**Pax3 is required for cardiac neural crest migration in the mouse: evidence from the splotch (Sp²H) mutant**

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**SUMMARY**

Neural crest cells originating in the occipital region of the avian embryo are known to play a vital role in formation of the septum of the cardiac outflow tract and to contribute cells to the aortic arches, thymus, thyroid and parathyroid. This ‘cardiac’ neural crest sub-population is assumed to exist in mammals, but without direct evidence. In this paper we demonstrate, using RT-PCR and in situ hybridisation, that Pax3 expression can serve as a marker of cardiac neural crest cells in the mouse embryo. Cells of this lineage were traced from the occipital neural tube, via branchial arches 3, 4 and 6, into the aortic sac and aorto-pulmonary outflow tract. Confirmation that these Pax3-positive cells are indeed cardiac neural crest is provided by experiments in which hearts were deprived of a source of colonising neural crest, by organ culture in vitro, with consequent lack of up-regulation of Pax3. Occipital neural crest cell outgrowths in vitro were also shown to express Pax3. Mutation of Pax3, as occurs in the splotch (Sp²H) mouse, results in development of conotruncal heart defects including persistent truncus arteriosus. Homozygotes also exhibit defects of the aortic arches, thymus, thyroid and parathyroids. Pax3-positive neural crest cells were found to emigrate from the occipital neural tube of Sp²H/Sp²H embryos in a relatively normal fashion, but there was a marked deficiency or absence of neural crest cells traversing branchial arches 3, 4 and 6, and entering the cardiac outflow tract. This decreased expression of Pax3 in Sp²H/Sp²H embryos was not due to down-regulation of Pax3 in neural crest cells, as use of independent neural crest markers, Hoxa-3, Crubp1, Prx1, Prx2 and c-met also revealed a deficiency of migrating cardiac neural crest cells in homozygous embryos. This work demonstrates the essential role of the cardiac neural crest in formation of the heart and great vessels in the mouse and, furthermore, shows that Pax3 function is required for the cardiac neural crest to complete its migration to the developing heart.

Key words: embryo, heart development, truncus arteriosus, malformations, neural crest, mutant mouse, splotch, gene expression, in situ hybridisation

**INTRODUCTION**

The neural crest is a migratory cell population derived from the dorsal neural tube that contributes to a wide variety of tissues throughout the embryo (Weston, 1970). Analysis of quail neural crest cells transplanted into chick embryos has identified a neural crest sub-population, termed the ‘cardiac’ neural crest that appears to be transitional in its properties between the neural crest of cranial and trunk regions (Kirby and Waldo, 1995). Cardiac neural crest cells emigrate from the occipital neural tube and migrate through the third, fourth and sixth branchial arches, making a cellular contribution to arch derivatives, including the aortic arch arteries, thymus, thyroid and parathyroid. Some neural crest cells migrate beyond the branchial arches, entering the aortic sac and cardiac outflow tract where they participate in formation of the aorto-pulmonary septum. Ablation studies in the chick have demonstrated that the cardiac neural crest is essential for normal heart morphogenesis. Cardiac defects of the conotruncal type are induced, including persistent truncus arteriosus (PTA, also called common arterial trunk) and double outlet right ventricle (DORV; Kirby et al., 1983). Additionally, the aortic arches are malformed and the thymus, thyroid and parathyroids are defective or absent in chick embryos following ablation of the cardiac neural crest (Bockman and Kirby, 1984; Kirby and Waldo, 1990).

Relatively little evidence has been presented to support the existence of a cardiac neural crest cell lineage in the mammalian embryo. Dil labelling experiments in the rat have demonstrated migration of labelled cells from the occipital neural tube to the cardiac outflow tract (Fukiishi and Morriss-Kay, 1992), but the identity of the labelled cells as neural crest was not confirmed by use of gene expression markers, nor was the differentiative fate of the putative cardiac neural crest cells determined. In no case has a conotruncal heart defect been demonstrated to result from failure of neural crest migration in a mammalian system. On the other hand, PTA and other conotruncal malformations are well recognised birth defects in both humans and teratogen-based rodent systems. For instance,
PTA is a component of the DiGeorge syndrome, where it results from anomalies of chromosome 22q11.2 (Scambler, 1993). Outflow tract defects also occur in humans and rodents exposed to excess retinoids during early embryonic development (Shenefelt, 1972; Lammer et al., 1985), frequently in association with malformations of the craniofacial region, thymus, thyroid and parathyroids. Extrapolating from the avian system, the general assumption has been that human and rodent conotruncal heart defects also result from disturbance of the cardiac neural crest.

