Development of a lethal congenital heart defect in the splotch (Pax3) mutant mouse

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Abstract

Objective: The splotch (Sp2H) mutation disrupts the Pax3 gene and is lethal in homozygotes. The aim of the present study was to investigate the cause of lethality. Methods and results: Using the splotch (Sp2H) mouse mutant, we demonstrated that approximately 60% of Sp2H homozygotes die in utero at 13.5–14.5 days of gestation. All these embryos have cardiac malformations involving partial or complete failure of septation of the outflow tract. Although the cause of death in utero is unknown, the dying embryos are edematous, their superior caval veins are over-expanded, and the fetal liver is enlarged and engorged with blood, all signs of cardiac failure. The remaining Sp2H homozygotes die around the time of birth, and these embryos have grossly normal hearts. All Sp2H homozygotes have neural tube defects, either spina bifida, exencephaly, or both. Although these defects clearly do not cause death in utero, they are very likely responsible for the perinatal death of homozygotes that survive to late gestation. There is no correlation between the presence or absence of a cardiac defect and the type of neural tube defect. On the other hand, there is a striking correlation between presence of a cardiac defect and reduction or absence of dorsal root ganglia, which are derivatives of the neural crest. Conclusions: In this paper, we show that the lethality has a biphasic pattern, and the data strongly suggests that mid-gestation lethality is due to cardiac defects and not the associated neural tube defects. This finding supports the idea that ‘conotruncal’ cardiac defects involving the ventricular outflow tracts develop as a result of failure of the ‘cardiac’ neural crest to colonise the developing heart in the mid-gestation embryo, and that the resulting heart defects are solely responsible for the observed mortality. © 1997 Elsevier Science B.V.

Keywords: Mouse mutant; Congenital heart disease; Neural tube defects; Pax; Splotch; Cardiac neural crest; Heart failure

1. Introduction

Congenital heart disease continues to be the most prevalent category of major birth defects in humans [1]. Although some cardiac defects can be diagnosed before birth, and later corrected by surgical intervention, there are no methods routinely available for preventing the development of these malformations. If we are to develop preventive measures, it is important to gain a detailed understanding of the embryonic mechanisms that underlie the abnormal formation of the heart. This goal is greatly facilitated by the availability of an increasing number of mutant mouse strains in which the causative genes are known, and in which heart defects develop resembling those seen in humans [2,3]. Such mouse mutants provide model systems in which to probe the molecular and cellular basis of abnormal cardiac development.

Mutations at the splotch locus are known to result from disruption or deletion of the Pax3 transcription factor [4]. Homozygotes have long been known to develop neural tube defects, both exencephaly and spina bifida, and defects in neural crest-dependent structures such as the dorsal root ganglia and melanocytes [5]. Moreover, homozygotes for the Sp1H allele have been described as developing defects within the ventricular outflow tract, specifically...
common arterial trunk (also known as persistence truncus arteriosus, PTA) [6]. In the avian embryo, a common trunk results when a specific sub-population of neural crest cells (the ‘cardiac neural crest’) fails to colonise the developing outflow tracts [7,8]. Since splotch embryos are known to have neural crest-related defects, this provides a possible explanation for the development of this cardiac defect in the homozygous mutants.

One of the most intriguing features of the Sp, Sp\(^{1H}\) and Sp\(^{2H}\) mutant alleles is the mortality of homozygotes around day fourteen of gestation [5]. In previous studies, it was observed that all homozygotes die prenatally [5,6], making it difficult to determine which aspect of the mutant phenotype is responsible for the lethal effect. However, we observed that only around 60% of Sp\(^{2H}\) homozygotes die at day 14, with the remainder surviving to term. This provided an opportunity for a detailed analysis of the developmental defects exhibited by both dying and surviving Sp\(^{2H}\) homozygous embryos, offering the potential to determine the cause of the prenatal mortality. The objective of the present study was to investigate the cause of lethality, and in this paper we show that there is no correlation between the type or severity of neural tube defect and death in utero of Sp\(^{2H}\)/Sp\(^{2H}\) embryos. On the other hand, there is a striking correlation between the presence of malformations within the ventricular outflow tract and mortality on days 13–14 of gestation, suggesting strongly that cardiac defects are the cause of prenatal death in the mutant embryos. Moreover, diminution in the size of the dorsal root ganglia, a marker of defective neural crest, is strongly associated with the presence of a common arterial trunk, providing support for a link between abnormal neural crest and cardiac malformations in splotch mice.

