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Insertion of Cre into the *Pax3* locus creates a new allele of *Splotch* and identifies unexpected *Pax3* derivatives

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Abstract

Pax3 is a transcription factor expressed in the dorsal neural tube and somite of the developing embryo. It plays critical roles in pre-migratory neural crest cells and in myogenic precursors of skeletal muscle. Pax3-deficient *Splotch* embryos display neural tube and neural crest defects and lack hypaxial muscles. We have created a new allele of *Splotch* by replacing the first coding exon with a gene encoding Cre recombinase. This functions as a null allele and no Pax3 protein is detected in homozygous embryos. Heterozygous $Pax3^{Cre/+}$ mice display a white belly spot, as do *Splotch* heterozygotes. Homozygous $Pax3^{Cre/Cre}$ embryos are embryonic lethal. We have used $Pax3^{Cre/+}$ mice to fate-map Pax3 derivatives in the developing mouse. As expected, neural crest and some somitic derivatives are identified. However, we also detect previously unappreciated derivatives of Pax3-expressing precursors in the colonic epithelium of the hindgut and within the urogenital system.

Keywords: Neural crest; Fate map; Cre recombinase; Enteric ganglia; Skeletal muscle

Introduction

Pax3 is a member of a small gene family characterized by the presence of a conserved 120 amino acid paired-type DNA binding domain (Chi and Epstein, 2002). Pax genes are expressed by various organs and tissues in the developing embryo, and they play critical roles in cell fate determination, survival, and proliferation of undifferentiated cells. In general, Pax gene expression diminishes during late stages of development in parallel with definitive differentiation. The precise functions of Pax genes within various precursor cell populations are the focus of ongoing studies, and the cell types derived from specific Pax gene-expressing precursors remain to be fully elucidated.

Pax3 is expressed by pre-migratory neural crest cells and by presomitic mesoderm (Goulding et al., 1991). Expression initiates at ~E8.5 and becomes restricted to the dorsal neural tube, where neural crest cells are specified, and to the maturing somites. Neural crest cells represent a multipotent population of migratory cells that give rise to the entire peripheral nervous system, to melanocytes, enteric ganglia, a subpopulation of vascular smooth muscle, bone, and cartilage of the face, and to various other cell types. *Pax3* plays a critical role during development of many neural crest derivatives. A spontaneous mutation in *Pax3* (Epstein et al., 1993), which results in absent Pax3 protein expression (Li et al., 1999), was identified in the 1950s because of a melanocyte defect in heterozygous mice (Auerbach, 1954). This phenotype accounts for the name "*Splotch*". Homozygous *Splotch* mice on a C57BL/6 genetic background display embryonic lethality by E14.5 due to cardiovascular defects, and numerous neural crest derivatives are absent or abnormal, including dorsal root ganglia, melanocytes, and enteric ganglia (Auerbach, 1954; Epstein et al., 1993; Lang et al., 2000). Severe neural tube defects are also present. Several alleles of *Splotch* have been described, including

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Sp, which is caused by a splicing mutation in intron 3 (Epstein et al., 1993), and Sp^{2H}, which is caused by an intragenic deletion (Epstein et al., 1991). A hypomorphic allele, Sp^{d}, caused by a missense mutation in the paired type DNA binding domain, is characterized by a less severe phenotype with homozygotes surviving until birth (Vogan et al., 1993). Neural crest deficiencies and related cardiovascular defects are reported to be less severe than those seen in loss-of-function alleles, while skeletal muscle defects are similar (Franz, 1993).

Several reports have examined the effect of Pax3 loss-of-function on neural crest migration and differentiation. Although initial reports suggested that Pax3 is required for proper neural crest migration (Conway et al., 1997a; Moase and Trasler, 1990), more recent studies indicate that Pax3 is not essential for migration. The absence of Pax3 may influence survival, proliferation, or differentiation of neural crest precursors and migrating neural crest cells which accounts for neural crest-related defects (Conway et al., 2000; Epstein et al., 2000). In *Splotch* embryos, cardiac neural crest cells migrate to the outflow tract of the heart, though outflow tract septation is deficient (Epstein et al., 2000). Enteric ganglia precursors migrate to the foregut, but *c-ret* expression is diminished and derivatives fail to populate the mid and hindgut (Lang et al., 2000). It is not clear if all neural crest derivatives are affected equally in the absence of Pax3, or if certain lineages are favored at the expense of others. Interestingly, facial derivatives of neural crest are largely spared in *Splotch* homozygotes, perhaps due to functional redundancy of the closely related *Pax7* gene (Borycki et al., 1999; Jostes et al., 1990; Mansouri et al., 1996). The complete repertoire of mature cell types derived from Pax3-expressing embryonic precursors has not been described.