The splotch (Sp$^{2H}$) mouse provides a model system in which to study directly the relationship between neural crest migration and cardiac development. A defect of neural crest migration is suggested by the pigment defects which characterise splotch heterozygotes and the small or absent dorsal root ganglia of splotch homozygous (Auerbach, 1954). More recently, the development of PTA, as well as defects of the thymus, thyroid and parathyroids, have been described in a small series of splotch homozygous embryos (Franz, 1989). Splotch embryos additionally exhibit defects of neural tube closure and of the limb musculature (Auerbach, 1954; Franz, 1990; Franz et al., 1993).

Five mutant alleles at the splotch locus are known, in which there is either a mutation (Sp, Sp$^a$), intragenic deletion (Sp$^{2H}$) or complete deletion (Sp$, Sp^{HH}$) of the Pax3 gene (Epstein et al., 1991, 1993; Goulding et al., 1993; Vogan et al., 1993). The homozygous phenotypes of the Sp and Sp$^{2H}$ alleles are closely similar, exhibiting defects of the neural crest, neural tube and limb musculature, whereas the milder Sp$^a$ allele has less severe neural crest defects, although manifesting closely similar anomalies of limb musculature (Franz, 1993). The Sp$^a$ (Beechey and Searle, 1986) and Sp$^{HH}$ (Goulding et al., 1993) alleles are early postimplantation lethals, probably because of deletion of genes adjacent to Pax3. In humans, mutations of the Pax3 gene occur in Waardenburg syndrome types I and III (Strachan and Read, 1994). Pax3 encodes a DNA-binding protein (Goulding et al., 1991) whose activity is disturbed by splotch and Waardenburg mutations (Chalepakis et al., 1994); the gene product probably controls a cascade of embryonic gene expression, although the down-stream regulated genes are, as yet, poorly understood (Edelman and Jones, 1995; Kioussi et al., 1995; Stuart et al., 1995).

Although Pax3 expression has been detected in mouse and chick embryos in the developing neural tube, early migrating neural crest and dermomyotomal cells entering the limb bud (Goulding et al., 1991; Bober et al., 1994; Goulding et al., 1994), there have been no reports of Pax3 expression specifically in the cardiac neural crest or in the developing heart. In this study, we show that cardiac neural crest cells can be detected by virtue of their expression of the gene markers Pax3, Hoxa-3, Crabp1, Ptx1, Ptx2 and c-met. These cells populate the aorto-pulmonary outflow tract of normal mouse embryos, whereas homozygous splotch embryos exhibit a marked reduction, or complete lack of cardiac neural crest prior to the development of PTA.

MATERIALS AND METHODS

Mouse strains and embryos

Sp$^{2H}$ is a radiation-induced allele at the splotch locus that arose on a C3H/101 hybrid background (Beechey and Searle, 1986). A heterozygous Sp$^{2H}$/+ female, obtained from the MRC Radiobiology Unit, Harwell, UK, was mated with a CBA/Ca male and the offspring were mated inter se to found a randomly bred colony. Sp$^{2H}$/+ heterozygotes, identified by the presence of a white belly spot, were mated together to produce Sp$^a$/Sp$^{2H}$ embryos. Noon on the day of finding a copulation plug was designated 0.5 days of gestation. Pregnant females were killed by cervical dislocation and the embryos were explanted into Dulbecco’s Modified Eagles Medium (DMEM, Flow Labs, UK) containing 10% fetal calf serum (FCS). The yolk sac and amnion were opened and the umbilical cord was cut. A pulsating stream of blood indicated ongoing contractions of the heart in live embryos. The yolk sac and/or a limb bud were processed for PCR genotyping using Pax3 primers to detect the 32 base pair intragenic deletion in the Sp$^{2H}$ allele, as described by Epstein et al. (1991); Estibeiro et al. (1993). Embryos were then immersed in fixative for in situ hybridisation, stored at −70°C for RT-PCR, or prepared for scanning electron microscopy (Vuillemin and Pexieder, 1989).

Neural crest cultures

Primary neural tube explants were prepared from 8.5-day embryos (4-10 somites) and cultured as described previously (Moase and Trasler, 1990). The portion of neural tube between the otic placode and the third somite was cultured for 72 hours, then each neural tube explant was removed and discarded. Neural crest cell outgrowths were either fixed for in situ hybridisation, or harvested by loosening their attachment to the culture dishes with 1% trypsin for 20 minutes at 37°C, then spun into a pellet and used for RT-PCR analysis.

Culture of isolated hearts and whole embryos

Embryonic hearts were dissected from early 9.5-day embryos (12-15 somites) in DMEM containing 10% FCS, washed twice in phosphate-buffered saline and cultured for 30 hours in 1 ml of 100% rat serum (Cockcroft, 1990) at 37°C in a humidified atmosphere of 5% CO$_2$ in air. The remainder of the embryo was used for PCR genotyping. Whole 9.5-day embryos in the same somite range were cultured (Cockcroft, 1990) for 30 hours in the same serum batch, then dissected to yield hearts for RT-PCR analysis and the remainder for PCR genotyping. At the end of culture, some hearts were fixed in 4% paraformaldehyde, prepared as paraffin sections and stained with haematoxylin and eosin.