2. Materials and methods

2.1. Mouse strains

Sp\(^{2H}\) is a radiation-induced allele at the splotch locus on the C3H/101 background [9]. 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 to produce Sp\(^{2H}/\)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 hearts were removed from embryos in cold PBS and then stored in the same buffer. Microdissection of the hearts was performed by the method of Pexieder [11]. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985).

2.2. Genotyping of embryos

Since splotch is a recessive lethal mutation, embryos must be generated by matings between Sp\(^{2H}/\) + individuals. This yields three categories of offspring: Sp\(^{2H}/\)Sp\(^{2H}\), Sp\(^{2H}/\) + and +/+ . In order to perform comparisons between mutant and normal embryos it is essential to be able to identify the various genotypes early in development, before the appearance of morphological abnormalities. Genotyping was performed using the polymerase chain reaction (PCR) to identify the 32 base pair deletion present in the Sp\(^{2H}\) mutant allele of Pax3 [4]. Genomic DNA was isolated from the yolk sac and/or a limb bud as described previously [10]. Oligonucleotide primers that flank the deletion [4] were used to amplify a DNA fragment of length 127 bp (wild type) or 95 bp (Sp\(^{2H}\)). PCR conditions were: initial denaturation, 95°C for 5 min; 30 cycles of amplification, 94°C for 30 s, 60°C for 1 min, 72°C for 30 s; final extension, 72°C for 10 min. Amplified Pax3 fragments were analysed by electrophoresis on a 2.5% agarose gel in TAE buffer: 0.04 M Tris-acetate, 1 mM EDTA (pH 8.0).

2.3. Histological examination

Embryos were fixed by immersion in Bouin’s fluid overnight, dehydrated through a graded series of alcohols and stored in absolute ethanol. Embryos were then cleared in Histoclear and embedded in paraffin wax. Serial sections of 10 μm thickness were cut on a Leica microtome, stained with haematoxylin and eosin, and mounted in DPX. The diameter of structures including the dorsal root ganglia and ventricular septal defect were measured using an eyepiece graticule on a Zeiss Axioskop microscope.

2.4. Scanning electron microscopy (SEM)

Hearts were removed from embryos in cold PBS and immersed overnight in a fixative comprising 2% glutaraldehyde, 1% formaldehyde in 0.1 M cacodylate buffer (300 mosmol/l). Following fixation, hearts were washed overnight in 0.1 M cacodylate buffer, postfixed for one hour in 0.1 M cacodylate buffer containing 1% osmium tetroxide, rinsed twice in isotonic 0.1 M cacodylate buffer, and then stored in the same buffer. Microdissection of hearts was performed by the method of Pexieder [11]. The hearts were then dehydrated through a graded series of ethanol and critical point dried using liquid CO\(_2\), at a temperature of 50°C and pressure of 1400 lb/m\(^2\).
2.5. Wet and dry weight measurements on embryos

All measurements were made on a Precisa 80A-200M balance. After measuring wet weight, the pericardium of the embryo was opened and the morphology of the outflow tract was recorded. Embryos were then dehydrated through a graded alcohol series and placed on paper filter discs for one week, prior to determination of dry weight.

3. Results

Our preliminary observations suggested that some Sp<sup>2H</sup>/Sp<sup>2H</sup> embryos survive to birth, in contrast to previous reports that all Sp<sup>5</sup> and Sp<sup>6</sup> homozygotes die in utero. We set out, therefore, to determine whether homozygosity for the Sp<sup>2H</sup> allele is lethal, and at which stage of gestation.

