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*Dkk*1 in the peri-cloaca mesenchyme regulates formation of anorectal and genitourinary tracts

Chaoshe Guo [a,1], Ye Sun [a,1], Chunming Guo [a,1], Bryan T. MacDonald [b], Joseph G. Borer [a], Xue Li [a,*]

[a] Department of Urology, Department of Surgery and Pathology, Boston Children's Hospital, Harvard Medical School, 300 Longwood Avenue, Boston, MA 02115, USA

[b] The F.M. Kirby Neurobiology Center, Department of Neurology, Boston Children's Hospital, Harvard Medical School, 300 Longwood Avenue, Boston, MA 02115, USA

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ABSTRACT

Anorectal malformation (ARM) is a common birth defect but the developmental history and the underlying molecular mechanism are poorly understood. Using murine genetic models, we report here that a signaling molecule Dickkopf-1 (*Dkk*1) is a critical regulator. The anorectal and genitourinary tracts are major derivatives of caudal hindgut, or the cloaca. *Dkk*1 is highly expressed in the dorsal peri-cloacal mesenchymal (dPCM) progenitors. We show that the deletion of *Dkk*1 causes the imperforate anus with rectourinary fistula. Mutant genital tubercles exhibit a preputial hypospadias phenotype and premature urethral canalization. *Dkk*1 mutants have an ectopic expansion of the dPCM tissue, which correlates with an aberrant increase of cell proliferation and survival. This ectopic tissue is detectable before the earliest sign of the anus formation, suggesting that it is most likely the primary or early cause of the defect. Deletion of *Dkk*1 results in an elevation of the *Wnt/ß-catenin* activity. Signaling molecules *Shh*, *Fgf*8 and *Bmp*4 are also upregulated. Furthermore, genetic hyperactivation of *Wnt/ß-catenin* signal pathway in the cloacal mesenchyme partially recapitulates *Dkk*1 mutant phenotypes. Together, these findings underscore the importance of *DKK*1 in regulating behavior of dPCM progenitors, and suggest that formation of anus and urethral depends on *Dkk*1-mediated dynamic inhibition of the canonical *Wnt/ß-catenin* signal pathway.

Introduction

The embryonic hindgut, or the cloaca, is the primordial organ of the anorectal structure as well as the genitourinary tract. Inborn errors resulting in the anorectal malformation (ARM) affect approximately 1/2500 of live births (Cuschieri, 2002). The process of normal anorectal development, however, remains poorly understood and a major subject of ongoing debate (Hynes and Fraher, 2004; Kluth, 2010).

Historically, the morphogenetic process that separates the anorectal structure from the genitourinary tract has been described as the cloacal septation (Rathke, 1832; Retterer, 1890; Tourneux, 1888). Theories underlying this prevailing view are that local expansion of the cloacal mesenchyme from the rostral end (Tourneux, 1888), bilateral sides (Rathke, 1832; Retterer, 1890) or both (Stephens et al., 2002) leads to formation of the putative urorectal septum (URS). The general thought is that the putative URS, consisting the intra-cloacal mesenchyme (ICM), divides the cloaca into the dorsal anorectal tract and the ventral genitourinary tract (Fig. 1A–C). However, careful examinations of developmental history of the normal cloaca morphogenesis (Paidas et al., 1999; Penington and Hutson, 2003) and animal models with the ARM defect have led to a rejection of the septation theory (Kluth et al., 1995; Liu et al., 2003; Nakata et al., 2009; Suda et al., 2011; van der Putte, 1986). Instead, these studies suggest that morphogenesis of the cloaca depends primarily on formation and shift of the cloacal membrane (CM) (Kluth et al., 1995; van der Putte, 1986).