RT-PCR reactions

RNA was isolated from Sp$^{2H}$/Sp$^{2H}$ embryos and their wild-type littermates at 9.5, 10.5, 11.5, 12.5, 13.5 and 14.5 days of gestation. Embryos were dissected in cold diethylpyrocarbonate (DEPC)-treated PBS, their hearts and neural tubes were dissected, snap frozen and stored at −70°C. In most experiments, RNA was isolated from pooled (2-11 per sample) dissected hearts using RNAzol (Biogenesis Ltd, UK), with 300 ng of total RNA forming the basis of the reverse transcriptase reaction. When single isolated hearts were used for RT-PCR, a crude nucleic acid extract was prepared: isolated hearts were placed in 300 mM glucose for 20 minutes at room temperature, the tissue was spun down and resuspended in 1 mg/ml proteinase K for 15 minutes at 37°C. The tissue was again spun down and the supernatant used for RT-PCR. The methodology of Kawasaki (1990) was followed for both the reverse transcriptase and PCR reactions. Pax3 primers were designed to amplify the region between base pairs 753 and 974 (Goulding et al., 1993), generating a fragment of 222 base pairs. The primers were designed to amplify the region between base pairs 753 and 974 (Goulding et al., 1993), generating a fragment of 222 base pairs. The amplified fragment was confirmed by sequencing using Sequenase version 2.0 (Amersham). Moreover, use of the fragment as a probe for in situ hybridisation yielded an expression pattern identical to that described for Pax3 (Goulding et al., 1991). Since the primers spanned an intron, bands generated by amplification from cDNA could be distinguished from bands.
amplified from genomic DNA. ß-actin primers (upstream primer, base pairs 105-126 and downstream primer, base pairs 621-642; Alonso et al., 1986) were used in the same PCR reactions to control for pipetting and amplification differences. A total of 26 cycles of amplification were carried out (such that products were still within the linear range), the products were run on a 1.5% agarose gel, blotted onto a nylon membrane (Merck) and hybridised with Pax3 and ß-actin cDNA probes to yield high sensitivity and specificity of product detection.

In situ hybridisation

In situ hybridisation on paraffin sections was performed as described previously (Conway et al., 1994). Single-stranded Pax3 RNA probes were synthesised from a Bluescript vector (Stratagene) containing a 516 base pair HindIII-PstI fragment cloned from the 3′ end (base pairs 1071 to 1590) of the Pax3 gene (Goulding et al., 1991). Hybridisations were as described previously (Goulding et al., 1991) except that probe concentration was 300 ng/ml and hybridisations were at 65°C. Whole-mount in situ hybridisation was performed essentially as described by Wilkinson (1992), except that a lower probe concentration was used. Probes were: Pax3 (as above); Hoxa-3, a 660 base pair HindIII-EcoRI fragment containing the homeobox (Gaunt et al., 1986); Crabl, a 769 base pair EcoRI fragment (Stoner and Gudas, 1989); Prxl, a 450 base pair SmaI fragment (Kern et al., 1994); Prx2, a 600 base pair HindIII fragment (Kongsuwan et al., 1988); c-met, a 2.1 kb EcoRI fragment (Andermarcher et al., 1996). In all cases sense-strand control probes were used in parallel with antisense probes, with no evidence of non-specific hybridisation.

RESULTS

We found PTA with ventricular septal defect in approximately 50% of Sp²H/Sp²H embryos at 12.5 and 13.5 days of gestation, but not in Sp²H/+ or +/- littermates (Fig. 1). To investigate the relationship of Pax3 expression to the development of these cardiac defects, we studied the time course of expression of Pax3 in developing mouse hearts using reverse transcriptase-polymerase chain reaction (RT-PCR).

Pax3 expression is developmentally regulated in the early embryonic heart

Pax3 mRNA was amplified from pools of hearts isolated from genotyped embryos at 9.5 to 14.5 days of gestation. In wild-type hearts at 9.5 days, Pax3 expression was barely detectable (Fig. 2B, lane 1) or undetectable (Fig. 2A, lane 1), but was strongly expressed at 10.5 days (Fig. 2A, lane 2; Fig. 2B, lane 3). Thereafter, expression again became barely detectable at 11.5, 12.5 and 13.5 days, and was undetectable in 14.5 day hearts (Fig. 2A, lanes 3-6). Homozygous Sp²H/Sp²H embryos, like wild-type controls, expressed Pax3 in their neural tubes (Fig. 3, lanes 7, 8) although the Sp²H mutant mRNA, which has a 32 base pair deletion (Epstein et al., 1991), is predicted to generate a truncated protein product. Pax3 expression in Sp²H/Sp²H embryos differed from wild-type embryos in that the strong expression at 10.5 days of gestation was not observed (Fig. 2B, lane 4). Since cardiac neural crest cells are reported to colonise the developing heart at 11.5 days in the rat (the equivalent of 10.5 days in the mouse; Fukishii and Morriss-Kay, 1992), the RT-PCR findings suggested that cardiac neural crest cells express Pax3 in the mouse, at least until the time of entry into the cardiac outflow tract. Thus, Pax3 could serve as a control for loading. Each experiment was performed on at least three occasions with a similar result in each case.