3.1. Variable stage of lethality of Sp<sup>2H</sup> homozygotes

We carried out a detailed study of the survival of embryos in litters from matings between Sp<sup>2H</sup>/q individuals. Litter size was found to be relatively constant, at an average of 9.2 embryos per litter, between 9.5 and 13.5 days of gestation, but from 14.5 days onwards there was a decrease in mean litter size and, between 16.5 and 19.5 days, litters contained an average of only 5.4 embryos (Fig. 1a). One way analysis of variance showed that the differences in the mean values among the 9.5 and 13.5 days of gestation group and the 16.5 and 19.5 days are greater than would be expected by chance, and are statistically significant (P < 0.001). These results indicated that a sub-population of embryos die in utero. Indeed, dying embryos (defined as lacking a pulsating stream of blood within the umbilical artery/vein and an absence of contractions of the heart) were detected between 11.5 and 15.5 days of gestation, comprising up to 20% of each litter (Fig. 1b), although dying embryos were not found prior to 11.5 days nor between 16.5 and 18.5 days. Dying fetuses could again be detected in litters at 19.5 days, just prior to birth. The number of dying embryos observed on any one day of gestation was smaller than the magnitude of the reduction in litter size. The cumulative effect of loss of

![Fig. 1. Histograms illustrating the loss of approximately 60% of Sp<sup>2H</sup> homozygotes around 14.5 days of gestation. (a) Mean number of embryos per litter is constant until 13.5 days when a steady decrease occurs until 16.5 days. Thereafter, litter size is relatively constant until birth. A total of 112 litters were analysed in this study. (b) Non-viable embryos are observed in Sp<sup>2H</sup> litters between 11.5 and 15.5 days, and further dying fetuses can be detected just prior to birth, at 19.5 days. A total of 59 non-viable embryos were found out of a total of 901 embryos studied. (c) Litters (viable embryos only) between 9.5 and 13.5 days contain wild type, heterozygous and homozygous mutant embryos in a 1:2:1 ratio. However, from 14.5 days onwards, there is a decline in the proportion of Sp<sup>2H</sup>/Sp<sup>2H</sup> embryos, suggesting a loss of approximately 60% of the homozygotes at this time. Total number of viable embryos studied: qrq 233, Sp<sup>2H</sup>/q 456, Sp<sup>2H</sup>/Sp<sup>2H</sup> 153.

<table>
<thead>
<tr>
<th>Embryo genotype</th>
<th>Neural tube defects (no. embryos)</th>
<th>Dorsal root ganglia mean area ± SEM (no. embryos)</th>
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<tr>
<td></td>
<td>Total embryos</td>
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<td>+/+</td>
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<td>70</td>
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<tr>
<td>Sp&lt;sup&gt;2H&lt;/sup&gt;/+</td>
<td>139</td>
<td>134</td>
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<td>0</td>
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<td>31</td>
<td>0</td>
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</tbody>
</table>

* Distribution of neural tube defects differ significantly among embryo genotypes (χ<sup>2</sup> = 255.4, P < 0.0001).
* Areas (in mm<sup>2</sup>) measured on ganglia at their maximal diameter in sections at the level of the cardiac outflow tract. Analysis of variance on ranks shows that ganglion areas differ significantly between embryo genotypes (H = 25.1, P = 0.000015). Sp<sup>2H</sup>/Sp<sup>2H</sup> embryos with heart defects have significantly smaller ganglia than +/+ and Sp<sup>2H</sup>/+ embryos (P < 0.05) although the difference is not significant between Sp<sup>2H</sup>/Sp<sup>2H</sup> embryos with and without heart defects.
embryos on several successive days of gestation can probably account entirely for the observed reduction in sizes of litters.

*Pax3* genotyping revealed that all embryos dying between 11.5 and 15.5 days were *Sp<sup>2H</sup>* homozygotes, never heterozygotes or wild type embryos. A striking finding, however, was that only around 60% of the *Sp<sup>2H</sup>* homozygotes died during this period of gestation, whereas the remaining 40% survived to birth (Fig. 1c). Since living *Sp<sup>2H</sup>* homozygotes have never been observed postnatally in our colony, we conclude that there is a second stage of mortality of *Sp<sup>2H</sup>*<sub>r</sub>*Sp<sup>2H</sup>* individuals, beginning just prior to birth (Fig. 1b) and continuing in the immediate postnatal period.

