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Pitx2 is a Critical Early Regulatory Gene in Normal Cecal Development

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Abstract

Purpose—The murine cecum is a critical digestive structure. Morphogenesis of the cecum involves several key genes including *Homeobox (Hox) d12*. Ectopic expression of *Hoxd12* has been shown to result in cecal agenesis and a down regulation of both Fibroblast growth factor 10 (Fgf10) and the Pituitary homeobox 2 gene (Pitx2). Homozygous null mutation of *Fgf10* or its cognate receptor *Fgfr2IIIb* results in severe cecal defects where there is the initiation of mesodermal budding, but a failure of the endoderm to grow and extend into this structure. We examined the expression of *Pitx2* in the cecum and hypothesized that homozygous null mutation of *Pitx2* would result in cecal agenesis.

Methods—IACUC Approval was obtained for these studies. Whole mount in situ hybridizations for *Pitx2* were performed on wild-type embryos between Embryonic days (E) 11.0 and E12.5. *Pitx2* −/− and *Fgfr2IIIb* −/− embryos were generated from *n/+* heterozygote breedings and harvested at E10.5, E11.5, and E13.5. Genotypes were confirmed by PCR. Morphology of *Pitx2* −/− cecae were compared to those of wild-type littermates and *Fgfr2IIIb* −/− embryos at identical stages. Embryos were fixed overnight and photographed the following day.

Results—*Pitx2* is expressed in the cecal mesoderm and endoderm as early as E11.0. Expression becomes increasingly more robust by E12.5. Homozygous null mutation of *Pitx2* results in agenesis of the cecum. In contrast to *Fgfr2IIIb* −/− embryos, which demonstrate a persistent mesodermal bud as late as E18.5, no mesodermal bud is present in *Pitx2* −/− embryos.

Conclusions—Our findings demonstrate that *Pitx2* is a critical regulatory gene in cecal morphogenesis and suggest that *Pitx2* is required for initiation of mesodermal budding and likely resides upstream of *Fgf10-Fgfr2IIIb* signaling in the normal development of this structure.

Introduction

The cecum is tubular gut structure that lies between the small intestine and colon on the antimesenteric side of the bowel. It is host to a number of gut microbes that assist in the digestion of complex carbohydrates (1, 2). Homozygous null mutations in specific genes such as *Fgf9* result in both cecal agenesis as well as congenital short gut in mice (3, 4). This

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indicates that the process of lengthwise intestinal growth and cecal development are linked and that cecal development can serve as a model for studying molecular pathways involved in lengthwise intestinal growth. The morphogenesis of the cecum can be described in three general steps: budding, elongation, and arrest. A number of genes have been implicated in the formation of this structure. For example loss of *Fgf9* expression in the endoderm results in cecal agenesis and also loss of *Fgf10* expression within the cecal mesoderm (3). Loss of *Fgf10* signaling via mutation in either *Fgf10* or the gene for its cognate receptor *Fgfr2IIIb* in the endoderm results in a phenotype in which a mesodermal aggregation or bud forms on the antimesenteric side of the intestine but the endoderm fails to bud into this structure (5). These findings suggest that the budding phase of cecal development can be subdivided into additional steps. The first step would appear to be the induction of a mesodermal aggregation or bud which is dependent on *Fgf9*. During the formation of the mesodermal bud, *Fgf10* is expressed in the mesoderm which appears to act upon its target receptor Fgfr2IIIb in the endoderm. Thereafter, the endoderm begins to bud into the aggregation of cecal mesoderm and then the elongation phase of the endodermal and mesodermal components of the cecum begins.

Pitx2 is a transcription factor that is expressed during cecal development (5). The role of this gene in cecal development and its tissue specific expression pattern (endoderm and mesoderm) has not been described. Over expression of the *Hoxd12* has been shown result in a loss of *Fgf10* and *Pitx2* expression in the cecal region as well as cecal agenesis (6). This phenotype is very similar to that described for *Fgf9* −/− mutants (3). To better understand the role of *Pitx2* in the budding phase of cecal development we chose to characterize the tissue specific expression of *Pitx2* during this phase and examined the morphology of the cecum in *Pitx2* −/− mouse embryos. These embryos show severe abnormalities as they fail to undergo proper turning and abdominal wall closure and are unable to progress beyond E 14.5 in development (7, 8).

We hypothesized that *Pitx2* would be expressed in both the endoderm and mesoderm of the cecum. Because *Pitx2* expression has been observed in the mesodermal bud of the cecum in *Fgf10* −/− and *Fgfr2IIIb* −/− mutants (5), we postulated that *Pitx2* resides upstream of the Fgf10 signaling pathway and therefore the cecal agenesis observed due to over expression of *Hoxd12* is a direct result of the loss of *Pitx2* expression. Accordingly we predicted that *Pitx2* −/− cecum would lack the mesodermal bud seen in Fgf10 −/− and Fgfr2IIIb −/− mutants.