Fig. 2. Expression of Pax3, detected by RT-PCR, during mouse heart development in vivo. (A) Expression in wild-type hearts is low or undetectable at 9.5 days of gestation (lane 1), markedly up-regulated at 10.5 days (lane 2), down-regulated at 11.5 (lane 3), 12.5 (lane 4) and 13.5 (lane 5) days and undetectable at 14.5 days (lane 6). (B) Expression in wild-type hearts (lanes 1,3,5,7) compared with Sp²H/Sp²H hearts (lanes 2,4,6,8) at 9.5 (lanes 1,2), 10.5 (lanes 3,4), 11.5 (lanes 5,6) and 12.5 days (lanes 7,8) of gestation. The dramatic up-regulation of Pax3 in wild-type hearts at 10.5 days (lane 3) is absent from mutant hearts (lane 4). Expression of ß-actin is shown as a control for loading. Each experiment was performed on at least three occasions with a similar result in each case.

Fig. 3. Expression of Pax3, detected by RT-PCR, in mouse embryonic hearts immediately after isolation early on 9.5 days of gestation (lanes 1,2) or after 30 hours culture, either in isolation (lanes 3,4) or as part of an intact embryo (lanes 5,6). Wild-type hearts (lanes 1,3,5) exhibit up-regulation of Pax3 only when cultured as part of an intact embryo (lane 5), whereas Sp²H/Sp²H hearts (lanes 2,4,6) fail to up-regulate Pax3 in all cases. Pax3 expression is detected similarly in wild-type (lane 7) and Sp²H/Sp²H (lane 8) neural tubes. Expression of ß-actin is shown as a control for loading. Each experiment was performed on at least two occasions with a similar result in each case.
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Elevation of Pax3 expression in the heart requires colonisation by neural crest

In order to validate the use of Pax3 as a marker of cardiac neural crest, we examined the onset of Pax3 expression in hearts grown in vitro. We wanted to determine whether the up-regulation in Pax3 expression in wild-type hearts from 10.5-day embryos in vivo results from colonisation by Pax3-positive neural crest cells or, alternatively, whether Pax3 expression increases intrinsically in the developing heart at 10.5 days. To distinguish between these possibilities, whole wild-type hearts were explanted from early 9.5-day embryos (12-15 somites), prior to the stage of neural crest cell immigration, and were cultured for 30 hours, then assayed for Pax3 expression by RT-PCR (Fig. 4A). The rationale was that hearts, deprived of a source of incoming Pax3-positive neural crest cells, should exhibit only basal expression of Pax3. Indeed, we could detect no Pax3 expression in these isolated hearts (Fig. 3, lane 3), in contrast to the hearts of control embryos that had been cultured intact for 30 hours under identical conditions (Fig. 4A). The hearts of these intact cultured embryos, dissected and assayed following culture, exhibited strong Pax3 expression (Fig. 3, lane 5). The hearts of Sp2H/Sp2H embryos failed to express Pax3 whether they were cultured in isolation or as part of an intact embryo (Fig. 3, lanes 4,6). The cultured isolated hearts did not differ markedly from hearts of cultured whole embryos in either gross morphology (Fig. 4B) or histological appearance (Fig. 5), showing that isolated 9.5 day hearts can undergo relatively normal development in vitro for a period of at least 30 hours. At this early stage, the mutant hearts do not exhibit outflow tract defects, as PTA can be detected morphologically only from 11.5 days of gestation, 24 hours later than the cultures were terminated.

Migrating cardiac neural crest cells express Pax3 in vitro

If Pax3 is a marker of cardiac neural crest, we would expect to see expression in cells emigrating from the occipital neural tube in vitro. Neural tubes, isolated from the region between the otic placode and the third somite of 8.5-day wild-type and Sp2H/Sp2H embryos, were dissected free from somites and other tissues and were cultured in vitro for 72 hours. This

Fig. 4. An experiment in which mouse hearts were cultured, either in isolation or as part of an intact embryo, in order to determine the dependence of Pax3 expression on immigration of neural crest cells. (A) Design of the experiment. Top row shows hearts isolated from early 9.5-day embryos and cultured for 30 hours, before RT-PCR assay for Pax3 expression. Bottom row shows control embryos cultured intact from early 9.5 days for 30 hours before dissection of hearts and assay for Pax3 expression. (B) Morphology of hearts before culture, and after culture either in isolation or as part of an intact embryo. Control (Sp2H/+ or +/+ ) embryos are shown at the top and Sp2H/Sp2H embryos at the bottom. Note the closely similar morphology of isolated hearts compared with those from intact embryos. Scale bar, 1 mm.

