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Transgenic rescue of congenital heart disease and spina bifida in *Splotch* mice

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SUMMARY

Pax3-deficient *Splotch* mice display neural tube defects and an array of neural crest related abnormalities including defects in the cardiac outflow tract, dorsal root ganglia and pigmentation. *Pax3* is expressed in neural crest cells that emerge from the dorsal neural tube. *Pax3* is also expressed in the somites, through which neural crest cells migrate, where it is required for hypaxial muscle development. Homozygous mutant *Splotch* embryos die by embryonic day 14. We have utilized the proximal 1.6 kb *Pax3* promoter and upstream regulatory elements to engineer transgenic mice reproducing endogenous *Pax3* expression in neural tube and neural crest, but not the somite. Over expression of *Pax3* in these tissues reveals no discernible phenotype. Breeding of transgenic mice onto a *Splotch* background demonstrates that neural tube and neural crest expression of *Pax3* is sufficient to rescue neural tube closure, cardiac development and other neural crest related defects. Transgenic *Splotch* mice survive until birth at which time they succumb to respiratory failure secondary to absence of a muscular diaphragm. Limb muscles are also absent. These results indicate that regulatory elements sufficient for functional expression of *Pax3* required for cardiac development and neural tube closure are contained within the region 1.6 kb upstream of the *Pax3* transcriptional start site. In addition, the single *Pax3* isoform used for this transgene is sufficient to execute these developmental processes. Although the extracellular matrix and the environment of the somites through which neural crest migrates is known to influence neural crest behavior, our results indicate that Pax3-deficient somites are capable of supporting proper neural crest migration and function suggesting a cell autonomous role for Pax3 in neural crest.

Key words: Transgenic mice, Congenital heart disease, Persistent truncus arteriosus, Neural tube defects, Spina bifida, Epaxial muscle, Hypaxial muscle, Diaphragm, Neural crest

INTRODUCTION

Neural crest cells emerge from the dorsal neural tube and migrate throughout the developing embryo (Hall and Horstadius, 1988). One subset of neural crest cells follows a dorsolateral route and populates the skin providing pigmented melanocytes. Another subset migrates ventrally along precise pathways, contributing to the dorsal root and sympathetic ganglia and populating multiple organs and tissues including the heart. Neural crest cells pass through the anterior portion of each somite and environmental signals within the segmented paraxial mesoderm influence migratory behavior via mechanisms that include repulsive signals emanating from the posterior somites (Teillet et al., 1987; Tosney et al., 1994; Wang and Anderson, 1997). Defects in neural crest migration or function result in a spectrum of human disorders referred to as neurocristopathies that include piebaldism, DiGeorge syndrome (Driscoll, 1994), Hirchsprung disease and Waardenburg syndrome (Read and Newton, 1997). The latter, characterized by pigmentation defects, deafness, and sometimes limb muscle abnormalities, can be caused by dominant mutations in the *PAX3* gene (Tassabehji et al., 1993) which is expressed in neural crest, the neural tube and the somite. In the mouse, regulation of *Pax3* expression is complex, involving both positive and negative regulatory elements within a 25 kb region upstream of the transcriptional start site (Natoli et al., 1997).

Several mouse models for neural crest-related disorders exist, including the *Splotch* mouse (Auerbach, 1954) named for the white belly spot characterizing heterozygotes. *Splotch* mice have mutations in *Pax3* (Epstein et al., 1991, 1993) and homozygotes die by embryonic day 14 (E14). These embryos display congenital heart disorders involving the outflow tract of the heart (Epstein, 1996; Franz, 1989) identical to those seen in human patients with DiGeorge syndrome and in chick embryos after ablation of premigratory cardiac neural crest (Kirby et al., 1983). In each case, the cardiac defects are characterized by a partial or complete failure of outflow tract septation which is required for the single great vessel emerging from the embryonic heart (the truncus arteriosus) to divide into the pulmonary artery and the aorta. In the mouse, this process is normally complete by E13.5. $Sp^{-/-}$ embryos also display neural tube defects predominantly affecting the lumbosacral region and serve as a model for understanding molecular and

environmental contributions to spina bifida (Fleming and Copp, 1998; Moase and Trasler, 1992).

In addition to its role in neural tube and neural crest development, Pax3 also plays critical roles in myogenesis. Limb myoblasts fail to migrate to the limb buds of *Sp*$^{-/-}$ embryos (Bober et al., 1994; Franz et al., 1993), limb muscle defects are seen in patients with *PAX3* mutations and Waardenburg syndrome (Hoth et al., 1993), and translocations involving *PAX3* in humans result in tumors of muscle (Barr et al., 1993; Shapiro et al., 1993). In the embryo, *Pax3* is expressed in the unsegmented paraxial mesoderm and throughout early somites (Goulding et al., 1991). Later, it becomes restricted to the dermomyotome and then to the ventrolateral region of the dermomyotome from which hypaxial limb muscles arise. In the absence of Myf5, Pax3 is required for the formation of all trunk muscles, suggesting that *Pax3* acts genetically upstream of *myoD* (Tajbakhsh et al., 1997). Pax3 is required for survival of premigratory limb myoblasts (Borycki et al., 1999) and may directly activate the myogenic gene program under some conditions (Maroto et al., 1997).

Limb muscle progenitors from *Sp*$^{-/-}$ embryos are able to differentiate after transplantation, but are unable to migrate normally (Daston et al., 1996). In contrast, neural crest cells from *Sp*$^{-/-}$ embryos are able to migrate when grafted onto normal chick embryos and follow appropriate migratory pathways (Serbedzija and McMahon, 1997). This result has led to the suggestion that the neural crest defect in *Splotch* is related to an abnormal migratory environment due to the absence of *Pax3* expression in somites, and is not caused by a cell autonomous defect in neural crest.

In this study, we have addressed the cell autonomy of Pax3 function in neural crest by engineering transgenic mice lacking Pax3 in the somite while expressing Pax3 in neural crest and the dorsal neural tube. We show that replacement of Pax3 in these domains is sufficient to rescue cardiac outflow tract septation, dorsal root ganglia formation, neural tube closure and full gestation. Muscular defects are not rescued, indicating that separable upstream regulatory elements control *Pax3* expression in the somite and in neural crest, and providing the unambiguous definition in a newborn mouse of Pax3-dependent and -independent muscle groups.