While signal(s) from cloaca endoderm actively participate growth and patterning of surrounding mesenchyme (Lin et al., 2009; Miyagawa et al., 2009a; Seifert et al., 2009, 2010), we believe that asymmetric growth of surrounding cloacal mesenchyme is the driving force in reshaping the cloaca and separating the anorectal and the genitourinary tract. Our previous genetic fate mapping and mouse mutant studies suggest that the process of

* Corresponding author at: Boston Children's Hospital, Harvard Medical School, Room 1061.4, Enders Research Building, 300 Longwood Ave, Boston, MA 02115, USA. Fax: +1 617 7300530.
E-mail address: sean.li@childrens.harvard.edu (X. Li).
[1] Equal contribution.

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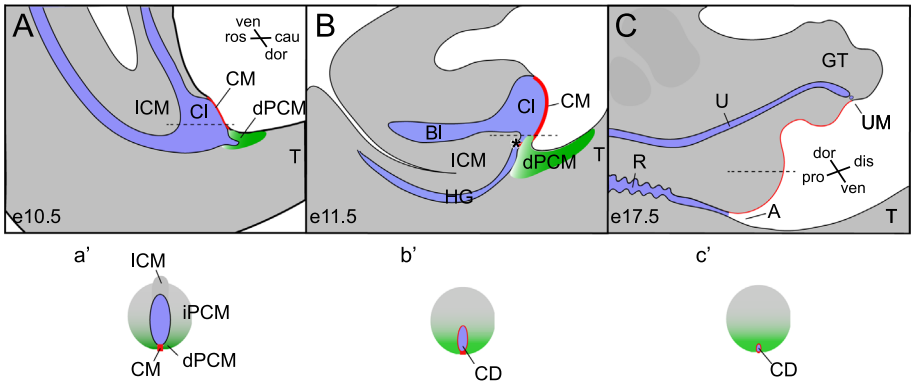

Fig. 1. The occlusion model of cloaca morphogenesis. The cloaca occlusion model suggests that the dorsal peri-cloacal mesenchyme (dPCM, green) and the cloacal membrane (CM, red) play prominent roles in morphogenesis of the cloaca (Cl). Blue, endoderm cavity. The CM and the dPCM function as two pivot points through which asymmetric growth of mesenchymal cells surrounding cloacal leads to separation of the hindgut (HG) and developing bladder (Bl), as well as outgrowth of genital tubercle (GT). Both midline sagittal views (A–C) and horizontal views (a′–c′, indicated in A–C as dash lines) are shown. A, anus; Bl, bladder; Cl, cloaca; cau, caudal; dis, distal; dor, dorsal; ICM, intra-cloacal mesenchyme; pro, proximal; R, rectum; ros, rostra; T, tail; ven, ventral; asterisk, juxtaposition of the ICM, the dPCM and the CM.

transforming the cloaca is reminiscent to the vascular occlusion (Fig. 1a′–c′) (Wang et al., 2011, 2013). This model is supported by the fact that some remnant of the cloaca, the cloacal duct (CD), can be found at the midline epithelial perineal surface (Seifert et al., 2008).

While these models are not mutually exclusive, each model has distinct features and implications. A key feature of the proposed occlusion model is the asymmetric growth of the cloacal mesenchyme along rostrocaudal and dorsoventral axes (Wang et al., 2011; Wang et al., 2013). Along these two axes, the CM and the dorsal peri-cloacal mesenchymal (dPCM) are two critical pivot points (Fig. 1). The dPCM refers to a cell population that locates at the caudal extreme of the cloaca between tail and developing genital tubercle. Specifically, the CM is devoid of mesenchymal cells, and the dPCM progenitors and the adjacent hindgut have a high rate of cell death and low rate of proliferation (Wang et al., 2013). Because of these two pivot points, asymmetric growth along the rostrocaudal axis results in the occlusion of the cloacal cavity, therefore, division of the cloaca (Fig. 1a′–c′). At the same time, asymmetric growth along dorsoventral axis leads to the genital tubercle outgrowth (Wang et al., 2013). This outgrowth results in a displacement of the cloacal membrane from a parallel to a perpendicular orientation to the body plan. Because of this displacement, a reversal of dorsoventral axis is adopted in the standard nomenclature to describe cloaca and genital tubercle, *i.e.* ventral of the cloaca becomes dorsal of the genital tubercle (compare Fig. 1A and C). The cloacal occlusion model suggests that the future anal orifice is prefigured at the juxtaposition of the ICM, the dPCM and the CM (Fig. 1B, asterisk). This working model predicts that the dPCM is critical for the anorectum formation.