3.2. Mortality in utero of *Sp<sup>2H</sup>* homozygotes is not caused by their neural tube defects

We found that the presence of a neural tube defect is invariable in *Sp<sup>2H</sup>/Sp<sup>2H</sup>* embryos, although the nature of the defect was variable (Table 1). Embryos can have either lumbosacral spina bifida, or exencephaly, or both (Fig. 2a,b). There was no correlation, between the type of neural tube defect and the in utero mortality of *Sp<sup>2H</sup>* homozygotes. For instance, if a particular type of neural tube defect were lethal in utero, then one would expect to see a reduction in the frequency of embryos with this defect late in gestation. In contrast, both spina bifida and exencephaly were equally frequent in embryos at 11.5–12.5 days of gestation and in fetuses near term (Fig. 2c), making it seem very unlikely that neural tube defects are the cause of death of around 60% of *Sp<sup>2H</sup>/Sp<sup>2H</sup>* embryos between 13.5 and 15.5 days. On the other hand, neural tube defects were very likely the cause of death in homozygotes surviving to birth, as is seen in other mouse mutants with comparable defects [12].

3.3. A proportion of *Sp<sup>2H</sup>* homozygotes have congenital cardiac defects

Since the presence of neural tube defects cannot easily explain the death of a proportion of *Sp<sup>2H</sup>* homozygotes, we next considered the possibility that the presence of congenital heart malformations may be the critical factor. We performed a detailed study of cardiac morphology in 12.5 day and 13.5 day embryos obtained from litters of *Sp<sup>2H</sup>/Sp<sup>2H</sup>* mice. Observations made by SEM on whole hearts, and by serial histological sectioning, revealed three main types of cardiac morphology. Normal hearts were found in all wild-type and heterozygous embryos examined. Moreover, 42.3% of *Sp<sup>2H</sup>/Sp<sup>2H</sup>* embryos at 12.5 days, and 40.6% at 13.5 days (Table 1), also had hearts with normal morphology. The remaining *Sp<sup>2H</sup>/Sp<sup>2H</sup>* embryos exhibited conotruncal heart defects that comprised either a common arterial trunk arising from the right ventricle (PTA; 50.0% of homozygotes at 12.5 days and 53.1% at 13.5 days) or double outlet of aorta and pulmonary trunk from the right ventricle (DORV; 7.7% of homozygotes at 12.5 days and 6.3% at 13.5 days).

Fig. 3 illustrates the appearance of these cardiac phenotypes at 13.5 days in scanning electron micrographs, and Fig. 4 shows representative histological sections through the region of the ventricular outflow tracts. In the normal 13.5 day embryo, the aorta and pulmonary trunk are separate vessels (Fig. 3a and Fig. 4a,b) connecting, respectively, with the left and right ventricles. This morphology was seen in all wild-type and heterozygous hearts and in around 40% of *Sp<sup>2H</sup>/Sp<sup>2H</sup>* hearts. In contrast, in the
majority of the $Sp^{2H}/Sp^{2H}$ embryos the ventricular outflow tracts were completely undivided (Fig. 3b and Fig. 4c), with a common arterial trunk arising solely from the right ventricle via a single arterial valve. Communication of the arterial trunk with the left ventricle was never observed other than via a co-existing ventricular septal defect. The pulmonary arteries were observed arising as separate vessels taking origin directly from the common trunk (Fig. 4d), but these arteries were invariably of smaller diameter than the pulmonary arteries of the normal embryo. In a smaller proportion of $Sp^{2H}/Sp^{2H}$ embryos, a less severe defect, double outlet right ventricle, was observed. In these mutant embryos the ventricular outflow tracts had divided to form the aorta and pulmonary trunk, but the vessels were abnormally positioned relative to each other, and both arose exclusively from the right ventricle via two separate arterial valves (Fig. 3c). In homozygotes with either common arterial trunk or double outlet right ventricle, the endocardium, myocardium and valves all appeared histologically normal, indicating the specific nature of the cardiac defects in $Sp^{2H}$ homozygotes.