MATERIALS AND METHODS

Production of transgenic mice

Splotch mice were obtained from Jackson Laboratories. P3proPax3 transgenic mice were produced on a B6SJLF1/J background. The 1.6 kb proximal *Pax3* upstream region (Natoli et al., 1997) was obtained by PCR amplification of genomic DNA using primers P3proF (ggccCAATTGCTCCTCCCCAAATGTGGG, a *Mun*I site is underlined) and P3proR (TATATCCAGGTGAAGGCGAGACG). The PCR fragment was restricted with *Mun*I and *Bst*EII and cloned into the *Eco*RI and *Bst*EII sites of pCMV-Pax3 (Epstein et al., 1995) replacing the CMV promoter. The resulting plasmid, P3proP3, contained the full length *Pax3* cDNA (GenBank Accession no. X59358) downstream of the *Pax3* promoter. P3proLacZ was derived by replacing *Pax3* with *lacZ* (derived from pCMV-β, Clonetech). An 818 bp sequence derived from a Toxoplasma gondii dihydrofolate reductase (DHFR) gene (GenBank accession no. L08489) was incorporated into the 3′ untranslated region of *Pax3* in P3proP3 by insertion into the *Sac*II site replacing the corresponding fragment of P3proPax3. The final plasmids, P3proPax3DHFR and P3proLacZ, were restricted with *Nar*I and *Sal*I to remove vector sequences, gel purified, and injected into fertilized eggs at the transgenic facility of the University of Pennsylvania. In the case of P3proPax3DHFR, resulting founders were genotyped by analysis of tail DNA using primers specific for the DHFR sequence. Positive founders were bred to wild-type B6SJLF1/J mice (Jackson Laboratories) and germline transmission confirmed.

In situ hybridization

^{35}S-labeled antisense and sense riboprobes were synthesized by in vitro run-off transcription of linearized plasmids, using SP6, T7 or T3 RNA polymerase and ^{35}S-UTP as described by Lutz et al. (1994). Slides were then dipped in Kodak NTB2 emulsion and exposed for 8-10 days at 4°C, developed and fixed in Kodak Dektol developer and fixer, counter stained in Hoechst 33258 (Sigma) for 2 minutes, washed in water, dried and mounted in Canada Balsam. Sense control probes gave no signal and the antisense DHFR probe gave no signal in non-transgenic embryos. Whole-mount in situ hybridization was performed as described by Borycki et al. (1999).

Histology and immunohistochemistry

Embryos and newborn mice were fixed in 4% paraformaldehyde in PBS for 24-48 hours. The skin was removed from newborn mice prior to fixation. After dehydration and embedding in paraffin wax, 8 μm sections were placed on glass slides. After dewaxing in xylenes and rehydration, sections were either stained with hematoxylin and eosin, or processed for immunohistochemistry. Bone and cartilage were stained in whole unfixed newborn pups using Alcian Red and Alcian Blue as described by Tremblay et al. (1998).

Polyclonal anti-Pax3 antibodies were prepared by immunization of rabbits (Cocalico Laboratories) with GST fusion proteins that included the carboxyl terminal 60 amino acids of Pax3, the carboxyl terminal 120 amino acids of Pax3, or the carboxyl penultimate 60 amino acids of Pax3. The protein used for each boost cycle was alternated such that each rabbit received each protein twice for a total of 6 injections over 5 months. The carboxy-terminal region of Pax3 was chosen as antigen since it is the least conserved region of the protein amongst Pax family members. Immunoglobulin was purified using protein A sepharose chromatography and diluted 1:3000 prior to use. MF20 anti-myosin antibody was obtained from the Developmental Studies Hybridoma Bank and diluted 1:100 prior to use. Antigen was unmasked using Vector Labs Unmasking Solution according to instructions. Detection of specific antibody binding was carried out using biotinylated horse anti-mouse or anti-rabbit secondary antibody (Vector Labs) at a dilution of 1:200, followed by developing with Vector Labs ABC reagent.

Corrosion casts

Newborn pups were killed and the heart exposed via a thoracic incision and rib removal. Batson no. 17 acrylic (Polysciences, Inc) colored either blue or red was injected into the right and left ventricles, respectively, and the entire embryonic vasculature was filled. After hardening, tissue was removed with Maceration Solution (Polysciences, Inc) at 50°C for 72 hours. Photographs were obtained digitally and processed with Photoshop software.

RESULTS

The proximal 1.6 kb *Pax3* regulatory element directs expression in neural tube and neural crest

In order to determine the specificity of the proximal *Pax3* promoter and upstream region, we produced transient transgenic mice in which the proximal 1.6 kb *Pax3* regulatory

