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Midline signaling regulates kidney positioning but not nephrogenesis through Shh

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ABSTRACT

The role of axial structures, especially the notochord, in metanephric kidney development has not been directly examined. Here, we showed that disruption of the notochord and floor plate by diphtheria toxin (DTA)-mediated cell ablation did not disrupt nephrogenesis, but resulted in kidney fusions, resembling horseshoe kidneys in humans. Axial disruptions led to more medially positioned metanephric mesenchyme (MM) in midgestation. However, neither axial disruption nor the ensuing positional shift of the MM affected the formation of nephrons and other structures within the kidney. Response to Shh signaling was greatly reduced in midline cell populations in the mutants. To further ascertain the molecular mechanism underlying these abnormalities, we specifically inactivated *Shh* in the notochord and floor plate. We found that depleting the axial source of Shh was sufficient to cause kidney fusion, even in the presence of the notochord. These results suggested that the notochord is dispensable for nephrogenesis but required for the correct positioning of the metanephric kidney. Axial Shh signal appears to be critical in conferring the effects of axial structures on kidney positioning along the mediolateral axis. These studies also provide insights into the pathogenesis of horseshoe kidneys and how congenital kidney defects can be caused by signals outside the renal primordia.

Introduction

The kidney derives from the intermediate mesoderm (IM) that is a narrow strip of mesodermal cells sandwiched between the paraxial mesoderm and the lateral plate mesoderm. During embryogenesis, three sets of nephric structures (pronephros, mesonephros, and metanephros) emerge from the nephric cord within the IM in succession from an anterior to posterior direction. In mammals, the metanephric kidney is the definitive kidney. Some components of the transient mesonephric kidney contribute to the development of the metanephric kidney. The pair of epithelial ducts connecting the mesonephros to the cloaca is called the mesonephric duct or Wolffian duct (WD). During midgestation, the ureteric bud (UB) emerges from the WD (at about E10.5 in mice) and invades the metanephric mesenchyme (MM) within the IM. MM expresses a combination of factors important for its differentiation and the induction of the UB from the WD (Dressler, 2006). *Lim1*, *Pax2*, and *Pax8*, expressed early in both the WD and the MM, are indispensible for renal development. A delicate regulatory network modulates the activity of the receptor tyrosine kinase Ret and its ligand Gdnf, ensuring that only one UB emerges from the correct axial location of the WD (Schedl, 2007). Once inside the MM, the UB undergoes extensive branching morphogenesis. The tips of the UB induce the mesenchymal–epithelial transformation of the adjacent MM to form functional segments of the nephron. The UB later becomes the ureteral epithelium and the collecting ducts within the kidney (Dressler, 2006).

The notochord is a flexible, rod-shaped structure composed of cells derived from the mesoderm and defines the primitive axis in embryos of all chordates. It persists as the axial structural support throughout life in lower vertebrates, but is replaced by the vertebral column in higher vertebrates. In higher vertebrates, the notochord is not just a transient structural support for the embryos, but is also an important organizer for embryogenesis. The notochord runs along the anterior–posterior (AP) midline of the embryo and provides cues to the surrounding tissues for positioning and fate determination. It is well established that the notochord is essential for the formation of the floor plate of the neural tube in which the gradient generated by the *Hedgehog* (*Hh*) proteins, particularly *Sonic Hedgehog* (*Shh*), from the notochord is instrumental (Placzek, 1995; Stemple, 2005). In addition, Bmp inhibitors Noggin and Chordin from the notochord have also been thought to have key roles in guiding the development of surrounding

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structures (Stemple, 2005). Previous studies have indicated that signals with unknown nature from axial and paraxial tissues are involved in patterning of the intermediate mesoderm (Barak et al., 2005; James and Schultheiss, 2003; Mauch et al., 2000). It is also suspected that the renal agenesis in *Danforth's short tail* (*Sd*) homozygous mutants is caused by notochord degeneration (Maatman et al., 1997). However, the role of the notochord and floor plate on metanephric kidney development has not been directly examined. It is still unclear what the key signals are for determining the position of the MM and the exact tissues where these key signals come from.

In this study, we examine how midline signals from axial structures, especially the notochord and floor plate, influence metanephric kidney development by using Diphtheria Toxin (DTA)-mediated cell ablation to disrupt the notochord and floor plate of the neural tube. To our surprise, we found that, instead of renal agenesis, disruption of the notochord and floor plate results in the formation of horseshoe kidneys. Further analyses indicate that Shh signaling is particularly important for the axial effects on mediolateral positioning of the kidneys.

