Published in final edited form as:

J Surg Res. 2009 October ; 156(2): 278–282. doi:10.1016/j.jss.2009.03.087.

WNT5A Knock-Out Mouse As A New Model of Anorectal Malformation

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Abstract

Background—Anorectal malformations (ARM) represent a variety of congenital disorders that involve abnormal termination of the anorectum. Mutations in *Shh* signaling and *Fgf10* produce a variety of ARM phenotypes. Wnt signaling has been shown to be crucial during gastrointestinal development. We therefore hypothesized that *Wnt5a* may play a role in anorectal development.

Methods—Wild type (WT), *Wnt5a*^{+/-}, and *Wnt5a*^{-/-} embryos were harvested from timed pregnant mice from E15.5 to E18.5 and analyzed for anorectal phenotype. Tissues were processed for whole-mount *in situ* hybridization and histology.

Results—*Wnt5a* is expressed in the embryonic WT colon and rectum. *Wnt5a*^{-/-} mutants exhibit multiple deformities including anorectal malformation. A fistula between the urinary and intestinal tracts can be identified as early as E15.5. By E18.5, the majority of the *Wnt5a*^{-/-} mutants display a blind-ending pouch of the distal gut.

Conclusions—The expression pattern of *Wnt5a* and the ARM phenotype seen in *Wnt5a*^{-/-} mutants demonstrate the critical role of *Wnt5a* during anorectal development. This study establishes a new model of ARM involving the *Wnt5a* pathway.

Introduction

Anorectal malformations (ARM) represent a variety of congenital disorders that involve abnormal termination of the anorectum. The clinical spectrum of these anomalies ranges from anal stenosis to imperforate anus to persistent cloaca. The overall incidence is approximately 1 in 5000 live births [1]. Although the anomalies can be surgically corrected, morbidity such as fecal incontinence continues to be a challenge in patient management. Our current understanding of the normal and abnormal development of the hindgut remains incomplete; however, recent studies of animal models of ARM have greatly enhanced our knowledge in this multifaceted developmental process.

Teratogenic agents such as all-trans retinoic acid and ethylenethiourea (ETU) have been used to induce ARM in both mice and rats [2,3]. Mice with ARM induced by ETU showed failure of the urorectal septum (URS) to fuse with the cloacal membrane, maldevelopment of the dorsal cloacal membrane, and absence of the tail groove [2]. The naturally occurring

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Presented at the 4th annual Academic Surgical Congress, Fort Myers, FL, February 3-6, 2009.

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Sd mouse (also known as Danforth's short-tail mouse) displays autosomal-dominant trait of imperforate anus; studies of these mice suggested that the ARM in this model resulted from the loss of the dorsal cloaca and the shortened cloacal membrane [4,5]. The specific genetic mechanism for ARM in *Sd* mouse has not been elucidated. Sonic hedgehog (Shh), an endoderm-derived signaling molecule critical in various aspects of development, has also been implicated to play an essential role in hindgut development. Mutations in *Shh* and its downstream mediators, *Gli2* and *Gli3* have been shown to recapitulate the spectrum of ARM seen in clinical setting [6]. The most severe form of the disease, persistent cloaca, is seen in *Shh*^{-/-} mutant [7]. Mutant mice lacking *Gli2* or *Gli3*, two zinc finger transcription factors involved in Shh signaling, respectively, display imperforate anus with recto-urethral fistula and anal stenosis [8,9]. Interestingly, persistent cloaca is also observed in *Gli2*^{-/-};*Gli3*^{+/-}, *Gli2*^{+/-};*Gli3*^{-/-}, and *Gli2*^{-/-};*Gli3*^{-/-} double mutant mice [6].

Previous work from our lab showed that inactivation of *Fibroblast growth factor 10* (*Fgf10*) results in imperforate anus in mice [10]. This is the first time that a member of the fibroblast growth factor family has been implicated in the development of the anorectum. The fibroblast growth factor family consists of 22 members of secreted signaling molecules important in many aspects of organogenesis [11]. *Fgf10* has been shown to be critical in gastrointestinal development, specifically in the stomach, duodenum, cecum, colon and anorectum [10,12-15].

Wnt signaling has emerged to be a critical player in embryonic development, and members of the Wnt family have been shown to be essential in regulating proliferation, differentiation, and organogenesis [16-20]. Recent studies have shown that *Wnt5a* regulates *Fgf10* and *Shh* signaling during lung development [21]. The interactions between these signaling pathways during gut development have not been defined. The current study investigates the role of *Wnt5a* in establishing anorectal continuity. We characterized *Wnt5a* expression in wild type embryos using whole mount *in situ* hybridization. We also analyzed the anorectal phenotype of *Wnt5a*^{-/-} mutants at an early developmental stage at E15.5, as well as near term at E18.5 to compare with the anomalies seen in infants.