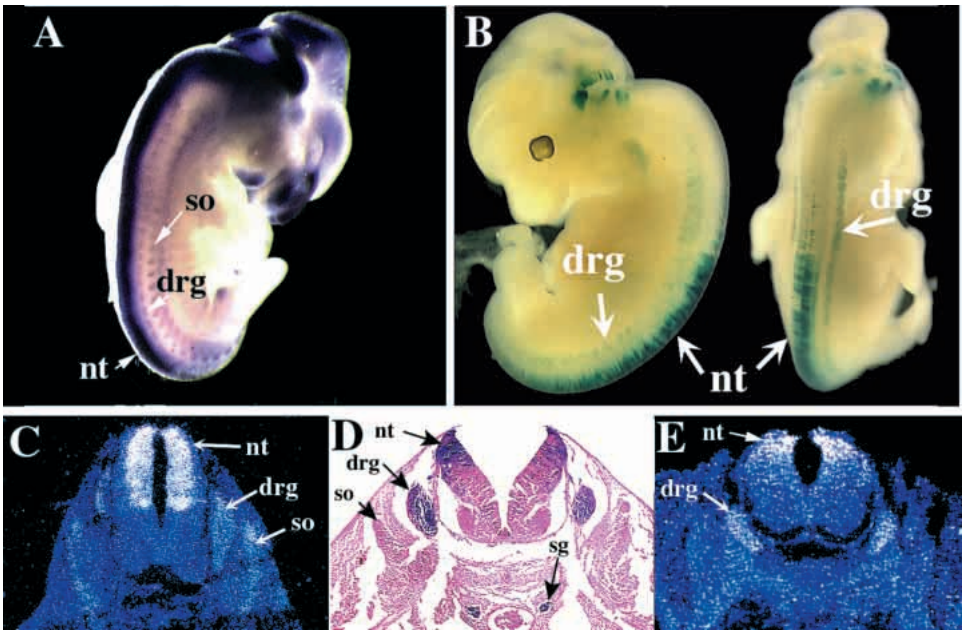

Fig. 1. The proximal 1.6 kb *Pax3* regulatory region directs expression in dorsal neural tube and neural crest derivatives. (A) Whole-mount in situ hybridization of a wild-type E11.5 mouse embryo with a *Pax3*-specific probe reveals expression in the neural tube (nt), the dorsal root ganglia (drg) and the somites (so). (B) Transient transgenic E12.5 embryo expressing *lacZ* from the 1.6 kb *Pax3* regulatory region shows expression in the neural tube and dorsal root ganglia. (C) Radioactive in situ hybridization of a wild-type E11.5 embryo transverse section shows normal *Pax3* expression in the dorsal neural tube, the dorsal root ganglia and the somites. (D) Transverse section of a transient transgenic embryo expressing *lacZ* from the 1.6 kb *Pax3* regulatory region (E11.5) shows transgene expression confined to the neural tube and dorsal root ganglia. Expression in the sympathetic ganglia (sg) is also evident. No expression in the somite is seen. (E) Radioactive in situ hybridization of a transverse section (E12.5) at the level of the heart and limb bud (lb) from a transgenic mouse derived from a stable line expressing *Pax3* from the 1.6 kb *Pax3* regulatory element. A DHFR probe specific for the transgenic transcript was used, and this probe produced no detectable signal in wild-type sections (not shown). In transgenic embryos, expression is seen in the dorsal neural tube (nt) and in the dorsal root ganglia (drg), but not in the somite.

region drove *lacZ* expression. *Pax3* is normally expressed in the dorsal neural tube, the dorsal root ganglia and the somite (Fig. 1A,C). In transgenic mice, we observed β-galactosidase expression in the dorsal neural tube and dorsal root ganglia (Fig. 1B), but not in the somites. Although a previous report suggested that expression lateral to the neural tube induced by the 1.6 kb *Pax3* regulatory region represented somitic expression (Natoli et al., 1997), we have confirmed that this represents expression in the dorsal root ganglia by sectioning embryos after staining for β-galactosidase (Fig. 1D). We never saw expression in the somites or in the limb buds, domains where endogenous *Pax3* is normally expressed (Goulding et al., 1991). Strong expression was seen in the dorsal neural tube, the dorsal root ganglia, and the sympathetic ganglia (Fig. 1D). Hence, the proximal 1.6 kb *Pax3* regulatory region is capable of reproducing the neural tube and neural crest subdomain of endogenous *Pax3* expression.

Production of transgenic mice over expressing *Pax3*

In order to examine the effect of over expression of *Pax3* in neural tube and neural crest, and to determine if neural crest expression of *Pax3* is capable of rescuing cardiac development in *Splotch* mice, we produced transgenic mice expressing *Pax3* from the 1.6 kb endogenous *Pax3* regulatory region by injection of the P3proPax3DHFR construct (see Materials and Methods). Twelve of 49 potential founders were found to harbor transgenic DNA and 10 of these 12 passed the transgene through the germline. E12.5 embryos from 9 of these 10 transgenic lines were examined by in situ hybridization for expression of the transgene. We used a probe specific for the transgenic transcript in order to differentiate transgenic from endogenous *Pax3*. The transgenic transcript included a 3′ untranslated sequence tag derived from the non-mammalian DHFR sequence. An antisense probe specific for the DHFR sequence did not hybridize to wild-type embryo sections (data not shown). Two lines showed no transgene expression and were not examined further. One line revealed expression in all tissues, probably due to the site of transgene insertion, and is not discussed further here. Specific transgene expression in the dorsal neural tube and the dorsal root ganglia similar to that seen for P3proLacZ mice was detected in 6 independent lines (for example, Fig. 1E). These lines were maintained for up to 18 months through 4-6 generations. Cardiac and musculoskeletal anatomy was examined in detail at E13.5, E17.5 and in newborn pups. No abnormalities were detected in any transgenic animals. Although over expression of *Pax* genes in cultured fibroblasts has been reported to result in transformation (Maulbecker and Gruss, 1993), we have not detected any tumors in *Pax3* over expressing transgenic mice.

Transgenic expression of *Pax3* rescues neural tube closure and cardiac outflow tract septation in *Splotch* embryos

Two of the transgenic lines over expressing *Pax3* in neural tube and neural crest were crossed to heterozygous *Splotch* mice. Heterozygous *Splotch* offspring carrying the transgene were identified by genotyping. In several cases we noted absence of the white belly spot characteristic of *Splotch* heterozygotes. White belly spots were always present in *Splotch* heterozygotes that did not inherit the transgene. This suggests that transgenic *Pax3* could rescue the melanocyte defect seen in *Splotch*. Heterozygous *Splotch* mice are otherwise normal, as were those containing the transgene.

Transgenic heterozygous *Splotch* mice were backcrossed to *Splotch* heterozygotes and litters were examined at E13.5. All non-transgenic *Splotch* homozygous embryos displayed neural