Sections through the ventricles of normal 13.5 day embryos revealed the ventricular septum to be intact (Fig. 5a). This was seen not only in $+/+$ and $Sp^{2H}/+$ embryos, but also in $Sp^{2H}/Sp^{2H}$ embryos with normally septated outflow tracts. By contrast, all $Sp^{2H}/Sp^{2H}$ embryos with abnormal ventriculo-arterial connections, either common arterial trunk or double outlet right ventricle,
exhibited a co-existing ventricular septal defect of variable size (Fig. 5b–d) which was not seen to close, even in Sp<sup>2H</sup> homozygotes that were dying at the time of dissection from the uterus. The interventricular communication between the left and right ventricles was directly adjacent to the membranous portion of the ventricular septum, the formation of the muscular outlet septum otherwise appears normal. To investigate in more detail the mechanics of closure of the ventricular septum, we measured the size of the interventricular communication at an earlier stage, 12.5 days, when the septum is open in both normal and malformed hearts (Table 2). Although there was no difference among the various embryo genotypes in diameter of the ventricles, the interventricular communication was approximately twice as large in Sp<sup>2H</sup>/Sp<sup>2H</sup> embryos with abnormal ventricular outflow tracts when compared to the embryos with normal outflow tract separation, demonstrating abnormal formation of the ventricular outlet components the defective hearts.

3.4. Death in utero results from cardiac failure in embryos with cardiac defects

Observations on embryos at 13.5 and 14.5 days of gestation revealed a striking correlation between the pres-
ence of cardiac abnormalities and mortality in utero. Embryos were judged as dying if pulsatile release of blood from the umbilical vessels was absent when the embryos were dissected from the uterus. The circulation in the non-viable Sp<sup>2H</sup> homozygotes appeared to ‘ebb and flow’, as in early development. Non-viable Sp<sup>2H</sup> homozygotes all had common arterial trunk or double outlet right ventricle, whereas Sp<sup>2H</sup> homozygotes with normal hearts appeared viable when removed from the uterus at this early fetal stage. Indeed, all the Sp<sup>2H</sup> homozygotes surviving past 14.5 days of gestation did not have any heart defects. Histological analysis of the Sp<sup>2H</sup> homozygotes with outflow tract defects revealed signs of cardiac failure: the superior caval veins were over-expanded Fig. 4b and the fetal liver was enlarged and engorged with blood. Moreover, measurement of wet and dry embryonic weights revealed that the wet/dry ratio was significantly higher in Sp<sup>2H</sup>/Sp<sup>2H</sup> embryos with cardiac defects (mean wet weight/dry weight in mg = 117.9/6.2 = 19.0) (P < 0.05) than in Sp<sup>2H</sup>/Sp<sup>2H</sup> embryos with normal hearts (134.5/8.2 = 16.4), in Sp<sup>2H</sup>/ + embryos (138.1/8.9 = 15.5) or in +/ + embryos (137.5/9.2 = 15.0). This finding, taken together with the edematous appearance of Sp<sup>2H</sup> homozygotes (Fig. 2b), suggests that Sp<sup>2H</sup>/Sp<sup>2H</sup> embryos

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<sup>a</sup> Maximum diameter measured across both ventricles. Values do not differ significantly between genotypes (one way analysis of variance, F = 1.13, P > 0.05).

<sup>b</sup> Distance from the free edge of the muscular part of the ventricular septum to the ridges of the atroventricular cushions. Values differ significantly between embryo genotypes (analysis of variance on ranks, H = 9.5, P = 0.023). Sp<sup>2H</sup>/Sp<sup>2H</sup> embryos with heart defects have significantly larger ventricular communications than +/+ embryos (P < 0.05) but do not differ significantly from the other genotypes.

Fig. 5. Transverse histological sections through the heart, at the level of the interventricular septum, in late 13.5 day embryos. (a) Sp<sup>2H</sup>/ + heart showing normal morphology. The right (R) and left (L) ventricles are separated by a thick, muscular septum (S). (b) Sp<sup>2H</sup>/Sp<sup>2H</sup> heart with common arterial trunk. There is a wide communication between the ventricles in the apical part of the septum (arrowhead). (c) Another Sp<sup>2H</sup>/Sp<sup>2H</sup> heart with common arterial trunk in which the ventricular septal defect (arrowhead) is very small. Higher magnification (d) shows that the defect is patent, as blood cells can be seen passing between the ventricles. Scale bar represents 0.2 mm.