Fig. 5. Histological sections of hearts from early 9.5-day embryos before (A) and after (B,C) culture for 30 hours, either in isolation (B) or as part of an intact embryo (C). The heart cultured in isolation (B) closely resembles the heart that developed as part of an intact embryo (C), both in the demarcation of right (R) and left (L) ventricles, and outflow tract (F), the cellularisation of the outflow tract endocardial cushion (arrows) and the development of ventricular trabeculations (V). Scale bar, 0.1 mm.
Pax3 and cardiac neural crest technique is known to yield cell outgrowths highly enriched for neural crest (Ito and Takeuchi, 1984). Migrating cells of both genotypes derived from these neural tube cultures expressed Pax3 transcripts when assayed by RT-PCR and by in situ hybridisation (data not shown), strongly suggesting that cardiac neural crest cells express Pax3 in the mouse.

**Initiation of cranial neural crest migration is unaffected in mutant embryos**

Having established the validity of Pax3 as a marker of cardiac neural crest cells, we examined control and splotch embryos for Pax3 expression by whole-mount in situ hybridisation. Embryos at 9.5 days of gestation expressed Pax3 not only in the neural tube but also in streams of neural crest cells that could be seen emerging from rhombomeres 2 and 4 and entering branchial arches 1 and 2 respectively (Fig. 6), as described previously (Lumsden et al., 1991; Serbedzija et al., 1992). Smaller streams of cells could also be seen emerging from the neural tube caudal to the otic vesicle, in the future occipital region (Fig. 6). There were no consistent differences between control and Sp2H/Sp2H embryos in Pax3 expression at 9.5 days, suggesting that the initiation of migration of cranial neural crest is relatively unaffected by the Sp2H mutation.

![Fig. 6. Pax3 expression detected by whole-mount in situ hybridisation in 9.5-day mouse embryos.](image)

In both (A) control (Sp2H/+ or +/+; n = 8) and (B) Sp2H/Sp2H (n = 4) embryos, expression is intense in the neural tube and there are clearly demarcated streams of cells emerging from rhombomeres 2 (large arrowheads) and 4 (small arrowheads), which can be seen entering the first (1) and second (2) branchial arches. Other, less intense streams of cells can be seen in both embryos (arrows) emerging from the occipital region opposite the most cranial somites. Although perioptic expression is visible in both embryos, expression in the frontonasal mass has not yet begun in the mutant embryo, reflecting its slightly earlier developmental stage. Scale bar, 0.1 mm.

![Fig. 7. Pax3 expression detected by whole-mount in situ hybridisation in 10.5-day (A) control (Sp2H/+ or +/+; n = 36) and (B) Sp2H/Sp2H (n = 11) embryos.](image)

Higher magnification of the heart and branchial arches of the control embryo is shown in (C). Expression is intense in the neural tube (N) and craniofacial region, and does not differ between control and mutant embryos. A stream of expressing neural crest cells can be seen in the control embryo emerging from the occipital neural tube, traversing the developing branchial arches 3, 4 and 6, and entering the aortic sac and aorto-pulmonary outflow tract (black arrow in A and white arrowheads in C). Note the close proximity of the Pax3-positive cells to the truncal ridges of the outflow tract (open arrows in C). There is a dramatic reduction in the stream of Pax3-positive cells in the mutant embryo (black arrow in B). Pax3 expression is also observed in the control embryo in the somitic region, representing dermomyotomes and dorsal root ganglia, and in the forelimb bud (arrowhead in a) showing immigration of myoblast precursors from the dermomyotome. There is severe disruption of expression in the mutant embryo in the somitic region (S), and in the limb (black arrowhead in B). Note the exencephaly in the mutant embryo (white arrowhead in B). A, common atrium; V, common ventricle. Scale bar, 1 mm in A and B; 0.4 mm in C.
**Pax3-positive neural crest cells enter the hearts of normal but not splenoch embryos**

