest cells, and arrowheads in D indicate SMA positive myocardium. (E,F) Adjacent sections from a *Sp<sup>-/-</sup>* heart stained for  $\alpha$ MLC (E) to indicate myocardial cells and SMA (F) to detect neural crest cells and myocardial cells. The arrows in F indicate cells within the compact mesenchymal layer of the outflow endocardial cushion that express SMA but not  $\alpha$ MLC, suggesting that neural crest cells are present in the mutant hearts.

**Table 1. Analysis of postotic neural crest cell migration**

<i>Spotch</i> genotype	Migration index		
	24 hours	48 hours	BrdU Incorp (%) ¶
+/+	0.415±0.036 (15)*	1.788±0.093 (15)	23.6±8.7% (8)
+/-	0.477±0.032 (39)	1.804±0.069 (30)	31.2±5.4% (14)
-/-	0.733±0.117 (3)‡	2.457±0.241 (3)§	18.7±14.2% (4)

\*Numbers in parenthesis = number of samples analyzed.

‡*P* values 0.0087 when compared to +/+ and 0.0247 compared to +/- embryos.

§*P* values 0.0072 when compared to +/+ and 0.0062 compared to +/- embryos.

¶No significant differences in rate of BrdU incorporation.

emergence may be altered in the *Sp*<sup>-/-</sup> embryos, we carried out time lapse videomicroscopy with images of the outgrowth cultures captured every 10 minutes over a 20-hour interval. Such analysis showed no difference in the rate of crest cell emergence from *Sp*<sup>-/-</sup> explants as compared to that of heterozygous or wild-type explants. Overall, these results are consistent with the hypothesis that Pax3 is not required for the emergence or migration of cardiac neural crest cells. They further suggest that Pax3 is nevertheless important for crest cell migration, possibly by fine tuning the migratory behavior of the cardiac neural crest cells.

## DISCUSSION

These studies provide a series of observations that alter our understanding of Pax3 function in cardiac neural crest development. Pax3 is expressed by neural crest progenitors in the dorsal neural tube and by other neural crest derivatives such as the dorsal root ganglia and various cranial ganglia (Goulding et al., 1991; Tremblay et al., 1995). However, our studies indicated that Pax3 is down-regulated in many neural crest lineages. Of particular significance for cardiac morphogenesis is the fact that in wild-type embryos, Pax3 expression was not detected in large regions of the pharyngeal arches or in the outflow tract of the heart. Instead, we observed the persistent expression of Pax3 by migrating hypoglossal myoblasts that previously were misinterpreted as Pax3-expressing neural crest cells (Conway et al., 1997). This unexpected finding compelled us to reexamine the role of Pax3 in the cardiac malformations of *Spotch* mutants, and its involvement in the modulation of neural crest cell migration.

### Pax3 expression and the perturbation of neural crest cells

Utilizing a binary Cre-lox system to activate  $\beta$ -galactosidase expression in Pax3-expressing cells and their descendants, we fate mapped Pax3-expressing cells without the need for tissue transplantation or other experimental manipulations. Such studies showed that Pax3-expressing neural crest cells are fated to populate pharyngeal arches 3, 4 and 6, the three caudal arches known to contribute crest cells involved in outflow tract septation (Phillips et al., 1987). Importantly, we also observed that the Pax3-expressing crest cells were fated to invade the cardiac outflow tract. These studies relied on the use of the proximal Pax3 promoter that we have previously characterized (Li et al., 1999) to direct expression of Cre recombinase. While this promoter was sufficient to direct Pax3 expression in *Spotch* embryos resulting in correction of gross congenital cardiac defects, it remains possible that regulatory elements not included in this transgene are important for directing expression in cranial pharyngeal arches or in cells that contribute to later cardiac development or function.

