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FGF9–Pitx2–FGF10 signaling controls cecal formation in mice

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ABSTRACT

Fibroblast growth factor (FGF) signaling to the epithelium and mesenchyme mediated by FGF10 and FGF9, respectively, controls cecal formation during embryonic development. In particular, mesenchymal FGF10 signals to the epithelium via FGFR2b to induce epithelial cecal progenitor cell proliferation. Yet the precise upstream mechanisms controlling mesenchymal FGF10 signaling are unknown. Complete deletion of *Fgf9* as well as of *Pitx2*, a gene encoding a homeobox transcription factor, both lead to cecal agenesis. Herein, we used mouse genetic approaches to determine the precise contribution of the epithelium and/or mesenchyme tissue compartments in this process. Using tissue compartment specific *Fgf9* versus *Pitx2* loss of function approaches in the gut epithelium and/or mesenchyme, we determined that FGF9 signals to the mesenchyme via *Pitx2* to induce mesenchymal *Fgf10* expression, which in turn leads to epithelial cecal bud formation.

Introduction

The cecum forms a pouch contiguous with the gastrointestinal (GI) tract, lying between the ileum and the large intestine or colon. Hosting a large reservoir of microbes, the cecum plays an important role in the digestion of small food particles and complex carbohydrates from plant matter. Thus, this part of the gut tends to be more prominent in herbivores and omnivores than obligate carnivores (Backhed et al., 2005; Eckburg et al., 2005). In mouse embryogenesis, the cecum starts to form at E10.5 as a mesenchymal expansion, followed by an epithelial evagination (Burns et al., 2004). Epithelial evagination initiation is then followed by elongation (growth), differentiation and arrest. To date, however, the signals defining cecal development remain incompletely understood.

The cecum, like other parts of the intestine, is composed of two layers: an endoderm-derived epithelium and the surrounding mesoderm-derived mesenchyme. Epithelial–mesenchymal interactions are required for proper budding morphogenesis and differentiation in many organs including the gut (Cardoso, 2001; Koike and Yasugi, 1999; Shannon and Hyatt, 2004). Fibroblast growth factors, key elements of epithelial–mesenchymal interactions in many tissues, have been described as major players in controlling cecum formation. We have shown that loss of FGFR2b signaling, in *Fgf10* or *Fgfr2IIIb* knock-out (K.O.) embryos, results in the formation of a mesenchymal expansion, but the epithelium fails to proliferate and bud (Burns et al., 2004; Fairbanks et al., 2004). Moreover, the guts of *Fgf9* null embryos display complete cecal agenesis, with the absence of both mesenchymal expansion and epithelial budding. This is accompanied with decreased mesenchymal proliferation as well as complete lack of *Bmp4* and *Fgf10* expression. In turn, absence of *Fgf10* resulted in decreased epithelial proliferation (Zhang et al., 2006). During embryonic development, *Fgf9* is mostly found in the epithelium of the cecum but is also detected at lower levels in the mesenchyme. However, compartment specific (epithelial vs. mesenchymal) deletion of *Fgf9* in the cecum has not yet been studied.

Pitx2 is a member of the homeobox gene family that encodes a transcription factor initially identified as a gene mutated in Axenfeld–Rieger Syndrome type I, a rare autosomal dominant disorder that affects the development of the teeth, eyes and umbilicus (Semina et al., 1996a). In the GI tract, *Pitx2* is mainly expressed in the mesenchyme of the developing cecum (Burns et al., 2004) and it was recently reported that classical *Pitx2* inactivation in mouse leads to cecal agenesis (Nichol and Saijoh, 2011). In addition, it has been shown that over-expression of *Hoxd12*, another homeobox gene, phenocopies the loss of *Fgf9* and

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leads to cecal agenesis and loss of *Fgf10* and *Pitx1* expression (Zacchetti et al., 2007).

Developmental studies of branching processes in different organs, e.g. cecum and lung, suggest that the mechanisms controlling branching are substantially conserved between organs. We have reported previously that deletion of mesenchymal *β-catenin* in the embryonic lung results in a loss of *Pitx2* and *Fgf10* expression. This was associated with impaired epithelial and mesothelial FGF9 signaling to the mesenchyme due to decreased expression of *Fgfr2-IIIc* (De Langhe et al., 2008). These results led us to propose that an FGF9/Pitx2/FGF10 signaling pathway controls lung bud formation. In the current study, we have used tissue specific *Fgf9* and *Pitx2* loss of function approaches in the gut epithelium and mesenchyme to show that this signaling axis is active in the developing gut, and demonstrate its importance for cecal formation.

Materials and methods

Transgenic embryos

Dermo1-Cre (C57Bl/6 background), and $Fgf9^{flox/flox}$ mice were obtained from Dr. David Ornitz (Washington University, Saint Louis, MO (Yu et al., 2003)) and Dr. Fen Wang (Institute of Biosciences and Technology, Houston, TX (Lin et al., 2006)) respectively. $Pitx2^{flox/flox}$ mice were previously described (Gage et al., 1999). *Dermo1-Cre* mice were crossed with $Pitx2^{flox/flox}$ mice to generate [*Dermo1-Cre*; $Pitx2^{flox/+}$] that were then crossed with $Pitx2^{flox/flox}$ mice to generate [*Dermo1-Cre*; $Pitx2^{flox/flox}$] mutant embryos (called hereafter $Pitx2^{Dermo1\text{-}Cre}$). *Shh-Cre* mice were purchased from The Jackson Laboratory and were used to inactivate *Pitx2* specifically in the epithelium. *Shh-Cre* mice were crossed with $Pitx2^{flox/flox}$ mice to generate [*Shh-Cre*, $Pitx2^{f/+}$] that were then crossed with $Pitx2^{flox/flox}$ mice to generate [*Shh-Cre;* $Pitx2^{flox/flox}$] mutant embryos (called hereafter $Pitx2^{Shh\text{-}Cre}$). To inactivate *Fgf9* in the mesenchyme, we used *Dermo1-Cre* as described above for *Pitx2* inactivation. *CMV-Cre* mice were also used to completely inactivate *Fgf9* throughout the embryo including both epithelium and mesenchyme of the gut. Animal experiments were performed under the research protocol (31-08) approved by the Animal Research Committee at Children's Hospital Los Angeles and in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The approval identification for Children's Hospital Los Angeles is AAALAC A3276-01.