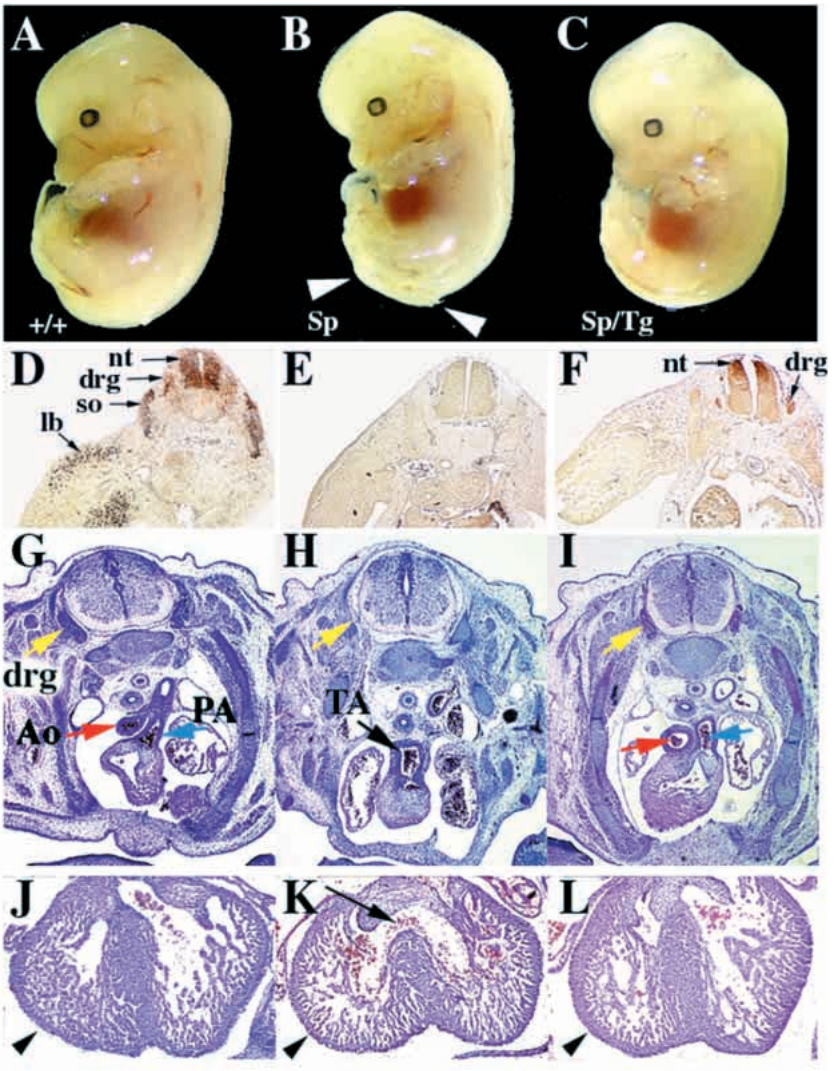

Fig. 2. Spina bifida and congenital heart disease is corrected by transgenic expression of *Pax3* in neural tube and neural crest. (A-C) E13.5 litter mates with a normal genotype (A), *Splotch* (B) or *Splotch* carrying the *Pax3* P3proPax3DHFR transgene (C) are shown. The *Splotch* embryo (B) has spina bifida (white arrowheads). The wild-type (A) and transgenic *Splotch* (C) embryos do not. (D-F) Pax3 protein expression revealed by immunohistochemistry using a Pax3-specific Ab reveals Pax3 protein expression in the dorsal neural tube (nt), the dorsal root ganglia (drg), the somite (so) and the limb bud (lb) in E11.5 wild-type embryos (D). No Pax3 protein is detected in homozygous *Splotch* embryos (E). Pax3 protein is detected in dorsal neural tube and dorsal root ganglia of transgenic homozygous *Splotch* embryos (F). (G-I) Transverse sections of E13.5 embryos stained with H&E at the level of the right ventricular outflow tract (RVOT) reveals transgenic correction of outflow septation and dorsal root ganglia development. In wild-type embryos (G) the RVOT is contiguous with the pulmonary artery (PA). A clearly distinct aorta (Ao) arises from the left ventricle below the plane of section. Dorsal root ganglia (drg) are easily identified. In *Splotch* embryos (H) there is persistent truncus arteriosus (black arrow); distinct aorta and pulmonary artery are not present. The dorsal root ganglia are absent (yellow arrow). In transgenic *Splotch* embryos (I) outflow tract septation is normal and the dorsal root ganglia are well formed (yellow arrow). (J-L) H&E sections of E13.5 cardiac ventricles from wild-type (J), *Splotch* (K) and transgenic *Splotch* (L) embryos reveals an abnormally thinned myocardium in *Splotch* hearts (arrowhead, K) and a ventricular septal defect (arrow, K). In transgenic *Splotch* embryos, the myocardium appears normal (arrowhead, L) and there is no ventricular septal defect.

tube defects in the lumbosacral region (compare Fig. 2A with 2B) and some also showed exencephaly. In striking contrast, all transgenic *Splotch* homozygous embryos (*n*=9) from both transgenic lines had normal closed neural tubes at E13.5 (Fig. 2C).

Immunohistochemical analysis using Pax3-specific antibodies confirmed expression of Pax3 protein in transgenic embryos. Pax3 protein expression was detected in the dorsal neural tube, the dorsal root ganglia, the somite and the limb buds of wild-type embryos (Fig. 2D). No Pax3 protein was detected in *Splotch* embryos (Fig. 2E). Pax3 protein was identified in the dorsal neural tube and the dorsal root ganglia of transgenic *Splotch* embryos, but Pax3 protein was not detected in the somite or in the limb bud (Fig. 2F). This result further confirms neural tube- and neural crest-specific expression of the proximal 1.6 kb *Pax3* regulatory region. Note also that at the protein level, the amount of Pax3 expressed in dorsal neural tube is roughly comparable between transgenic *Splotch* embryos and wild-type litter mates.

Histologic analysis of E13.5 litter mates revealed additional abnormalities in non-transgenic homozygous *Splotch* embryos consistent with prior reports (Auerbach, 1954; Franz, 1989; Franz and Kothary, 1993; Franz et al., 1993). Fig. 2G reveals the normal anatomy of a wild-type embryo at E13.5. The dorsal root ganglia are well formed (yellow arrow). The cardiac outflow tract has septated forming two great vessels: the aorta (red arrow, Ao), and the pulmonary artery (blue arrow, PA) that is emerging from the right ventricular outflow tract. In homozygous *Splotch* embryos (Fig. 2H), the dorsal root ganglia are absent or very small (yellow arrow). Septation of the cardiac outflow tract has failed to occur leaving a single great vessel, a persistent truncus arteriosus, emerging from the right ventricle (black arrow, Fig. 2H). Fig. 2I shows that in transgenic homozygous *Splotch* embryos the dorsal root ganglia develop normally (yellow arrows). In all cases there was septation of the outflow tract of the heart as demonstrated by a distinct aorta (red arrow) and pulmonary artery (blue arrow). In one case (not shown) outflow tract septation occurred, but a small defect was detected in the aorto-pulmonary septum leaving a connection between the aorta and the pulmonary artery distal to the aortic and pulmonary valves, indicating a subtle residual defect of outflow tract septation.