were dissected from the uterus. The circulation in the non-viable Sp<sup>2H</sup> homozygotes appeared to ‘ebb and flow’, as in early development. Non-viable Sp<sup>2H</sup> homozygotes all had common arterial trunk or double outlet right ventricle, whereas Sp<sup>2H</sup> homozygotes with normal hearts appeared viable when removed from the uterus at this early fetal stage. Indeed, all the Sp<sup>2H</sup> homozygotes surviving past 14.5 days of gestation did not have any heart defects. Histological analysis of the Sp<sup>2H</sup> homozygotes with outflow tract defects revealed signs of cardiac failure: the superior caval veins were over-expanded Fig. 4b and the fetal liver was enlarged and engorged with blood. Moreover, measurement of wet and dry embryonic weights revealed that the wet/dry ratio was significantly higher in Sp<sup>2H</sup>/Sp<sup>2H</sup> embryos with cardiac defects (mean wet weight/dry weight in mg = 117.9/6.2 = 19.0) (P < 0.05) than in Sp<sup>2H</sup>/Sp<sup>2H</sup> embryos with normal hearts (134.5/8.2 = 16.4), in Sp<sup>2H</sup>/ + embryos (138.1/8.9 = 15.5) or in +/ + embryos (137.5/9.2 = 15.0). This finding, taken together with the edematous appearance of Sp<sup>2H</sup> homozygotes (Fig. 2b), suggests that Sp<sup>2H</sup>/Sp<sup>2H</sup> embryos

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Table 2

Closure of the interventricular septum in normal and malformed splotch hearts at 12.5 days of gestation

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with heart defects are fluid-overloaded and supports the idea that they die in utero from cardiac failure.

3.5. Sp2H cardiac defects correlate with neural crest defects, but not neural tube defects

We examined the association between abnormal ventriculo-arterial connections, neural tube defects and size of the dorsal root ganglia in Sp2H/Sp2H embryos. Reduced size of dorsal root ganglia probably results from defective migration of the neural crest in splotch homozygotes [5]. There was no discernible correlation between the presence or type of cardiac defect and the type of neural tube defect: for instance, combined spina bifida and exencephaly was equally common in Sp2H homozygotes with cardiac defects as in those with normal hearts (Table 1). In addition, around 4% of Sp2H/+ embryos exhibited neural tube defects, although combined spina bifida and exencephaly was not seen in heterozygotes. In contrast to the lack of correlation between cardiac and neural tube defects, there was a strong association between cardiac and neural crest defects (Table 1). Wild-type and heterozygous embryos had closely similar sized dorsal root ganglia whereas Sp2H/Sp2H embryos had significantly smaller ganglia. Most strikingly, Sp2H homozygotes with cardiac defects had very much smaller ganglia than homozygotes with normal hearts. Indeed, a number of homozygotes with abnormal ventriculo-arterial connections had no recognisable dorsal root ganglia at all, at the level of the anticipated contribution to the cardiac outflow tracts (Fig. 4b). These findings support the idea that the malformations of the ventricular outflow tracts result from failure of colonisation of the putative aorto-pulmonary septum by cells from the cardiac neural crest. It may be that severe diminution, or total absence, of the cardiac neural crest is necessary to yield a common arterial trunk or double outlet from the right ventricle, whereas persistence of sub-normal numbers of neural crest cells is sufficient to promote normal septation of the outflow tract. Indeed, it was shown that neural crest ablations of only part of the ‘cardiac neural crest’ region gives rise to predominantly double outlet, whereas complete ablation gives rise to predominantly common arterial trunk [12].

4. Discussion

We have examined litters of mouse embryos segregating the Sp2H mutant allele in order to determine the cause of in utero mortality of splotch homozygotes. Our results demonstrate that although Sp2H/Sp2H embryos invariably have neural tube defects, there is no evidence that the nature or severity of the defect has any influence upon whether the embryo dies around 13.5–14.5 days of gestation. On the other hand, we find that presence of either a common arterial trunk or double outlet from the right ventricle, is strongly associated with in utero death of Sp2H homozygotes.