We also examined the distribution of neural crest cells using the *Cx43-lacZ* transgene marker. This *lacZ* marker was chosen for our studies as it is well characterized with regard to the fidelity with which it labels cardiac neural crest cells (Waldo et al., 1999). Moreover, our in situ hybridization studies showed that *Cx43* promoter activity is not altered by the loss of Pax3 function, and thus validated the use of this transgene marker in the *Spotch* mutants. These studies showed that

cardiac neural crest cells do emerge and migrate to the heart outflow tract in *Spotch* mutant embryos. In addition, we observed a reduction in the size of the three caudal arches, and in the abundance of  $\beta$ -gal-expressing crest cells in the outflow tract. Similar studies are ongoing using our Cre-lox system to examine the distribution of crest cells in *Spotch* mutants. As this involves a three way cross, a large number of embryos in several generations would have to be screened in order to obtain the desired  $\beta$ -gal-expressing *Spotch* mutant embryos. Furthermore, the ability of Pax3 to regulate its own promoter will have to be evaluated in order to interpret the results that emerge.

Studies with the *Cx43-lacZ* marker also showed that trunk neural crest cells in *Spotch* embryos emerge and participate in the formation of the DRG, albeit the DRG are greatly reduced in numbers and size, and appear to be malpositioned and abnormally organized. We also observed crest cells participating in the formation of the cranial ganglia in *Spotch* mutants. Notable was the absence of the facial nerve in *Spotch* embryos. Although this finding has not been previously reported, our results are generally consistent with previous observations obtained by others using three different *lacZ* transgenic markers in *Spotch* embryos (Kothary, 1991; Serbedzija and McMahon, 1997; Tremblay et al., 1995). Variations among the results of these studies may in part reflect strain background differences, as has been described for the DRG deficiency seen in the *Sp* vs. *Sp<sup>d</sup>* mutants (Tremblay et al., 1995).

### Pax3 and cell migration

The cardiac outflow tract defects and other neural crest related abnormalities in *Spotch* mutants are strikingly similar to defects seen in chick embryos after ablation of premigratory neural crest cells, and a neural crest migration defect secondary to Pax3 deficiency has been postulated (Conway et al., 1997; Franz, 1989). By examining the expression of the *Cx43-lacZ* transgene in wild-type and *Spotch* embryos, we demonstrated that Pax3 is not required for cardiac neural crest migration to the heart. However, the distribution of neural crest cells within the cardiac outflow tract was not normal in *Spotch*. In normal embryos, two parallel columns of neural crest cells invade the truncus arteriosus and form a spiral structure that progresses in a rostral to caudal direction. In *Spotch*, these two columns of cells are also present, but they are abnormally positioned, and in some embryos, the intensity of  $\beta$ -gal staining is also reduced, suggesting that there is a marked reduction in the abundance of cardiac crest cells. Interestingly, the degree to which the intensity of staining was reduced appeared to be correlated with the severity of the cardiac defect, such that embryos with PTA exhibited fewer cardiac neural crest cells in the outflow tract region than those exhibiting DORV. Whether these differences in crest cell abundance are causally related to the cardiac defect remains to be determined. In this regard, our findings are generally consistent with those of Conway et al. (1997) who used a series of markers in addition to Pax3 itself to demonstrate a reduction in neural crest cells within the outflow tract in severely affected *Spotch* embryos. Some differences between those results and ours may also be related to the use of different *Spotch* alleles.

A role for Pax3 in regulation of neural crest migration has been supported by analogy to the role of Pax3 in migratory



muscle precursors. In limb muscle progenitors, compelling evidence indicates that Pax3 can regulate migratory processes. Pax3 is required to mediate migration of myoblasts to the developing limb bud (Bladt et al., 1995; Bober et al., 1994; Daston et al., 1996; Epstein et al., 1996; Goulding et al., 1994). This process involves the direct activation of the *c-met* gene by Pax3, a process that is required for myoblasts to migrate in response to the secreted ligand for c-Met, scatter factor, which is expressed by limb mesenchyme (Brand-Saber et al., 1996). In the absence of Pax3 (or c-Met), limb myoblasts fail to delaminate from the ventral-lateral lip of the somite and there is a complete absence of myoblasts invading the limb bud (Bladt et al., 1995; Daston et al., 1996; Epstein et al., 1996; Franz et al., 1993).