A defect in myocardial development has been reported in homozygous *Splotch* mice consisting of a thinned compact layer and defective excitation-contraction coupling (Conway et al., 1997b; Creazzo et al., 1998). A similar defect is seen in chick

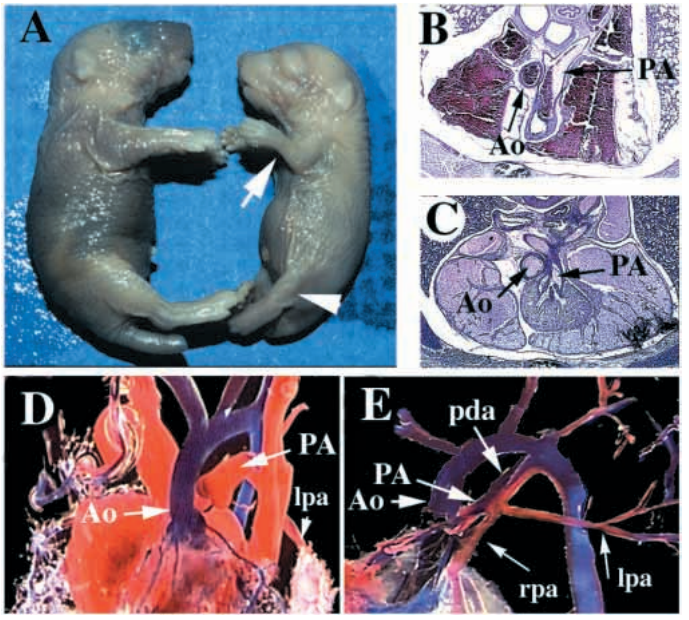

Fig. 3. Transgenic *Splotch* mice survive until birth, have normal cardiac outflow tract septation and have a persistent ductus arteriosus. (A) Transgenic homozygous *Splotch* pups (shown on the right side of A) died immediately after delivery and were smaller than wild-type litter mates (left side of A). The upper extremities were thin, flexed and partially supinated (arrow, A). The proximal hindlimb region was also thin (arrowhead, A). (B,C) Transverse H&E sections at the level of the right ventricular outflow tract from wild-type (B) and *Splotch* transgenic newborn (C) pups reveals normal septation of the great vessels in both cases. The proximal pulmonary artery (PA) is clearly distinct from the proximal aorta (Ao) and can be seen to divide into right and left pulmonary arteries. (D,E) Corrosion casts were prepared (see Methods) from wild-type (D) and transgenic *Splotch* newborn pups by injection of blue acrylic into the left ventricle and red acrylic into the right ventricle. Complete septation of the outflow tract producing distinct aortae (Ao) and pulmonary arteries (PA) is present in both cases. In transgenic *Splotch* pups, a patent ductus arteriosus (pda) connecting the pulmonary artery to the descending aorta is present and mixing of blue and red colors is evident. Lpa, left pulmonary artery; rpa, right pulmonary artery.

embryos after cardiac neural crest ablation (Creazzo et al., 1997; Nosek et al., 1997). At E13.5, myocardial histology was clearly abnormal in all homozygous *Splotch* hearts examined (compare Fig. 2J with 2K), but was normal in all transgenic homozygous *Splotch* hearts (e.g. Fig. 2L). Hence, transgenic expression of *Pax3* in neural tube and neural crest rescues the myocardial defect associated with the *Splotch* phenotype.

Transgenic *Pax3* expression rescues embryonic lethality in *Splotch* mice

After matings of heterozygous transgenic *Splotch* mice, pregnant mothers were allowed to come to term. Resulting offspring were genotyped. We never identified surviving pups that were homozygous *Splotch*, with or without the transgene. However, we frequently noted 1 or 2 pups per litter that died immediately after birth. In all cases (n=12 from 2 independent lines) these pups were *Splotch* homozygotes that carried the *Pax3* transgene. The mutant newborn pups were smaller than wild-type or transgenic wild-type litter mates and had smaller limbs that were partially supinated (Fig. 3A). Necropsy revealed normal cardiac anatomy. Fig. 3B shows a transverse section from a wild-type pup at the level of the right ventricular outflow tract. The aorta (Ao) is clearly distinguished from the pulmonary artery (PA) that is seen dividing into right and left pulmonay arteries. Fig. 3C reveals similar normal appearing anatomy in a transgenic homozygous *Splotch* newborn pup. Normal septation of the outflow tract was confirmed by preparation of corosion casts of the vasculature of newborn wild-type (Fig. 3D) and transgenic *Splotch* (Fig. 3E) pups. In both cases, injection of red dye into the right ventricle filled the pulmonary artery, while injection of blue dye into the left ventricle filled the aorta (Fig. 3D,E). However, in transgenic *Splotch* pups, mixing of red and blue dye in the great vessels was evident because of the presence of a patent ductus arteriosus. The ductus arteriosus allows blood to bypass the lungs during embryogenesis and connects the pulmonary artery to the descending aorta. This vessel normally closes in the mouse within a few hours of birth, as it had in the wild-type embryo shown in Fig. 3D which was killed between 6 and 12 hours after birth. The patent ductus arteriosus in the transgenic *Splotch* pup is consistent with perinatal demise shortly after birth and suggested that these newborn pups were not able to increase arterial oxygen tension upon delivery, a process that triggers closure of the ductus arteriosus.

Absence of diaphragm and hypaxial muscles in transgenic rescued newborn *Splotch* mice

The cause of perinatal demise and failure of oxygenation was evident upon immunohistologic analysis of newborn pups. Fig. 4A shows a sagittal section of a wild-type newborn pup after identification of muscular tissue using a myosin-specific monoclonal antibody. The muscular diaphragm is clearly present (white arrows) and the lungs (Lu) are expanded. However, a similar section of a newborn transgenic *Splotch* pup (Fig. 4B) reveals absence of a muscular diaphragm. Instead, a thin fibrous membrane is present preventing herniation of abdominal contents into the thorax. The lungs (Lu) are not expanded. Absence of any muscular component to the diaphragm was confirmed by in situ hybridization analysis using a probe specific for myogenin RNA. Myogenin was expressed abundantly by the diaphragm and intercostal muscles of wild-type embryos (Fig. 4C). No myogenin expression was seen in the diaphragm of transgenic *Splotch* mice (arrow, Fig. 4D), while myogenin was expressed in the intercostal region of transgenic *Splotch* mice in a pattern similar to that seen in wild-type litter mates.