At 10.5 days, control and Sp^{2H}/Sp^{2H} embryos continued to express Pax3 (i.e. the full length and truncated transcripts, respectively) in a similar pattern in their neural tubes (Fig. 7A,B). However, the two types of embryo differed markedly in their expression of Pax3 in migrating cardiac neural crest. In control embryos, neural crest migration from rhombomeres 2 and 4 was no longer evident, whereas a prominent stream of Pax3-positive cells was detected passing through the developing third, fourth and sixth branchial arches and entering the aorto-pulmonary outflow tract (Fig. 7A,C). Sp^{2H}/Sp^{2H} embryos either failed completely to exhibit this stream of Pax3-positive cells (8/11 embryos examined) or showed marked reduction in the number of expressing cells at this site (Fig. 7B; 3/11 embryos). In situ hybridisation on serially sectioned embryos confirmed these observations: Pax3-positive cells were observed in control embryos within pharyngeal arches 4 and 6, at the junction with the aortic sac, whereas we were unable to detect labelled cells at this site in Sp^{2H}/Sp^{2H} embryos despite detailed examination of twelve serially sectioned 10.5-day embryos in the 30-42 somite range (Fig. 8). From 11.5 days onwards, when the outflow tract becomes partitioned into aortic and pulmonary channels (Fananapazir and Kaufman, 1988), Pax3 expression was undetectable in the heart by in situ hybridisation (data not shown), consistent with the down-regulation noted by RT-PCR.

**Use of independent neural crest markers**

Diminished expression of Pax3 in Sp^{2H} homozygous embryos could result from specific down-regulation of Pax3 in the mutant cardiac neural crest, rather than a reduction in number of neural crest cells per se. To examine this possibility, we performed in situ hybridisation with probes for a series of other genes, Hoxa-3 (Manley and Capocchi, 1995), CrabpI (Leonard et al., 1995), Prx1 and 2 (Leussink et al., 1995) and c-met (Tsarfaty et al., 1992), that we found to be expressed in the cardiac neural crest of 10.5-day embryos. Hoxa-3 (Fig. 9A), CrabpI(Fig. 9C), Prx1 (Fig. 10A,B), Prx2 and c-met (data not shown) were all expressed in a stream of cells that appeared to traverse the third, fourth and developing sixth branchial arches, entering the aortic sac and outflow tract in a similar fashion to Pax3. Although none of these genes are expected to be mis-regulated as a result of the Pax3 mutation, nevertheless, the stream of cells expressing Hoxa-3 (Fig. 9B), CrabpI (Fig. 9D), Prx1 (Fig. 10C,D), Prx2 and c-met (data not shown) was considerably reduced or absent in Sp^{2H}/Sp^{2H} embryos, strongly suggesting that the cardiac neural crest is diminished, or missing, in mutant embryos.

Expression of these genes in other tissues was generally comparable in control and mutant embryos. The anterior limit of Hoxa-3 expression in the neural tube, at the boundary between rhombomeres 4 and 5, was present in both controls and mutants (Fig. 9A,B), although the intensity of expression in the occipital and cervical regions, and in branchial arches 1 and 2, was reduced in the mutants. Comparable levels of CrabpI expression were observed in neural tube, frontonasal mass and limb bud of control and mutant embryos, but expression observed in the first branchial arch of control embryos was absent from the mutants (Fig. 9C,D).
Fig. 9. Expression of Hoxa-3 (A,B) and CrabpI (C,D) in 10.5-day embryos, detected by whole-mount in situ hybridisation. Hoxa-3 expression is visible in (A) control embryo (Sp2H/+ or +/+; n =16) in a stream of neural crest cells traversing the third, fourth and developing sixth branchial arches, and migrating towards the aortic sac and cardiac outflow tract (arrow in A). Expression in (B) Sp2H/Sp2H embryo (n=4) is markedly reduced at this site (arrow in B), as well as in the first and second branchial arches (see Discussion), and in the occipital neural tube (C). Nevertheless, the anterior limit of Hoxa-3 expression, at the boundary between rhombomeres 4 and 5, is maintained in mutant embryos (arrowheads in A,B). Expression in the fore limb bud (L) is similar in control and mutant embryos. CrabpI expression in the neural tube (N), frontonasal region (F) and fore limb buds (L) is closely similar in (C) control (Sp2H/+ or +/+; n=17) and (D) Sp2H/Sp2H embryos (n=8). However, as with Pax3 and Hoxa-3, a stream of expressing cells can be seen in the control embryos emerging from the occipital neural tube, traversing the third and fourth branchial arches (black arrowheads in C) and migrating towards the aortic sac and cardiac outflow tract, whereas this stream is absent from the mutant embryos, as is expression in the first branchial arch. Note that both mutant embryos have exencephaly (white arrowheads in D). Scale bar, 1 mm.

Fig. 10. Expression of Prx1 in 10.5-day (A,B) control (Sp2H/+ or +/+; n =9) and (C,D) Sp2H/Sp2H embryos (n = 4) detected by whole-mount in situ hybridisation. (B) and (D) are higher magnifications of the cardiac region of the embryos shown in (A) and (C) respectively. Prx1 expression is visible in the control embryo in a stream of neural crest cells traversing the third, fourth and developing sixth branchial arches, and migrating towards the aortic sac and cardiac outflow tract (arrows in A,B). In the mutant embryo (large arrowhead in C indicates the exencephaly), Prx1 expression is markedly reduced at the site of cardiac neural crest cell migration (small arrowheads in C,D). Nevertheless, expression in the maxilla (M), branchial arches 1 and 2 and in the fore (F) and hind (H) limb buds is closely similar in control and mutant embryos. Abbreviations: O, cardiac outflow tract; 1, 2, branchial arches 1 and 2. Scale bar, 1 mm in A,C; 0.5 mm in B,D.
of both genes in the limb bud appeared closely similar in control and mutant embryos. Most strikingly, Prx1 showed an almost identical pattern of expression in the maxilla, branchial arches 1 and 2 and in the limb buds of control and mutant embryos, whereas the neural tube and somite derivatives were negative in both embryo types (Fig. 10).