In this study, we test the cloaca occlusion hypothesis by functionally characterizing a signaling molecule Dickkopf-1 (*Dkk*1) during anorectal and genitourinary tract development. We demonstrate that *Dkk*1 is highly expressed in the dPCMs but not the CM epithelial cells. Functionally, *Dkk*1 mutants exhibit malformations including an imperforate anus with rectourinary fistula, a preputial hypospadias and a premature canalization of the genital urethra. *Dkk*1 deletion results in a concerted upregulation of *Wnt*, *Shh*, *Fgf*8 and *Bmp*4 signals in the genital tubercle. Furthermore, genetic hyperactivation of the *Wnt/ß-catenin* signaling pathway partially recapitulates *Dkk*1 mutant phenotypes. Together, these findings highlight the importance of the dPCM progenitors and establish *Dkk*1 mutant allele as a valuable animal model to investigate etiology of the ARM. These findings further suggest that development of the anogenital structures depends on a dynamic *Dkk*1-mediated inhibition of the canonical *Wnt/ß-catenin* signal pathway.

Materials and methods

Mice

All animal studies were performed according to protocols reviewed and approved by the institutional animal care and use committee at Boston Children's Hospital. Mouse lines, including *Dkk*1 (MacDonald et al., 2004; Mukhopadhyay et al., 2001), *Six1*^{Cre} (Wang et al., 2011), the *ß-catenin* GoF allele (Harada et al., 1999; Sun et al., 2012), *Shh*^{Cre} (Harfe et al., 2004) and R26R^{mTmG} (Muzumdar et al., 2007) have previously been reported. Genotyping of the mice was performed as described.

Histology, Immunohistochemistry and in situ hybridization

We have described these procedures previously (Wang et al., 2011, 2013). In brief, staged embryos were dissected in cold phosphate buffer and fixed in 4% paraformaldehyde for subsequent analyses. Serial cryostat sections were used for histological analyses. Immunohistological staining was performed using cryostat sections. Whole-mount and section *in situ* hybridization were performed as described previously (Guo et al., 2011; Wang et al., 2011). Images were acquired with an Axioplan2 fluorescence microscope (Zeiss) or an Olympus SZX16 fluorescence dissection microscope equipped with a DP71 digital camera.

Cell proliferation and cell death analyses

Mitotic cells were labeled with the phospho-histone H3 Ser10 antibody (Upstate) and counter stained with a DNA-binding DAPI fluorescent dye (Molecular probe). Whole mount LysoTracker Red® (Invitrogen) vital dye labeling was performed according to the manufacturer's protocol. Stained embryos were fixed with paraformaldehyde and sectioned before image acquisition.

Real-time quantitative PCR

Microdissected genital tubercle tissue of e13.5 embryo was used for quantitative analyze gene expression level. RNA was extracted according to manufacturer's protocol (Qiagen RNAeasy mini). Relative gene expression levels were measured using SYBR Green Master Mix (Affymetrix) on an ABI-7500 detector (Applied BioSystems) and normalized to an internal control (18S RNA). The following oligos were used: 18S F: GTA ACC CGT TGA ACC CCA TT; 18S R: CCA TCC AAT CGG TAG TAG CG; Bmp4 F: GCC GAG CCA ACA CTG TGA GGA; Bmp4 R: GAT GCT GCT GAG GTT GAA GAG G; Fgf8 F: GGG AAG CTA ATT GCC AAG AG; Fgf8 R: TGT ACC AGC CCT CGT ACT