Pax3 plays an important role in developing skeletal muscle (Maroto et al., 1997; Tajbakhsh et al., 1997). In particular, *Pax3* is required for proper development of hypaxial muscles, which derive from the lateral somite and undergo long-range migration to the ventral body wall, limbs, diaphragm, and tongue prior to differentiation (Ordahl and Le Douarin, 1992). Although *Pax3* is expressed widely in the presomitic mesoderm, expression becomes restricted to the hypaxial domain and, to a lesser extent, to the most medial portions of the somite, during mid-gestation. Migrating myoblasts initially continue to express *Pax3* and later down-regulate mRNA and protein expression as myogenic markers such as *MyoD* become apparent (Daston et al., 1996; Tremblay et al., 1998; Williams and Ordahl, 1994). *Pax3* functions genetically upstream of *MyoD*, since mice lacking *MRF4*, *Myf5*, and *Pax3* fail to express *MyoD* in any muscles below the neck (Kassar-Duchossoy et al., 2004; Tajbakhsh et al., 1997). Pax3-deficient embryos exhibit skeletal muscle defects and transgenic rescue experiments have demonstrated an exquisite requirement for *Pax3* during development of the diaphragm and limb musculature (Li et al., 1999).

Recently, we have described enhancer sequences located in the upstream genomic region of the *Pax3* gene that are sufficient to mediate dorsal neural tube and neural crest expression in transgenic mice (Li et al., 1999; Milewski et al., 2004). We have described transgenic mice in which the proximal 1.6 Kb of upstream genomic sequence directs expression of Cre recombinase (Li et al., 2000). Crosses with reporter mice indicate that many neural crest derivatives below the neck are labeled in mice carrying the Cre transgene and a Cre reporter. However, some potentially ectopic expression was noted in the lumbosacral region, and unexpected labeling of derivatives not previously thought to derive from neural crest, such as a small number of myocardial cells within the heart, suggested that this transgene might be "leaky" and/or might express in cells that do not normally express *Pax3* (Li et al., 2000). In addition, significant regions of the dorsal neural tube that normally express *Pax3* are not labeled by this transgene. Hence, we sought to develop a more faithful Pax3-Cre mouse by gene targeting. Here, we report successful targeting of Cre recombinase to the *Pax3* locus, and we demonstrate both expected and unexpected derivatives of *Pax3*-expressing precursors.

Materials and methods

Generation of $Pax3^{Cre/+}$ mice

The targeting vector backbone was based on the pPNT vector (Tybulewicz et al., 1991) modified to include a LoxP flanked PGK-neo cassette and a PGK-HSV thymidine kinase gene. The 5′ arm was 6.2 kb of the murine *Pax3* upstream genomic sequence fused with Cre and a bovine growth hormone polyadenylation site (derived from Li et al., 2000), a LoxP flanked PGK-neo cassette and 1.4 kb of *Pax3* 3′ flanking sequence derived from intron 1. Most of the first *Pax3* exon downstream of the transcription start site including the initiating ATG and 69 5′ flanking nucleotides was replaced.

The *Pax3CreKI* targeting vector was linearized by digestion with *Asc*I and electroporated into 2×10^7 R1 embryonic stem (ES) cells (Nagy et al., 1993) in a 0.4-mm cuvette at 300 V for 1 ms using a BTX electro cell manipulator. Five targeted clones out of 350 were identified by PCR and DNA from three of these PCR-positive ES cell clones was analyzed by Southern blotting using a 3′ flanking probe. One of three ES cell clones positive for homologous recombination by both PCR and Southern blotting was injected into C57BL/6J mouse embryos. Five (129/Sv × C57BL/6J) chimeras were generated. The $Pax3^{Cre}$ allele was maintained on a mixed 129/Sv × C57BL/6J genetic background. After passing through the male germline, the PGK-neo cassette was no longer present within the targeted locus.