An equivalent role for Pax3 in the regulation of neural crest migration has not been reported. Our analysis of cardiac crest cell migration in explant cultures showed no evidence of a defect or delay in the emergence of neural crest cells. This result contrasts with a previous study showing delayed emergence of trunk neural crest cells from neural tube explants derived from *Spotch* embryos (Moase and Trasler, 1990). This discrepancy may reflect the fact that the previous study involved the analysis of trunk neural crest cells rather than vagal or postotic crest cells that give rise to the cardiac neural crest. However, another *in vivo* study using a *lacZ* reporter transgene controlled by a *wnt1* promoter element reported normal emergence of trunk neural crest cells in *Spotch* embryos (Serbedzija and McMahon, 1997). Surprisingly, these authors also reported absence of labeled neural crest cells in the caudal trunk region. This result conflicts with our findings and that of others using different *lacZ* reporter constructs (Kothary, 1991; Tremblay et al., 1995). The failure to detect *wnt1-lacZ*-expressing crest cells in the caudal trunk could reflect the down regulation of *wnt1* promoter activity in *Spotch* embryos. Interestingly, in this same study, labeled neural crest cells derived from the caudal trunk level of *Spotch* embryo neural tube fragments were observed to migrate after transplantation into chick embryos. This was interpreted to indicate that the migration defect in *Spotch* embryos is non-cell autonomous, perhaps secondary to the absence of Pax3 in non-neural crest tissues (such as the somite) that indirectly affected neural crest migration. However, this interpretation is probably incorrect, as we recently showed that reconstitution of Pax3 expression only in neural crest populations is sufficient to rescue cardiac septation and dorsal root ganglia formation in *Spotch* embryos (Li et al., 1999). An alternative interpretation of the chimera studies is that in the presence of wild-type Pax3-expressing chick cells, *wnt-1* promoter activity may be up regulated via a non-cell autonomous mechanism. The resulting up regulation of  $\beta$ -gal expression would then give rise to the appearance of neural crest cell "rescue" in these embryos.

Our studies also indicate that there is a significant elevation in the apparent rate of crest migration in the absence of Pax3. This is based on the quantitative analysis of the outgrowth area in the neural crest outgrowth cultures. This result contrasts with the finding of fewer crest cells in the outflow tract. These apparently conflicting results suggest that the targeting of neural crest cells to the heart is compromised. For example, the loss of Pax3 function may perturb the directionality or persistence of cardiac crest cell movement, or alter the

chemotropic response of crest cells to migratory cues. To address these questions, we are currently examining neural crest migration by time lapse videomicroscopy, and analyzing their migratory response to various growth factors known to be expressed along the cardiac crest migratory route.

### Pax3 and cell survival

It is also possible that Pax3 may have other roles in neural crest cells unrelated to migration, including modulation of cell survival. In limb myoblasts, we have noted increased apoptosis in the absence of Pax3 (Borycki et al., 1999). We also noted clusters of apoptotic cells in the regions usually occupied by DRGs, suggesting programmed cell death of Pax3-deficient neural crest cells. We were unable to detect apoptotic cardiac neural crest cells in the hearts of *Spotch* embryos, but the normal high levels of apoptosis in mid-gestation endocardial cushions and cardiac mesenchyme makes such studies technically difficult. Given the documented ability of Pax3 to suppress apoptosis in other systems (Bernasconi et al., 1996; Phelan et al., 1997), it is possible that changes in the level of apoptosis may contribute to the altered distribution of neural crest cells in the hearts of *Spotch* embryos.

In summary, our studies indicate that Pax3 is normally expressed by cardiac neural crest progenitors and is down-regulated prior to cardiac neural crest population of the outflow tract of the heart. Pax3-dependent neural crest cells are also fated to populate pharyngeal arches 3, 4 and 6, and in the absence of Pax3, these arches are hypoplastic. While neural crest defects including cardiac outflow tract disorders characterize Pax3-deficient *Spotch* embryos, Pax3 is not required for postotic neural crest cell migration in explant cultures or cardiac neural crest cell migration into the heart outflow tract *in vivo*. Taken together, our results suggest that Pax3 is not required for the emergence or migration of neural crest cells. Rather, the role of Pax3 is likely more subtle, perhaps involving the fine tuning of the migratory behavior of crest cells or in mediating other critical roles in neural crest function.

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