Materials and Methods

Mutant Mice

This research project was approved by the animal ethics committee, and the animals were cared for in accordance with the institutional guidelines (Institutional Animal Care and Use Committee protocol #142). *Wnt5a*^{-/-} embryos were generated by crossing *Wnt5a*^{+/-} mice, and WT littermates were used as controls. Embryos were harvested from timed pregnant *Wnt5a*^{+/-} mice at E15.5 and E18.5, referring to embryonic day or post-conception day 15.5 and 18.5, respectively.

Tissue Collection and Histology

Embryos were removed from the uteri and examined grossly for anorectal phenotype. Embryos were fixed in Methacarn (120 mL Methanol, 60 mL Chloroform, 20 mL Acetic Acid) overnight, washed in 100% ethanol twice, and kept in 100% ethanol overnight prior to embedding in paraffin. Sagittal sections were obtained at 5 µm using a microtome and mounted on coated glass slides. Specimens were deparaffinized and rehydrated. Hematoxylin and eosin (H&E) staining was performed using standard protocol.

In Situ Hybridization

The gastrointestinal tract (GIT) was isolated from the embryos and fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) at 4°C. The fixation time was

adjusted for the age of gestation. The samples were washed twice in PBS for 5 minutes, kept in 70% ethanol overnight, and stored in 100% ethanol. The murine cDNA used as templates for riboprobes was a 360 base-pair *Wnt5a* fragment, previously described by Li et al [22]. Whole mount in situ hybridization was performed using the protocol described by Winnier et al [23].

Results

Wnt5a Expression in wild type (WT)

Wnt5a is expressed in the WT embryonic colon and rectum, shown in Figure 1 using whole mount *in situ* hybridization. In this photomicrograph, expression of *Wnt5a* is demonstrated by the dark purple staining. Level of *Wnt5a* expression is very prominent in the proximal colon, decreases in the mid-portion, and increases dramatically in the very distal portion and in the rectum.

Wnt5a^{-/-} Embryos Exhibit ARM

Compared to the WT littermates, *Wnt5a*^{-/-} mutants are smaller in size and display shortened limbs and tails, as described previously by Yamaguchi et al [24]. Figure 2 shows that *Wnt5a*^{-/-} mutant exhibits severe anorectal malformation near term at E18.5. In the WT embryo at E18.5, the anus is patent and is found between the tail and the genital tubercle (Fig 2A). *Wnt5a*^{-/-} mutant exhibits imperforate anus, a truncated tail, and a hypoplastic genital tubercle (Fig 2B). Mid-sagittal sections of corresponding tissues from Fig 2A and 2B were obtained to better delineate the anatomy. H & E staining of these sections reveal mature continuity of the epithelium of the rectum and the anus in the WT embryo (Fig 2C). *Wnt5a*^{-/-} mutant fails to show continuity of the rectal and anal epithelium (Fig 2 D); in fact, no rectal tissues were identified in the *Wnt5a*^{-/-} mutants. The liver is enlarged and displaced caudally. The phenotype has 100% penetrance with all analyzed *Wnt5a*^{-/-} mutants displaying anorectal malformation (n=16).

At E15.5, a fistula between the bladder and the colon is typically present in the *Wnt5a*^{-/-} mutant (n=3, Figure 3B). By E18.5, we were only able to identify the fistula in 25% of the *Wnt5a*^{-/-} mutants (n=4, Figure 3C). In the majority of the cases, a blind-ending pouch without a fistula was seen (n=12).

Discussion

Anorectal malformations represent a spectrum of congenital disorders that involve abnormal termination of the anorectum. The clinical presentation ranges from anal stenosis to anorectal malformation (ARM) with fistula to persistent cloaca. The clinical management of ARM continues to be a challenge for pediatric surgeons, and our current understanding of the normal and abnormal development of the hindgut remains incomplete. Mutations in *Shh* and its downstream mediators, *Gli2* and *Gli3*, have been shown to resemble various forms of anorectal malformation, suggesting a role of Shh signaling in anorectal development [6]. Previous work from our lab also showed that *Fgf10*^{-/-} mutant displays imperforate anus [10]. Our current study establishes a new model of ARM involving the *Wnt5a* pathway.

Wnt5a^{-/-} mutant, first described by Yamaguchi et al., exhibits multiple congenital anomalies, including dwarfism, truncated limbs and tail, hypoplastic genitals, impaired distal lung morphogenesis, and defective pancreatic islet cell migration [22,24,25]. Here we report for the first time ARM in the absence of *Wnt5a*. Figure 1 shows that *Wnt5a* expression is very prominent in the WT rectum, suggesting a role in normal anorectal development. In Figure 2, we show that *Wnt5a*^{-/-} mutant fails to establish anorectal continuity. A fistula between the urinary and intestinal tract can be identified as early as E15.5 in all the *Wnt5a*^{-/-} mice

