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Cardiovascular malformations with normal smooth muscle differentiation in neural crest-specific type II TGFβ receptor (Tgfbr2) mutant mice

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Abstract

Previous studies have demonstrated that TGFβ induces a smooth muscle fate in primary neural crest cells in culture. By crossing a conditional allele of the type II TGFβ receptor with the neural crest-specific *Wnt1cre* transgene, we have addressed the in vivo requirement for TGFβ signaling in smooth muscle specification and differentiation. We find that elimination of the TGFβ receptor does not alter neural crest cell specification to a smooth muscle fate in the cranial or cardiac domains, and that a smooth muscle fate is not realized by trunk neural crest cells in either control or mutant embryos. Instead, mutant embryos exhibit with complete penetrance two very specific and mechanistically distinct cardiovascular malformations—persistent truncus arteriosus (PTA) and interrupted aortic arch (IAA-B). Pharyngeal organ defects such as those seen in models of DiGeorge syndrome were not observed, arguing against an early perturbation of the cardiac neural crest cell lineage. We infer that TGFβ is an essential morphogenic signal for the neural crest cell lineage in specific aspects of cardiovascular development, although one that is not required for smooth muscle differentiation.

Keywords: Neural crest; TGFβ; Type II receptor; Persistent truncus arteriosus; Interrupted aortic arch; DiGeorge syndrome

Introduction

Multipotent progenitor cells generally receive instructional cues that direct their differentiation and choice of fate. The neural crest cell lineage is one such progenitor population. Neural crest cells arise at all axial levels of the dorsal neural tube just prior to neural tube closure, and then migrate to peripheral locations. Fate mapping studies in avian embryos (LeDouarin, 1982) and more recently in mouse embryos (Jiang et al., 2000; Chai et al., 2000) have demonstrated that a variety of ectodermal and mesodermal fates are assumed by this cell lineage in normal development. Transplantation studies have demonstrated that neural crest cells isolated from the neural tube retain considerable plasticity when reintroduced into new locations (LeDouarin, 1982), leading to the inference that signals in the local environments into which the neural crest cells migrate direct the differentiation of these cells.

Among the many factors which are believed to instruct pleuripotent neural crest cells to differentiate to specific fates, TGFβ has for several years been suggested to promote smooth muscle differentiation. Thus, primary embryonic neural crest cells cultured in vitro will differentiate to smooth muscle with very high frequency when exposed to TGFβ, but not to a variety of other factors (Shah et al., 1996). Recent studies have suggested that TGFβ has differing effects on neural crest cells that arise at different axial levels: trunk neural crest cells assume a smooth muscle fate when exposed to TGFβ, whereas this fate is suppressed by TGFβ in cranial neural crest cells (Abzhanov et al., 2003). Because these studies have been undertaken with cells cultured in vitro, the in vivo relevance of these approaches has been uncertain.

As conventionally understood (Massague, 2000), TGFβ signals are received through cell surface receptors that obligatorily include the type II TGFβ receptor. This receptor

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is encoded by one gene (*Tgfbr2*), although other type II receptors that mediate other ligand signals are well known. In the presence of ligand, the type II receptor phosphorylates a type I receptor to initiate intracellular signal transduction. ALK5 is the conventional type I receptor for TGFβ signaling, although ALK1 has been suggested as an additional type I TGFβ receptor in endothelial cells, and ALK2 may be involved in mediating TGFβ signals in the endocardial cushions of the heart. In many cases, TGFβ signals are transduced via activation of Smad proteins, although several Smad-independent alternative pathways may also be important in certain processes (Derynck and Zhang, 2003).

Conventional knockout of the *Tgfbr2* gene in mice results in yolk sac hematopoietic and vasculogenic defects, causing visibly disrupted embryonic development by E9.5 and lethality by E10.5 (Oshima et al., 1996). A similar phenotype is obtained in mice lacking the type I receptor (ALK5) (Larsson et al., 2001). To circumvent this early phenotype, and to address the role of TGFβ and its receptors in neural crest biology, we crossed a conditional *Tgfbr2* allele (Chytil et al., 2002) with the *Wnt1cre* transgene (Danielian et al., 1998). This transgene directs highly efficient recombination of target genes specifically in the neural crest cell lineage at all axial levels of the embryo (Jiang et al., 2000; Chai et al., 2000). Homozygous *Wnt1cre/Tgfbr2* embryos are delivered at full term at normal size, although with severe cranial (Ito et al., 2003) and cardiovascular (see below) malformations, and die in the immediate postnatal period. We find that TGFβ signal reception by neural crest is essential for two very specific morphogenic processes in cardiovascular development – formation of the aorticopulmonary septum and preservation of the arch of the aorta – but is dispensable for smooth muscle specification and differentiation.