Other muscular abnormalities were also noted consistent with the normal expression of *Pax3* in the lateral somite from which migratory hypaxial muscles arise. The *Pax3* transgene utilized in these studies did not produce *Pax3* expression in the somite (Fig. 2F). Normally, by E11.5, limb muscle progenitors expressing *Pax3* migrate from the somite to the limb buds (Fig. 2D) and begin to express myoD, followed by myogenin and myosin (Bober et al., 1994; Goulding et al., 1994; Williams and Ordahl, 1994). In *Splotch* embryos, myoD is never detected in the limb buds, and limb musculature fails to

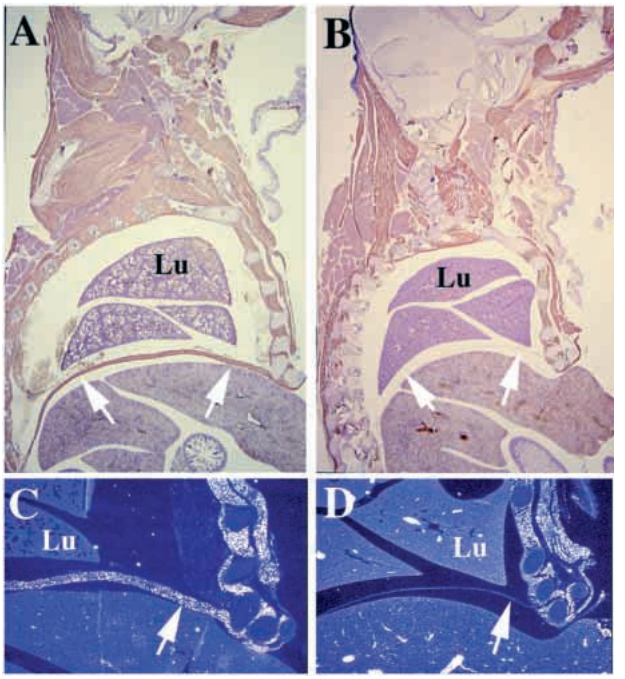

Fig. 4. Newborn transgenic *Splotch* mice lack a muscular diaphragm. (A,B) Immunohistochemical staining for myosin (brown staining) reveals a muscular diaphragm in sagittal sections of wild-type (arrows, A) newborn pups, but a complete absence of myosin expression in the diaphragm of transgenic *Splotch* litter mates (arrows, B). Other muscles of the neck, back and intercostal region can be seen to have developed normally. Note that the lung (Lu) is expanded in wild-type (A) but not in transgenic *Splotch* (B) pups. (C,D) Radioactive in situ hybridization of adjacent sagittal sections to those shown in A and B using a myogenin-specific probe confirms a complete absence of myogenesis in the diaphragm of transgenic *Splotch* pups (D) compared to wild type (C).

develop. Transgenic *Splotch* embryos also failed to express myoD in the limb buds during gestation and at E17.5 myogenin expression was specifically excluded from the limbs and the diaphragm (data not shown). Immunostaining for myosin expression and in situ hybridization for myogenin expression in newborn pups allowed for specific evaluation of all muscle groups and therefore a definitive assignment of Pax3-dependent and -independent muscles. At the shoulder girdle region, the supraspinatus, the infraspinatus, the subscapularus and the terres minor were absent as were all distal limb muscles (Fig. 5A-D), indicating that shoulder (rotator cuff) as well as more distal limb muscles require Pax3. The trapezius, the lattisimus dorsi, the intercostals, the rectus abdominus and the three layers of abdominal musculature were present. In ventral regions the abdominal wall muscles appeared less well developed than those of wild-type litter mates (Fig. 5E,F), suggesting that ventral body wall muscles also have a partial requirement for Pax3. All muscles of the head and deep back were present. Of particular note was the normal development of the hypoglossal muscles and the tongue (Fig. 5G,H). *Pax3* is expressed in the hypoglossal cord during gestation and the derivative muscles, like the diaphragm, represent migratory muscle groups (Hazelton, 1970; Mackenzie et al., 1998). Unlike the diaphragm, however, the tongue and hypoglossals can develop in the absence of *Pax3* expression in myoblasts.