4.1. Why are abnormal ventriculo-arterial connections associated with mortality in splotch embryos?

Mortality in utero is not normally considered a feature of common arterial trunk or double outlet right ventricle either in humans [14] or in the chick following neural crest ablation [7]. However, in recent video-microscopy studies of 12.5 and 13.5 day Sp2H/Sp2H hearts, we found poor myocardial contractility compared with normal litter mates. In Sp2H/Sp2H hearts with outflow tract defects, the left ventricular area ejection fraction was less than 50% when compared with normal hearts. However, mutant hearts exhibited normal contractile force in detergent-skinned ventricular muscle strips but, when excitation-contraction coupling was examined, Ca2+ transients were found to be undetectable. Ca2+ currents were examined using the perforated patch clamp technique on isolated ventricular myocytes, and the magnitude of the Ca2+ current was found to be reduced in Sp2H/Sp2H hearts with common arterial trunk (Conway, Greene, Godt et al.; unpublished). These findings, together with our observations of edema, fluid-overload, expanded caval veins and liver engorgement, suggest that Sp2H/Sp2H embryos with abnormal ventriculo-arterial connections are dying of cardiac failure in utero.

Lethality in Sp2H/Sp2H embryos seems most likely to result from a functional haemodynamic defect of the cardiovascular system that shares a common developmental origin with the abnormal ventriculo-arterial connections. Altered hemodynamics could arise, for instance, from a restrictive ventricular septal defect, as occurs with small septal defects in humans [15], or from alteration in the pattern of aortic arch arteries, as has been described in splotch homozygotes [6]. An alternative mechanism is suggested by studies in the chick, where a reduction in myocardial contractility has been detected in neural crest-ablated embryos prior to the appearance of structural heart defects [16,17], suggesting that the neural crest may exert an influence on the developing myocardium from an early stage in its development. A similar mechanism could operate in splotch embryos, although the nature of this putative interaction is currently unknown. What may be of significance is that all the embryos with abnormal hearts showed the arterial segment connected exclusively to the right ventricle. Clearly, further studies are required to determine the cause of cardiac failure in Sp2H/Sp2H embryos with abnormal hearts.

4.2. Requirement of the neural crest for normal development of the ventricular outflow tracts

The neural crest is an embryonic stem cell population that arises from the edges of the neural plate and migrates...
ventrally and laterally through the embryonic mesoderm, differentiating into a variety of cell types [18]. Labelling of pre-migratory neural crest cells, using the chick/quail cytological marker [19] has revealed that the aortopulmonary septum contains derivatives of the neural crest. Moreover, Dil labelling in the rat embryo [20] and tracing of neural crest migration using gene expression markers in the mouse [21] have both confirmed the existence of a ‘cardiac’ neural crest cell sub-population in mammals. Ablation of the chick pre-migratory cardiac neural crest leads to defective septation of the outflow tract in avian embryos [19] and it seems likely that defective neural crest migration in splotch embryos is similarly responsible for the presently observed cardiac defects. Indeed, we observed a marked reduction or absence of cardiac neural crest cells, as recognised by their expression of Pax3, Hoxa3, Crabp1, Prx1, Prx2 and c-met in 10.5 day Sp 2H/Sp 2H embryos [21]. In the present study, we found a close correlation between reduced size of dorsal root ganglia and presence of abnormal ventriculo-arterial connections, also pointing to a relationship between defective neural crest migration and failure of septation of the cardiac outflow tract. It should be noted, nonetheless, that the common feature of the abnormal hearts was origin of the arterial segment exclusively from the right ventricle rather than complete failure of septation, since the hearts with double outlet right ventricle had separate arterial trunks, in part, to the left ventricle. This is a most unusual finding within humans with this malformation, and points to more complex factors being involved. Similarly, within the chick neural crest-ablation system, the truncus arises predominantly over-riding the ventricular septum [16]. These differences may reflect different outflow tract architecture within the different species (Anderson, Kirby and Conway; unpublished observations).

The fact the that only approximately 60% of Sp 2H homozygotes die in utero at 13.5–14.5 days of gestation, and have cardiac malformations involving partial or complete failure of septation, suggests that there is sufficient neural crest colonisation of the outflow tract within those Sp 2H homozygotes that survive until birth. Thus, there appears to be a minimum threshold number of cardiac neural crest cells that are required for complete septation of the outflow tract.