Whole mount in situ hybridization (WISH)

Microdissected guts were fixed in 4% paraformaldehyde (PFA) for 20 min and dehydrated in ethanol. The samples were washed twice in PBS for 10 min, transferred and stored in 70% ethanol until use. Whole-mount in situ hybridization protocol was performed as described (Winnier et al., 1995). The following mouse cDNAs were used as templates for the synthesis of digoxigenin-labeled riboprobes: a 528 bp fragment of *Fgf9* (provided by Dr. Ornitz), a 642 bp fragment of *Shh* (a kind gift from Dr Andrew McMahon, Harvard University, Boston, MA), a 584 bp fragment of *Fgf10* (Bellusci et al., 1997), a 1.5 kb full-length mouse *Bmp4* (Winnier et al., 1995), and a 559 bp fragment of *Pitx2* present in all 3 *Pitx2* isoforms (De Langhe et al., 2008). Sense probes were used for negative controls on E12.5 wild type ceca.

Proliferation analysis

Intraperitoneal injection of 0.2 mL bromodeoxyuridine (BrdU, Amersham Biosciences UK) was given to pregnant females (4 pregnant females) carrying mutant and littermate control embryos at E12.5. The females were sacrificed 15 min later and the embryos were immediately placed in ice-cold Hank's solution. The ceca were dissected from the embryos, fixed in 4% PFA, gradually dehydrated in ethanol and processed for paraffin sectioning. The ceca ($n=6$) were uniformly dissected and oriented away from the label of the embedding cassette or slide, with a short segment of the Ileum and a segment of the colon towards label. The embedded specimens were sectioned at 5 μm. The sections were re-hydrated and the antigen was retrieved by boiling the slides for 15 min in a microwave in 10 mM sodium citrate (pH 6.0). The slides were incubated for 1 h with monoclonal anti-BrdU antibody (Clone BU-1) RPN 202 as recommended by the manufacturer (Amersham Biosciences, UK). Cy3-labeled anti-mouse secondary antibodies were used. The slides were then mounted using Vectashield containing DAPI and photographed. The epithelial and mesenchymal cells of the cecum were counted separately for the number of total cells and BrdU-labeled cells. The boundaries of the mutant ceca were defined by the mesenchymal thickness and the curvature angles on each side, as illustrated in Fig. 4, panel K. The results are reported as the percentage of BrdU-positive cells. Tissues from females not injected with BrdU, and sections stained with secondary antibody alone were used as negative controls. No staining was observed in these specimens.

Quantitative PCR analyses

RNA was extracted from individually microdissected ceca from $Pitx2^{Dermo1\text{-}Cre}$ mutant and littermate control embryos at E12.5 ($n=8$). One microgram of RNA was reverse-transcribed into cDNA using Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science, Indianapolis, USA) according to the manufacturer's instructions. cDNA (2 μL) was used for dual color Hydrolysis Probe—Universal probe library based real time PCR, using the LightCycler 480 from Roche Applied Science. Mouse GAPDH gene assay (Roche applied Science) was used as the reference gene. The sets of primers and probe used for each gene examined are *Bmp4* (Forward: GAGGAGTTTCCATCACGAAGA, Reverse: GCTCTGCCGAGGAGATCA, Probe 89), *Fgf9* (Forward: GGGGAGCTGTATGGATCAGA, Reverse: TCCCGTCCTTATTTAATGCAA, Probe 12), *Fgf10* (Forward: CGGGACCAAGAATGAAGACT, Reverse: AACAACTCCAGATTTCCACTGA, Probe 80), *Pitx2* (Forward: CCTTACGGAAGCCCGAGT, Reverse: CCAAGCCATTCTTGCACA, Probe 40), *Fgfr2b* (Forward: CCCTACCTCAAGGTCCTGAA, Reverse: CATCCATCTCCGTCACATTG, Probe 21), *Fgfr2c* (Forward: TGCATGGTTGACAGTTCTGC, Reverse: TGCAGGCGATTAAGAAGACC, Probe 60). mRNA and water were used as negative controls.

In vitro cecum culture

Ceca were microdissected from wild type C57Bl/6 mice at E12.5, and placed atop polycarbonate filters (13 mm diameter, 8 μm pore size, Whatman) in 1 mL DMEM/F12 supplemented with 5% Fetal Bovine Serum and 1% Penicillin/Streptomycin. Ceca were then incubated with or without 250 ng/mL of human recombinant FGF9 ($n=4$ in duplicates) at 37 °C for 12 h in a moist atmosphere (5% CO_2).

Ectopic Pitx2 expression in cultured fibroblasts

NIH 3T3 murine fibroblasts (ATCC, #CRL-1658) were grown in DMEM with 10% FBS to 80% confluence. Cultures were transfected with plasmid encoding human *Pitx2* (pCI-HA*Pitx2a*) (Kozlowski and Walter, 2000) or empty vector (pCI) in Opti-MEM (Gibco Life Technologies) using Lipofectamine 2000 (Invitrogen), following the manufacturer's recommended protocol. Forty-eight hours