In conclusion, our results suggest that occipital neural crest cells colonise the developing cardiac outflow tract of the mouse embryo and that, although initiation of cardiac neural crest migration occurs in Sp²H/Sp²H embryos, these cells fail to arrive at their destination, leading to the development of conotruncal heart defects.

DISCUSSION

In this paper we describe transient expression of Pax3 in mouse hearts at 10.5 days of gestation. Several lines of evidence suggest that this expression results from colonisation of the aorto-pulmonary outflow tract by Pax3-positive cardiac neural crest cells. Firstly, the up-regulation of Pax3 expression, detected by RT-PCR, correlates temporally with the arrival of neural crest cells at the outflow tract in the rat (Fukushima and Morrisey-Kay, 1992), although the timing of colonisation has not previously been studied in the mouse. Secondly, up-regulation of Pax3 expression is not detected in cultured isolated hearts, deprived of a source of colonising neural crest cells in vitro, even though their overall development closely parallels that of hearts within intact cultured embryos. This strikingly normal development of embryonic hearts in organ culture has also been noted by other workers (Sorokin et al., 1994).

Thirdly, isolated neural tubes from the occipital region of 8.5-day embryos give rise to neural crest cell outgrowths that express Pax3. Fourthly, in situ hybridisation studies reveal a stream of Pax3-positive cells migrating along the pathway described for cardiac neural crest in the chick: from the occipital region, through branchial arches 3, 4 and 6 and into the aortic sac and outflow tract.

This evidence strongly suggests that cells of the cardiac neural crest express Pax3 during their migration to the developing mouse heart. Indeed, recent studies demonstrate that chick cardiac neural crest cells also express Pax3 (Conway, Kirby and Copp, unpublished). Upon arrival at their destination, cardiac neural crest cells appear to cease expression of Pax3, at least at the level of detection of RT-PCR and in situ hybridisation. We speculate that down-regulation of Pax3 marks the onset of cytodifferentiation in the cardiac neural crest which, in the chick, has been shown to form ectomesenchymal components of the outflow tract septum and walls of the great vessels (Kirby and Waldo, 1990).

It is important to consider the possibility that the Pax3-positive cells we observe are not, in fact, neural crest but another cell type. Migratory muscle progenitor cells that originate from the dermomyotome express Pax3 and have been shown to be depleted in splotch embryos (Bober et al., 1994; Goulding et al., 1994; Fig. 7A,B) concomitant with a loss of c-met expression, which is also expressed by the muscle progenitors (Daston et al., 1996; Yang et al., 1996). However, muscle precursors are not known to follow the pathway of migration observed in the Pax3 whole-mount in situ preparations. Moreover, although these cells express CrabpI, they do not appear to express either CrabpI (Gustafson et al., 1993) or Prx1 (Fig. 10A,B). In contrast, the migratory cells observed in the present study were positive for CrabpI and Prx1, but negative for CrabpI (data not shown). Another possibility is that the Pax3-positive cells represent the vagal nerve supply to the developing heart and great vessels. This seems unlikely in view of the findings with in situ hybridisation which clearly indicate that Pax3 expression is present in a stream of migrating cells, rather than within nerve axons. Moreover, use of an anti-neurofilament antibody at 10.5 to 12.5 days of gestation has revealed that the vagus nerve is present in Sp²H/Sp²H embryos, and appears to innervate the heart in a relatively normal fashion (Conway, Oey and Copp, unpublished). We conclude, therefore, that Pax3 expression is likely to mark the cardiac neural crest cell lineage in the mouse.

Although Pax3, Hoxa-3, CrabpI, Prx1, Prx2 and c-met all appear to be expressed in migrating cardiac neural crest, there were notable variations in the pattern of their expression in the neural crest as a whole. For instance, Hoxa-3, CrabpI and Prx1 appeared to mark a wider range of cranial neural crest cells than Pax3. Although Pax3 transcripts were present in neural crest emerging from rhombomeres 2 and 4 at 9.5 days, there was no expression in branchial arches 1 and 2 at 10.5 days, suggesting rapid down-regulation in these neural crest sub-populations. In contrast, Hoxa-3 and Prx1 were expressed in branchial arches 1 and 2, and CrabpI in arch 1, at 10.5 days. This suggests that Pax3 expression is transient in most neural crest cells, and may persist only in the cardiac neural crest which emerges from the neural tube between rhombomeres 6 and the third somite (Kirby and Waldo, 1990). The finding of low level Hoxa-3 expression in branchial arches 1 and 2 was unexpected, since it had not been reported in previous studies (Manley and Capocci, 1995). It is interesting to note, however, that targeted mutations in Hoxa-3 cause defects of branchial arch 2 (Chisaka and Capocci, 1991), so our finding could provide an explanation for this observation.