4.3. Relationship between neural crest migration and neural tube closure

Homozygous Sp 2H embryos are characterised by defects of both closure of the neural tube and migration from the neural crest. This led Auerbach [5] to suggest that both types of developmental defect may result from an abnormality of dorsal neuroepithelial cells that comprise the presumptive neural crest and the site of fusion of the apposing neural folds during neurulation. However, there is no obligatory relationship between neural crest migration and neural tube closure. For instance, neural crest cells emigrate from the neuroepithelium prior to closure in the cranial region but following closure in the spinal region of avian and mammalian embryos. Moreover, neural tube defects have been observed in the presence of apparently normal dorsal root ganglia in the cranial region of splotch mice [22] and in the lumbosacral region of splotch/curly tail double mutant mice [23]. In the present study we show that there is no particular association between the type or severity of neural tube defect and the presence of neural crest-related heart defects. It seems most likely, therefore, that defects due to closure of the neural tube and migration from the neural crest are independent effects of a lack of the Pax3 transcription factor. Deficiency in the migration of somite-derived myoblast precursors to form the limb musculature is also observed in splotch homozygotes [24–26] and this may represent a third requirement for the Pax3 gene product. Recently, it has been shown that Pax3 is a key regulator of skeletal myogenesis, in that Pax3 expression is sufficient to induce expression of MyoD, Myf-5 and myogenin basic helix-loop-helix myogenic transcription factors [27]. There is no expression of MyoD, Myf-5 and myogenin in cardiac muscle, but as many of the genes that are regulated by them are expressed in both skeletal and cardiac muscle [3], this indicates that MyoD, Myf-5 and myogenin are not the targets of Pax3-regulation in the heart. Currently, it is not known if Pax3 also plays a role in cardiac myogenesis or even if it is expressed in the embryonic heart.

4.4. Developmental role of the Pax3 transcription factor

The Sp 2H mutation involves a 32 base pair deletion in the homeodomain, one of the two main DNA-binding regions, of the Pax3 gene [4]. This deletion leads to formation of a stop codon so that the C-terminal portion of the homeodomain is missing from the mature protein. DNA-binding studies in vitro have shown that an expressed protein with structure similar to the predicted truncated Pax3 protein in Sp 2H has reduced DNA-binding activity with an altered specificity for target sequences [28]. The other splotch alleles disrupt the Pax3 gene in varying ways: Sp’ and Sp 4H involve deletion of the entire gene, Sp involves a point mutation at a splice acceptor site that leads to aberrantly spliced transcripts and Sp d has a point mutation in the paired box region. Sp 1H is probably an identical mutation to Sp 2H, since both mutations were isolated from the same mutagenised male [9]. It seems likely that each mutation results in loss of some or all of the functions of the Pax3 gene in controlling downstream development.
gene expression. Until the downstream targets of Pax3 are determined, it will not be possible to explain the variation in phenotype, for instance, between Sp2H in which homozygotes develop exencephaly, spina bifida and heart defects, and Sp2I in which only spinal neural tube defects are seen in homozygotes.

Pax3 is expressed in the dorsal part of the neural tube, throughout the early somite, in the dermomyotome, in muscle precursors that migrate from the ventrolateral portion of the somite to colonise the limb, and in the cardiac and other early migrating neural crest [21,25,26,29]. Recent studies have suggested several likely targets for Pax3 gene regulation at these sites, including N-cadherin, c-met, Msx2 and NCAM. For instance, N-cadherin expression co-localises with Pax3 in the developing somite and dermomyotome [30], while c-met expression is absent from the dermomyotome and limb muscle precursors in splotch mutant embryos [30,31]. Similarly, in the neural tube, Msx2 exhibits a closely similar pattern of expression to Pax3 [32], whereas NCAM expression is altered in splotch mutants [33]. It is difficult to determine from gene expression studies of this type whether the putative regulatory interactions are direct or mediated via intermediate gene products. Further insight is likely to emerge from cell transfection experiments in which the action of Pax3 in regulating target gene expression can be tested directly.

There is an ever increasing list of different gene mutations that show similar embryonic heart and neural tube defects (e.g., RXR alpha knockout mice [34]) which also exhibiting different combinations of neural crest-related heart defects [2,3], so that it should be possible in future to gain insight into the mechanisms underlying human cardiovascular development and congenital heart defects.

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References