Methods

Animals

IACUC approval for these studies was obtained from the University of Wisconsin School of Medicine and Public health (P.F.N. protocol # M02258, YS #10-05008). All animals were maintained in a clean facility with ad libitum access to fresh food and water, and kept on a 12 hour alternating light/dark cycle. *Fgfr2IIIb* −/− and *Pitx2* −/− embryos were generated through traditional heterozygous +/n breedings (9, 10) of established lines. Genotyping of embryos for *Fgfr2IIIb* −/− and *Pitx2* −/− alleles was performed as described previously (9, 10).

Morphologic studies

Embryos were harvested at either (E) 10.5, E11.0, E11.5, E12.0, E12.5, E13.5 or E18.5. The thorax was opened and the embryos were fixed overnight in 4% paraformaldehyde at 4°C. The intestines were dissected out and photographs of the cecal regions were obtained under a standard dissecting microscope.

Histological studies

E12.5 embryos were dissected free of the yolk sac and embryos were fixed overnight at 4°C in 4% paraformaldehyde. They were then dehydrated through a series of escalating Methanol/PBST washes, isopropyl alcohol and xylene. They were washed 3 times in paraffin at 65 °C in a vacuum oven and then embedded in paraffin. Sagittal sections were taken a 10μm thickness. Sections were stained for standard H&E and photographed under a standard light microscope.

Whole mount in situ hybridization

Samples were dehydrated through a series of escalating PBS-Tween Methanol steps then stored overnight at −20 °C. The following day they were rehydrated into PBS-Tween, treated with hydrogen peroxide and proteinase K, and in situ hybridization was performed at 70 °C with antisense probes for *Pitx2* (11). Specimens were stained at 37°C, washed in PBS-Tween and fixed with 4% PFA. Photographs were taken under a dissecting light microscope.

Results

Morphogenesis of the cecum

We examined the morphogenesis of the cecum in detail. The cecum can be seen forming as early as E10.5 just distal to the bend in the intestinal tube (Figure 1A). This is a full day earlier than had been previously reported (5). As the intestine increases in length at E11.0, the mesoderm continues to thicken in the cecal region (Figure 1B). By E11.5 the endoderm can be seen extending into the mesodermal bud (Figure 1C). After this, the mesoderm and endoderm extend outward from the antimesenteric side of the intestine (Figure 1D and E). On sagittal section at E12.5, the endoderm near the distal tip of the cecum is cuboidal (Figure 1F, white arrow) where as that in the proximal region is stratified pseudo-columnar (black arrow). The basement membrane in the distal region is thinned compared to the proximal region indicating invasion of the endoderm bud into the mesoderm at this stage.

Pitx2 expression during intestinal development

Examination of *Pitx2* expression during intestinal development demonstrates that this gene is expressed in distinct regional patterns. In the small intestine its expression is restricted to the endoderm and is seen as early as E11.0 (Figure 2A). In the cecum, *Pitx2* is expressed in *both* the endoderm and the mesoderm at E11.0 (Figure 2A). In contrast no expression is seen in the colon. These distinct regional patterns persist through at least E12.5 (Figures 2B and C). The expression in the mesoderm of the cecum is asymmetric in that there is more intense staining of the side of the cecum that is towards the intestine or rostral (white arrows). By E11.5 the endodermal bud can be seen extending into the mesoderm and the mesodermal staining for *Pitx2* remains rostral (Figure 2B). The distribution of the mesoderm at this stage is also more rostral than caudal as well. At E12.5 in development, *Pitx2* is robustly expressed throughout the entire cecum and discrepancies in rostral-caudal staining are absent (Figure 2C). The data indicate that *Pitx2* is expressed within the endoderm and mesoderm of the developing cecum. In contrast, expression in the small intestine appears limited to the endoderm whereas in the colon expression is absent at all stages examined.

Loss of *Pitx2* expression results in an absence of a mesodermal cecal bud at E13.5

We next examined the morphology of cecal development in *Pitx2* −/− embryos. We compared this morphology to that of wild-type embryos and *Fgfr2IIIb* −/− embryos which have a defect in cecal development, but *do* form a mesodermal bud (5). At E 13.5, the wild type cecum has extended and the distribution of the mesoderm continues to be asymmetric with more being distributed on the rostral side (Figure 3A). By E18.5, the cecum has

lengthened substantially and the asymmetric distribution of mesoderm is absent although the cecum curves rostrally towards the small intestine (Figure 3D). At E13.5, the *Fgfr2IIIb* −/− embryo has a mesodermal bud (Figure 3B). This bud persists until term, yet no endoderm is present and the bud has elongated only minimally (Figure 3E). Similar to the other specimens, the *Pitx2* −/− embryo at E13.5 has a clear angulation of the intestine at the boundary between the small intestine and colon. In contrast, however, there is no significant accumulation of the mesoderm in this area and no endodermal budding (Figure 3C, white arrow). These findings indicate that *Pitx2* is required for in the induction and development of the mesodermal bud of the cecum.

Discussion

Our findings demonstrate that *Pitx2* is a critical regulatory gene in cecal morphogenesis. Expression of *Pitx2* in the cecum is observed in both the endoderm and mesoderm which is a unique staining pattern compared to the remainder of the intestine. Our data also suggests that *Pitx2* is required for initiation of mesodermal budding which appears to be a prerequisite for budding and extension of the endoderm into this structure.