We found that not only Pax3, but also Hoxa-3, CrabpI, Prx1 and 2, and c-met all exhibit reduced expression in the cardiac neural crest region of 10.5 day Sp²H/Sp²H embryos strongly suggesting a severe reduction or absence of this neural crest sub-population in the splotch mutant. More than 50% of the 10.5 day Sp²H/Sp²H embryos studied had no detectable Pax3-positive cardiac neural crest cells, and we suggest that these embryos go on to develop PTA, in which septation of the outflow tract is entirely absent (Franz, 1989). On the other hand, Pax3-positive cardiac neural crest cells could be detected, albeit at severely reduced levels, in other Sp²H/Sp²H embryos, and these homozygotes may be destined to achieve some degree of outflow tract septation, either developing DORV or a completely divided outflow tract (Conway, Henderson, Kirby, Anderson and Copp, unpublished). Thus, the mammalian heart appears to require a source of immigrating neural crest cells for the outflow tract to undergo normal septation, as demonstrated previously in avian systems (Kirby and Waldo, 1990).

The Sp²H mutant phenotype could result from either faulty separation of neural crest cells from the neural tube or defective migration and/or colonisation of target tissues by neural crest following the initiation of migration. Our results support the latter mechanism since we could detect neural crest cells initiating migration in an apparently normal manner from the
occipital neural tube of mutant embryos both in vivo and in vitro. This is consistent with a previous study (Moase and Trasler, 1990) of the Sp and Sp
d alleles, in which mutant and wild-type neural crest cells were found to be present in similar numbers in 72-hour cultures, as in the present study. Moase and Trasler (1990) found delayed initial emigration of neural crest from splotch homozygous neural tubes in vitro, although we have not observed this at 24 and 48 hour time points in our cultures (unpublished data).

Apart from Pax3, a number of other genes appear to play a role in the development of cardiac neural crest. Candidate genes for DiGeorge syndrome have recently been isolated from the 22q11.2 region (Halford et al., 1993a,b; Budarf et al., 1995). Although Pax3, which maps to human chromosome 2q, is not a candidate for the DiGeorge gene, it will be interesting to determine whether it is regulated during development by the DiGeorge causative gene(s). In the mouse, cardiac neural crest to determine whether it is regulated during development by the other tissues (Ruberte et al., 1991; Rowe et al., 1994). A targeted mutation in NF-1 has been reported to produce double outlet right ventricle (Branman et al., 1994; Jacks et al., 1994) and this defect is also seen at low frequency in mice homozygous for a null mutation of the mouse. Mice doubly homozygous for RAR mutations exhibit cardiac anomalies, ventricular septal defects and aortic arch defects (Mendelsohn et al., 1994). RARs, particularly a and g, are expressed in the neural crest as well as in other tissues (Ruberte et al., 1991; Rowe et al., 1994). A targeted mutation in ET-1, where the predominant abnormalities affect the aortic arches and ventricular septum (Kurihara et al., 1995). In the Sox-4 knockout mouse (Schilham et al., 1996), common arterial tract defects occur in a similar fashion to the splotch mutant. However, the Sox-4 gene is strongly expressed in the endocardial ridges of the outflow tract (Schilham et al., 1996), unlike Pax3, suggesting a quite different mechanism underlying outflow tract defects in the two mutants.

In conclusion, we have confirmed that a cardiac neural crest lineage exists in the mouse, as previously demonstrated in avians, and have demonstrated that mutant splotch mice exhibit abnormalities of this lineage during development of conotruncal heart defects. Our studies demonstrate a number of genes that are expressed by migrating cardiac neural crest cells, and studies of other spontaneous and targeted mouse mutants reveal further genes that appear to be required for normal development of this cell lineage. These findings provide an opportunity for an in-depth analysis of the molecular mechanisms underlying development of the cardiac neural crest.

We thank Margaret Kirby and Bob Anderson for valuable advice, and Peter Scambler for reading a draft of the manuscript. cDNA probes were kindly provided by Peter Gruss (Pax3), Paul Hunt and Stephen Gaunt (Hoxa-3), Pierre Chambon (CrabpI), Michael Kern (Ptx1 and 2) and Ermanno Gherardi (c-met). This work was supported by the British Heart Foundation and the Wellcome Trust.

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