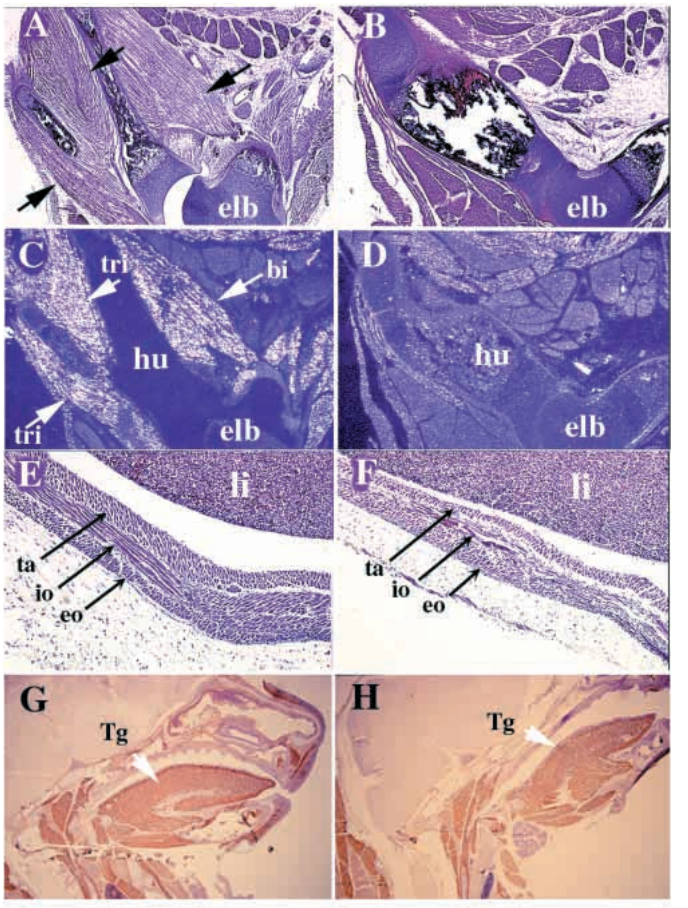

Fig. 5. Transgenic *Splotch* mice lack limb muscles. (A,B) H&E staining of sagittal sections through the upper limb and elbow (elb) of wild-type (A) and transgenic *Splotch* (B) pups reveals an absence of proximal limb muscles (arrows) including bicepts and tricepts in transgenic *Splotch* mice. Epaxial muscles are present. (C,D) Adjacent sections to those shown in A and B were used for in situ hybridization with a myogenin-specific probe and confirm the absence of myogenesis in the limbs of transgenic *Splotch* (D) pups compared to wild type (C). bi, bicepts; tri, tricepts; hu, humerus bone. (E,F) Transverse H&E sections of wild-type (E) and transgenic *Splotch* (F) litter mates at the level of the liver (li) reveals three layers of abdominal wall muscles (black arrows). Each layer is thinner in transgenic *Splotch* mice than in wild-type litter mates. Ta, transversus abdominis; io, internal oblique; eo, external oblique. (G,H) Myosin immunohistochemistry reveals normal development of the tongue (Tg) and hypoglossal muscles in transgenic *Splotch* pups (H) compared to wild type (G).

We also observed skeletal defects in transgenic *Splotch* newborn pups (Fig. 6). Multiple fusions of ribs in the ventral region were evident, as were abnormalities of the cervical vertebral column similar to those described previously (Tremblay et al., 1998). Facial bones appeared normal (data not shown). These results suggest that *Pax3* expression in neural tube and neural crest is not sufficient to allow for normal skeletal development, and suggest that *Pax3* expression in the somite is required for normal formation of the skeleton.

DISCUSSION

We have utilized the proximal 1.6 kb *Pax3* regulatory region

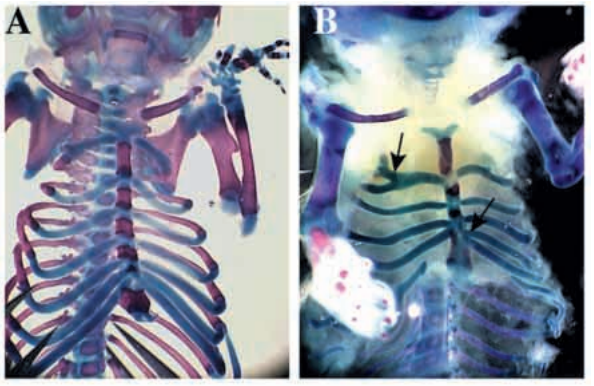

Fig. 6. Rib fusions in transgenic *Splotch* mice**.** Alcian red and blue staining of wild-type (A) and transgenic *Splotch* (B) newborn pups reveals multiple rib fusions in transgenic *Splotch* mice (arrows, B).

to engineer mice over expressing *Pax3* in the dorsal neural tube and in neural crest. Analysis of transgenic mRNA and protein expression conclusively demonstrates that distinct *Pax3* regulatory elements direct somitic and neural expression domains. Homozygous *Splotch* mice that carry this neural-specific transgene survive until birth whereas homozygous *Splotch* litter mates without the transgene die by E14. Spina bifida and congenital heart disease involving the outflow tract of the heart and the myocardium are corrected by the transgene, though muscular defects including formation of the diaphragm and limb muscles are not. Failure of myogenic differentiation within the diaphragm accounts for the perinatal lethality observed in homozygous *Splotch* transgenic newborns.

Cell autonomous Pax3 function in neural crest

Homozygous *Splotch* embryos display structural heart defects including persistent truncus arteriosus and abnormal myocardial maturation (Franz, 1989). These defects mimic some aspects of DiGeorge syndrome, vitamin A deficiency and retinoic acid toxicity in humans (for review see Epstein, 1996) and are remarkably similar to defects seen in experimental chick embryos after ablation of pre-migratory neural crest (Kirby et al., 1983). Therefore, structural heart disease in *Splotch* mice has been attributed to deficient *Pax3* expression in cardiac neural crest. Neural crest cells normally arise from the dorsal neural tube and form two columns of migratory cells that invade the outflow region of the heart populating the truncus arteriosus and the outflow endocardial cushions (Creazzo et al., 1998). They orchestrate, through mechanisms not well understood, the division of the truncus arteriousus into the aorta and pulmonary arteries, a process that takes place in the mouse between E11.5 and E13.5. Septation is accompanied by rotation and shortening of the outflow tract that allows the aorta to juxtapose and communicate with the left ventricle. Complete failure of this process results in persistent truncus arteriosus (a single great vessel emerging from the right ventricle), while partial failure results in an aorto-pulmonary window (in which incomplete septation leaves an inappropriate communication between the aorta and pulmonary artery) and related disorders. In humans, these cardiac malformations can be tolerated until birth, and sometimes repaired surgically. In mice, such defects are usually accompanied by malformed myocardium, heart failure, edema, and embryonic lethality by about E13.5 (Franz, 1989; Jacks et al., 1994; Schatteman et al., 1995; Sucov et al., 1994). In *Splotch* embryos, truncus arteriosus is associated with an abnormally thinned myocardium and specific loss of the compact zone where proliferating myocytes reside (Kochilas et al., 1999). Abnormal excitation-contraction coupling has been demonstrated in *Splotch* myocardium (Conway et al., 1997b). Our results indicate that replacement of Pax3 in neural crest rescues both outflow tract septation and myocardial development.

The precise cell type in which the loss of Pax3 function results in myocardial and septation cardiac defects has remained unclear, though neural crest has been strongly implicated in the abnormal outflow tract phenotype. Surprisingly, *Pax3*-expressing cells have not been conclusively identified in the myocardium or outflow tract. Pax3 may be required by cardiac neural crest cells long before these cells migrate to the heart region. Only very low levels of *Pax3* have been detected in the heart and the cell type expressing *Pax3* in these studies was unclear (Conway et al., 1997a). Although myocardial development is abnormal in *Splotch* embryos, we have not detected *Pax3* expression in myocardial cells by in situ hybridization or by RT-PCR (data not shown). The myocardial defect may therefore be secondary to neural crest deficiency or secondary to hemodynamic alterations caused by structural heart disease. Likewise, septation defects may be the result of cell autonomous defects in cardiac neural crest (analogous to neural crest ablation studies in the chick) or may be the result of molecular defects in tissues with which neural crest cells must interact along the migratory pathway or in the outflow tract itself. The expression of *Pax3* within neural crest cells and also in somitic cells lining the migratory pathway has previously made distinguishing between these possibilities difficult.