Materials and methods

Mouse (Mus musculus) strains and sample collection

All animal studies have been approved by IACUC (Institutional Animal Care and Use Committee) at Washington University School of Medicine and conducted as per the NIH guidelines. The *NFP* (Notochord and floor plate)-*Cre* transgene uses a *Foxa2* enhancer element to drive Cre expression in the notochord and floor plate (Kumar et al., 2007). Mice carrying this transgene were crossed with mice carrying the $ROSA^{DTA}$ allele (Wu et al., 2006) to produce *NFP-Cre;*$ROSA^{DTA/+}$ mice that would result in DTA-mediated apoptosis in notochord and floor plate. In Cre-positive cells, Cre-mediated *loxP* recombination removes a transcriptional "STOP" cassette in the $ROSA^{DTA}$ allele, leading to the expression of DTA under the control of the ROSA promoter (Fig. 1C) (Jia et al., 2008; Wu et al., 2006). DTA can cause translational arrest and apoptosis through its inhibition of the eukaryotic elongation factor 2 (Wu et al., 2006). Some of the *NFP-Cre;*$ROSA^{DTA/+}$ mice survived to adulthood and were used to generate *NFP-Cre;*$ROSA^{DTA/DTA}$ mice. Littermates with no cell ablation were used as controls. We also combined the *NFP-Cre* transgene and the Shh^{loxP} allele (Yu et al., 2002) to produce *NFP-Cre;*$Shh^{loxP/+}$ mice. Mice carrying Shh^{loxP} were obtained from the Jackson laboratory (Bar Harbor, Maine). Further crosses produced the *NFP-Cre;*$Shh^{loxP/loxP}$ mice with homozygous deletion of *Shh* in cells expressing the *NFP-Cre* transgene. Littermates with no homozygous *Shh* deletion in any cells were used as controls. The $ROSA^{LacZ}$ allele (Soriano, 1999) was used as a reporter to fate map cells expressing Cre. Mice carrying $Gli1^{lacZ}$ were obtained from the Jackson laboratory (Bar Harbor, Maine) and were crossed with *NC-DTA* mutants to reveal cells responding to Shh signaling.

Histological analysis, apoptosis assay, and skeletal preparations

For histological analyses, embryos were either fixed with 4% paraformaldehyde and embedded in paraffin or were cryopreserved in OCT. 7 μm paraffin sections or 10 μm cryosections were collected and stained by H&E following standard protocol (McDill et al., 2006). β-Galactocidase assays on whole-mount preparations and sections were performed as described (Wang et al., 2009). Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) analysis was performed on paraffin-embedded sections by using the ApopTag plus peroxidase *in situ* apoptosis detection kit (Roche, NutleY, NJ) (Jia et al., 2008). Skeletal preparations of newborn pups were made as described (Chen and Capecchi, 1997). Briefly, after removal of skin, newborn mice were eviscerated and fixed in 100% ethanol. The samples were then stained with 0.03% Alcian blue and 0.03% Alizarin Red. After staining, the samples were treated with 2% potassium hydroxide and cleared in a series of solutions with increasing concentration of glycerol.

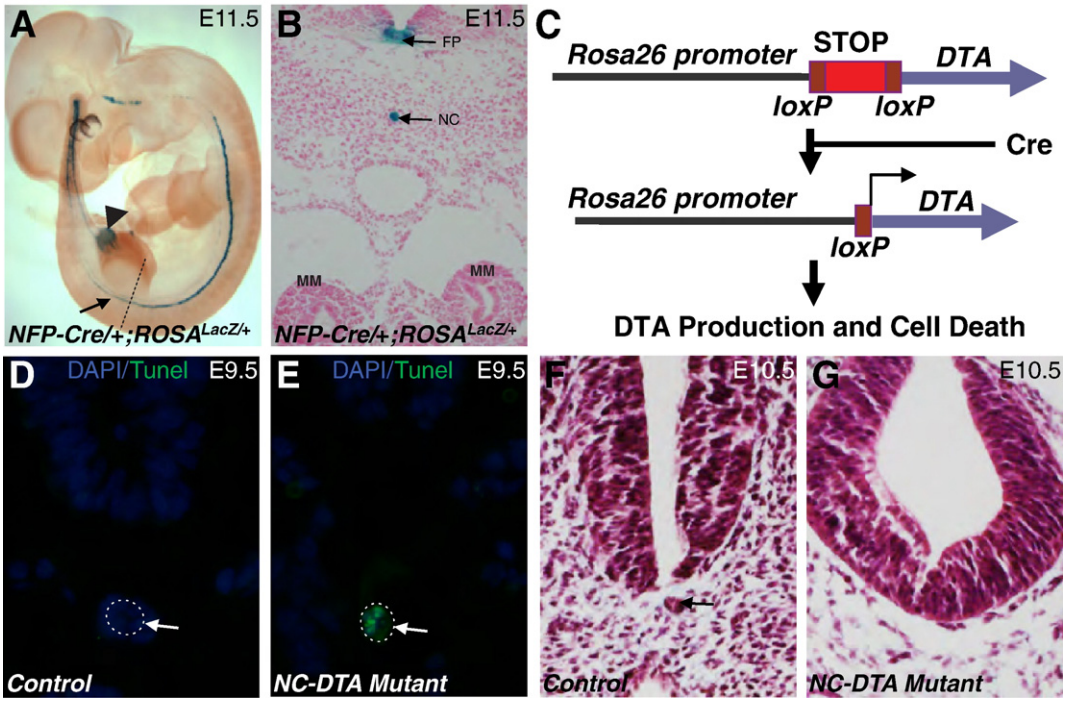

Fig. 1. Transgenic ablation of the notochord and floor plate by diphtheria toxin (DTA). A, the $ROSA^{LacZ}$ reporter revealed the expression of *NFP-Cre* in notochord and floor plate (arrow). The weak expression was also observed in part of the hindgut (arrowhead). B, A transverse section at the level of hind limb bud (shown by a dashed line in A) showed specific X Gal staining in the notochord and floor plate. C, when combined with a $ROSA^{DTA}$ allele, *NFP-Cre* induces the production of DTA, translational arrests, and apoptosis in Cre-positive cells. TUNEL assay on transverse sections of E9.5 control (D) and mutant (E) embryos at the level of the MM. Notochords are outlined by dotted circles. Apoptotic cells were seen only in the notochord of the mutant (E). F and G, transverse sections of the control (F) and mutant (G) embryos revealed notochord degeneration in the mutants at E10.5. Arrow in D–F points to the notochord. FP: floor plate; NC: notochord.