Materials and methods

The *Wnt1cre* (Danielian et al., 1998), *R26R* (Soriano, 1999) and conditional *Tgfbr2* (Chytil et al., 2002) alleles have been previously described. Xgal staining of embryos in whole mount and of frozen sections was as previously described (Jiang et al., 2000). Primary antibodies used were against smooth muscle α actin (monoclonal 1A4, Sigma), phospho-histone H3 (Upstate), and active caspase 3 (Promega), with HRP-conjugated secondary antibody and DAB/H_2O_2 detection. Rabbit polyclonal antibody against mouse tropoelastin (6–17) was kindly provided by Robert Mecham. For BrdU detection, pregnant females were injected with 100 μg BrdU/g body weight 2 h before sacrifice, followed by fixation and paraffin embedding, with immunodetection using reagents from Zymed. For in situ hybridization detection of parathyroid hormone, a digoxygenin-labeled antisense probe (corresponding to 97–534 of NM_020623.1) was generated by transcription in vitro from a template kindly provided by Nancy Manley, and following hybridization to paraffin sectioned tissue was detected with anti-digoxygenin alkaline phosphatase-coupled antibody (Roche) and BCIP/NBT substrate kit (Zymed). For detection of *Tgfbr2* transcript, a ^{35}S-UTP-labeled antisense probe representing exon 2 (422–590 of NM_009371.2) was hybridized to paraffin sections and visualized by exposure to photographic emulsion. Whole mount in situ hybridization utilized probes for CRABPI (285–435 of NM_013496) and AP2α (559–1559 of NM_011547). India ink visualization of embryonic vasculature was achieved by intracardiac injection, followed by fixation and clearing with methyl salicylate.

Results

Neural crest contribution to cranial smooth muscle

Neural crest constitutes the vast majority of cranial mesenchyme (Jiang et al., 2000; Chai et al., 2000), although paraxial mesoderm also contributes mesenchyme in the head. To assess the efficiency of recombination of the conditional *Tgfbr2* allele by the *Wnt1cre* transgene, we undertook in situ hybridization studies using a probe specific for exon 2 of the *Tgfbr2* gene, the exon that is deleted after cre-mediated recombination. The *Tgfbr2* gene is expressed in midgestation embryos in the first pharyngeal arch ectoderm and mesenchyme; when crossed to the *Wnt1cre* allele, signal is still readily detectable in ectoderm, but is reduced to background in neural crest-derived mesenchyme (Figs. 1A–B).

As vasculogenesis is initiated in development, endothelial cells of nascent vessels recruit mesenchymal cells in their vicinity to a smooth muscle fate, as exemplified by immunoreactivity against smooth muscle α actin (SMA). The neural crest origin of smooth muscle was assessed in control (*Wnt1cre* transgenic) and mutant (*Wnt1cre/Tgfbr2*) embryos via the

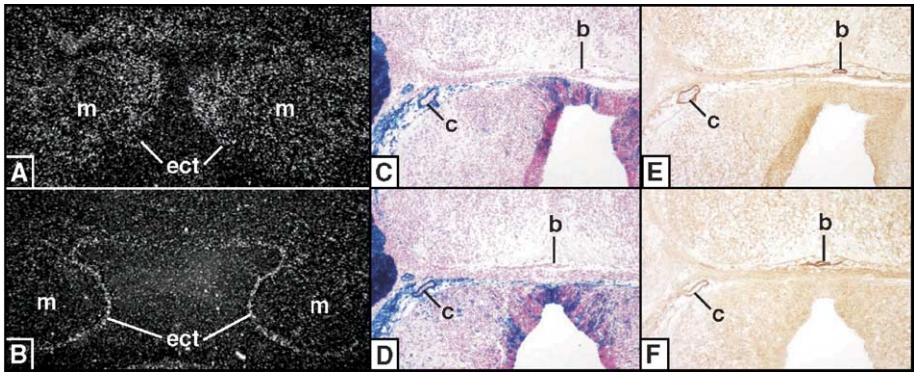

Fig. 1. Cranial neural crest contribution to smooth muscle. (A–B) Evaluation of *Wnt1cre*-mediated recombination of the conditional *Tgfbr2* allele. In situ hybridization using a probe specific for exon 2 of the *Tgrfbr2* gene shows expression in first arch mesenchyme (m; primarily neural crest-derived) and ectoderm (ect) in control E12.5 embryos (A), whereas in *Wnt1cre/Tgrfbr2* mutants (B), mesenchymal expression is abolished but ectodermal expression persists. (C–F) Near adjacent sections through control (C, E) or *Wnt1cre/Tgfbr2* (D, F) E13.5 embryos stained for Xgal (C–D) or anti-smooth muscle actin (E–F), emphasizing the basilar (b) and middle cerebral (c) arteries. Regardless of *Tgfbr2* status, the smooth muscle layer of the cerebral artery is neural crest-derived (Xgal-positive), whereas the smooth muscle layer of the basilar artery is mesodermal in origin (Xgal-negative).