(Figure 3B), suggesting that the fistula formation in anorectal malformation may be part of an early developmental abnormality seen in these mutants and not a result of mechanical obstruction as previously thought. The fistula can only be identified in 25% of the cases by E18.5 (Figure 3C), with the majority of the mutants displaying a blind-ending distal gut without a fistula. The progressive loss of fistula may represent a delayed developmental progression. In normal development, the cloaca disappears when the urorectal septum descends and divides the cloaca into the urogenital sinus and the rectum. This process is perhaps altered and delayed in the *Wnt5a*^{-/-} mutants. However, given the fact that the fistula is still present in 25% of the *Wnt5a*^{-/-} embryos at birth, the disappearance of the fistula may be the result of an imbalanced apoptosis and proliferation in the *Wnt5a*^{-/-} mutants. This theory is further supported by findings from a recently published article by Cervantes et al. where they demonstrated reduced cell proliferation in the *Wnt5a*^{-/-} mutant [26].

Studies of targeted gene deletion and over-expression of *Wnt5a* using lung-specific promoter showed that *Wnt5a*, FGF10, and Shh signaling pathways are functionally interactive [21,22]. These studies suggest that *Wnt5a* negatively regulates Shh signaling which leads to abnormal *Fgf10* expression in the lung [21]. Although both *Fgf10*^{-/-} and *Wnt5a*^{-/-} mutants display various GI anomalies including imperforate anus, we speculate that the defect occurs at a later developmental stage in the *Wnt5a*^{-/-} mutant. In the *Fgf10*^{-/-} mutant, the colon appears normal initially at E10.5 but completely disappears by E14.5 [14]. In the *Wnt5a*^{-/-} mutant, however, there is no loss of colonic integrity in the proximal colon where *Wnt5a* expression is highest. Although the overall length of the colon in the *Wnt5a*^{-/-} is shorter compared to the WT, the colon is still present at birth. Compared with *Fgf10*^{-/-} mutant, the ARM phenotype seen in *Wnt5a*^{-/-} mutant recapitulates much more closely the human phenotype. ARM seen in *Wnt5a*^{-/-} is not simply the result of loss of colonic tissue as seen in the *Fgf10*^{-/-} mutant but result of a more specific developmental defect.

Mutations affecting Shh signaling produce a spectrum of anorectal malformations in mice. *Shh*^{-/-} mutants exhibit persistent cloaca, the most severe form of ARM, whereas *Gli3*^{-/-} mutants display only anal stenosis [6]. We have demonstrated here that the phenotype of ARM seen in *Wnt5a*^{-/-} mutants is similar to that seen in *Gli2*^{-/-} mice with imperforate anus and a fistula generally found between the urinary tract and the intestinal tract. *Wnt5a* is likely downstream of *Shh* and *Gli2*, given the fact that the ARM seen in *Wnt5a*^{-/-} is a less severe phenotype than that seen in *Shh*^{-/-}. Additionally, *in situ* hybridization using riboprobe specific for *Shh* shows no altered expression in the *Wnt5a*^{-/-} mutants, further suggesting that *Shh* is upstream of *Wnt5a* (data not shown).

This study provides the first evidence of the critical role of *Wnt5a* in normal anorectal development. A network of signaling pathways including Wnt, Shh and FGF10 is involved in the proper development of the anorectum. Animal models of ARM such as the one described here provide a powerful tool to enhance our knowledge in the pathogenesis of ARM.

Acknowledgments

This work was supported by the training grant from the California Institute for Regenerative Medicine.

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Figure 1. *Wnt5a* is strongly expressed in the WT anorectum

The expression of *Wnt5a* is shown by *in situ* hybridization. There is prominent *Wnt5a* expression in the anorectum (a) in the WT embryo at E16.5. *Wnt5a* expression is also noted in the small intestine (si), cecum (c), and proximal colon.

Figure 2. *Wnt5a*^{-/-} mutant exhibits anorectal malformation (ARM)

A landscape view of the perineum of the wild type (WT) and *Wnt5a*^{-/-} mutant at E18.5 is shown in (A, B). In the WT embryo (A), the anus is widely patent (arrow) and is found between the tail and the genital tubercle (gt). In the *Wnt5a*^{-/-} mutant, the anus is absent (arrow), the tail is truncated, and the genital tubercle (gt) is hypoplastic. The tissues shown in (A, B) were processed, embedded, and sectioned as previously described and shown in (C, D). H & E staining of a mid-sagittal section of the WT embryo at E18.5 shows continuity of the rectal epithelium (arrow) and the anus (C). *Wnt5a*^{-/-} mutant fails to show anorectal continuity (D).

Figure 3. *Wnt5a*^{-/-} mutant displays ARM with fistula between the urinary and intestinal tracts (A) Wild type colon, bladder (b) and genital tubercle (gt) at E15.5. (B) *Wnt5a*^{-/-} mutant has overall shortening of the colon compared to WT and an abnormal connection between the colon and the bladder (b) at E15.5. Although the fistula is almost always seen at E15.5, it is only identified in 25% of the cases by E18.5 (C). This connection is demonstrated by the white arrow between the bladder (b) and the colon.