Disruptions in Fgf10 signaling either through mutation of the *Fgf10* gene or the gene for its receptor (Fgfr2IIIb) result in the formation of a cecal mesodermal aggregation or bud and expression of *Pitx2* within this structure (5) however subsequent endodermal budding fails to occur after this. Our observation that *Pitx2* −/− embryos fail to form a mesodermal cecal bud suggests that *Pitx2* resides upstream of *Fgf10-Fgfr2IIIb* signaling in the normal development of this structure.

We have observed that the expression of *Pitx2* in the cecal region in both the endoderm and mesoderm is unique to this part of the intestine. In contrast *Pitx2* expression in the small intestine is limited to the endoderm and *Pitx2* is to be absent from the colon. The cecal region has previously been described as having a unique permissivity to *Fgf* gene expression (3). This underscores the likelihood that *Pitx2* regulates Fgf signaling. However, at least 2 Fgfs are in involved in the morphogenesis of the cecum: Fgf9 and Fgf10 (3, 5). *Pitx2* could have a role in regulating both of these genes. For example, expression of *Fgf10* in the mesoderm appears to depend on the formation of a mesodermal bud (3). And mesodermally expressed *Pitx2* could be a key regulator of *Fgf10* expression after formation of the mesodermal bud. *Pitx2* is also expressed in the endoderm of the cecum as is *Fgf9* (3). Since mutations in either *Pitx2* or *Fgf9* results in an identical phenotype of cecal agenesis with failure of the mesoderm to form a bud, it is possible that endodermal *Pitx2* is regulating *Fgf9* expression and mesodermal bud formation as well. We are undertaking studies to examine these possibilities using a tissue specific Cre strategy.

Examination of other structures in development also suggests a link between Pitx2 and Fgf10. For example In fact *Pitx2* −/− embryos have massive abdominal wall defects. We have previously reported that mutations in gene encoding the cognate receptor isoform for Fgf10, *Fgfr2IIIb*, result in with mouse omphalocele (12). The greater severity of abdominal wall defect in *Pitx2* −/− embryos compared compound *Fgfr1/Fgfr2* embryos suggest that as in the cecum *Pitx2* resides upstream in regulating Fgf10 signaling. We predict based the expression pattern of *Pitx2* in the mesoderm that as a transcription factor is critical in regulating Fgf10 levels.

The cecum is a good model for studying lengthwise intestinal growth. In fact it appears to share many of the temporal and physical growth characteristics of the small intestine. Growth of the cecum begins with the budding phase which corresponds to the beginning of looping morphogenesis of the small intestine (E10.5): the most rapid phase of lengthwise

intestinal growth. Similar to the small intestine, the cecum appears to bend or loop. Additionally mutations in genes that disrupt cecal development such as Fgf9 also result in foreshortened intestine (3, 4). However, one of the major advantages of this organ as a model for intestinal growth is that it can be manipulated by in vitro culture, enabling one to study the affects of various exogenously applied growth factors (5). In future studies, we intend to employ this strategy to further delineate the role of Fgfs in the development of this organ and in the mechanisms of linear intestinal growth. It would be better to mention communication between mesoderm and endoderm since Fgfr2b mutant develop mesoderm but no endoderm. These interaction would be very interesting.

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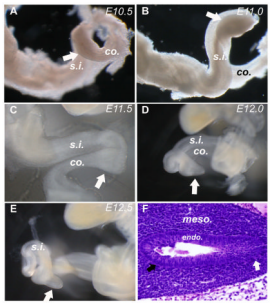

Figure 1.

A–E. Morphogenesis of the murine cecum. Whole mount photographs to the murine cecum as indicated by white arrows between E10.5 to E12.5.

F. A sagittal section through the cecum at E12.5. Black arrow indicates stratified pseudo-columnar epithelium and thick basement membrane of the proximal cecum. White arrow indicates distal cecum with cuboidal endoderm and a thinned basement membrane. Small intestine (s.i.), colon (co.).

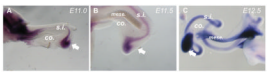

Figure 2.
Expression of *Pitx2* in the early murine cecum. Whole mount in situ hybridization for *Pitx2* at **A.** E11.0, **B.** E11.5 and **C.** E12.5. White arrows indicate rostral side of the cecum. Small intestine (s.i.), colon (co.) and intestinal mesentery (mese.).

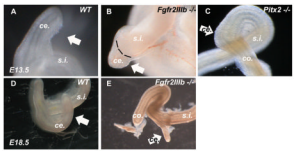

Figure 3.

Pitx2 −/− embryos fail to develop a cecum. **A.** Wild-type cecum at E13.5 **B**. *Fgfr2IIIb* −/− cecum at E13.5. **C.** *Pitx2* −/− intestine demonstrating a lack of a cecum at E13.5. **D.** Wild-type cecum at E18.5. exhibiting loss of asymmetric distribution of mesoderm and rostral looping of the cecum **E.** *Fgfr2IIIb* −/− cecum at E18.5 demonstrating the persistence of a mesodermal bud without elongation. Small intestine (s.i.), colon (co.) and cecum (ce.).