Our results support a cell autonomous role for Pax3 in neural crest during cardiac development. Replacement of Pax3 function in the dorsal neural tube and the neural crest was sufficient to rescue outflow tract septation. Also, myocardial development was rescued. We saw no evidence of transgene expression in myocardial cells or in tissues outside of the neural tube and neural crest either by in situ hybridization or by the use of the more sensitive *lacZ* reporter gene. This result strongly argues that the myocardial defect of *Splotch* embryos is secondary to neural crest dysfunction that is caused directly by a lack of Pax3 in neural crest cells.

Transgenic expression of *Pax3* in neural tube and neural crest also resulted in the correction of spina bifida and the development of histologically normal dorsal root ganglia. Neural tube closure is a complex process affected by multiple genes and environmental factors (Corcoran, 1998). *Splotch* has served as an important animal model for the study of spina bifida. As in humans, the incidence of neural tube defects in *Splotch* mice can be greatly ameliorated by dietary folate administration (Fleming and Copp, 1998). Of the many genes known to affect neural tube closure and neural crest cell migration, some are expressed within the neural tube and some in adjacent structures. *Pax3* is expressed both within the dorsal neural tube and in the adjacent somites. Experiments in which wild-type or *Splotch* neural tube explants were transplanted into normal chick embryo hosts have suggested that *Splotch* neural crest is capable of proper migration (Serbedzija and

McMahon, 1997). This has suggested that the migration defect in *Splotch* embryos may be due to the mutant environment, i.e. the abnormal somite which is lacking *Pax3* expression. Our results, however, demonstrate that neural tube closure and neural crest-derived structures develop normally in the complete absence of Pax3 in somites.

Pax3 dependent and independent muscles

The absence of somitic expression of *Pax3* in newborn transgenic *Splotch* mice allowed us to confirm the essential role for Pax3 in hypaxial muscle development. *Pax3* is normally expressed in the presomitic mesoderm and becomes restricted to the lateral dermomyotome of maturing somites. Migratory limb myoblasts expressing *Pax3* de-epithelialize from the lateral dermomyotome of limb level somites at about E10 and populate the dorsal and ventral muscle masses of the limbs (Bober et al., 1994; Goulding et al., 1994; Williams and Ordahl, 1994). This process appears to involve the direct activation of *c-met* by Pax3 in migratory myoblasts (Epstein et al., 1996; Yang et al., 1996). In the absence of Pax3, the somite is foreshortened, *c-met* expression is reduced, and limb muscles fail to form (Daston et al., 1996; Epstein et al., 1996). Pax3-deficient limb muscle precursors are capable of differentiating into muscle when transplanted into normal chick limbs suggesting a primary migration defect (Daston et al., 1996). Tracking of myoblasts in *Splotch* embryos with the use of a muscle-specific reporter transgene indicates that Pax3 is required for proper development of many ventral muscles that depend upon extensive migration of myogenic progenitors (Tremblay et al., 1998). In addition, skeletal abnormalities involving the ribs, vertebral column and limbs have been noted (Tremblay et al., 1998).

Our results confirm and extend these observations. We noted muscular defects in newborn transgenic *Splotch* mice that involved essentially the identical muscle groups that are abnormal at mid-gestation. This confirms that the earlier defect is not the result of developmental delay and cannot be compensated for by redundant processes at later times of gestation. Limb muscles, including those of the shoulder, were absent and ventral abdominal muscles were disorganized and thin. The muscular diaphragm was absent, although a thin fibrous diaphragm was present preventing herniation of the abdominal viscera into the chest. Although *Pax3* is strongly expressed in the hypoglossal chord during mid-gestation, we found that myogenic differentiation in the tongue was robust in the absence of somitic *Pax3* expression. Perhaps the closely related *Pax7* gene can compensate for loss of Pax3 function in some myogenic cells (Borycki et al., 1999).

Over expression of *Pax3* did not produce tumors

Previous reports have demonstrated that over expression of *Pax* genes in tissue culture can result in cellular transformation (Maulbecker and Gruss, 1993). In addition, an activating translocation involving *PAX3* causes pediatric alveolar rhabdomyosarcoma (Barr et al., 1993; Galili et al., 1993; Shapiro et al., 1993). The PAX3/FKHR fusion produced by this translocation is a more potent transcriptional activator than Pax3 itself suggesting that a gene dosage effect may be responsible for enhancing cellular proliferation (Fredericks et al., 1995), though altered target gene specificity of PAX3/FKHR compared with Pax3 has also been demonstrated (Epstein et al., 1998). A gene dosage effect for *PAX6* in humans has been reported (Glaser et al., 1994) and transgenic over expression of *Pax6* in mice results in ocular defects similar to those seen in *Pax6*-deficient *Sey* embryos (Schedl et al., 1996). Mis-expression of *Pax3* in the ventral neural tube results in dorsalization of ventral structures (Tremblay et al., 1996). In light of these results, it is interesting that in our studies transgenic mice over expressing *Pax3* in the dorsal neural tube were phenotypically normal and did not develop tumors.

Conclusions

The functional demonstration that a relatively small genomic fragment (1.6 kb) is capable of directing tissue-specific neural crest expression has allowed us to demonstrate the cell autonomy of Pax3 function in neural crest cells. Pax3 in neural tube and neural crest is required for septation of the outflow tract of the heart, for development of the dorsal root ganglia and for neural tube closure. Pax3 in the somite is dispensable for these processes. On the other hand, somitic Pax3 expression is required for limb muscle development and formation of the muscular diaphragm. Pax3 in the somite also affects, to a lesser degree, ventral body wall muscles and the developing skeleton. Separable regulatory elements direct Pax3 expression in somitic versus neural expression domains. Further analysis of these regulatory elements should allow for the identification of upstream effectors of neural crest and muscle development.

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