Abstract

Epithelial–mesenchymal interactions that govern the development of the colon from the primitive gastrointestinal tract are still unclear. In this study, we determine the temporal–spatial expression pattern of Fibroblast growth factor 10 (Fgf10), a key developmental gene, in the colon at different developmental stages. We found that Fgf10 is expressed in the mesenchyme of the distal colon, while its main receptor Fgfr2-IIIb is expressed throughout the entire intestinal epithelium. We demonstrate that Fgf10 inactivation leads to decreased proliferation and increased cell apoptosis in the colonic epithelium at E10.5, therefore resulting in distal colonic atresia. Using newly described Fgf10 hypomorphic mice, we show that high levels of FGF10 are dispensable for the differentiation of the colonic epithelium. Our work unravels for the first time the pivotal role of FGF10 in the survival and proliferation of the colonic epithelium, biological activities which are essential for colonic crypt formation.

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Keywords: Fgf10; Fgf10 hypomorph; Colon development; Atresia; Proliferation; Survival; Differentiation; Crypt formation; Progenitor cells

Introduction

The development of the gastrointestinal (GI) tract starts 2 days after gastrulation when the endoderm germ layer, consisting of an unspecified group of cells, turns into a patterned gut tube surrounded by the mesoderm (Grapin-Botton and Wells and Melton, 1999). Morphologically and functionally, the developing gut tube is subdivided into three regions: foregut, midgut, and hindgut. The foregut gives rise to the esophagus, lung, pancreas, stomach and liver. The midgut and hindgut give rise to the small intestine and colon, respectively, with the cecum forming the boundary between the small intestine and the colon (Mathan et al., 1976; Wells and Melton, 1999).

Regionalization of the primitive gut tube is orchestrated by mesenchymal–epithelium interactions (Haffén et al., 1987; Kedinger et al., 1998; Koike and Yasugi, 1999; Roberts, 2000). The proliferation of the intestinal epithelium and mesenchyme is tightly coordinated with the specification of the different GI tract domains along the proximal–distal axis. Interestingly, the control of cell proliferation and differentiation along the proximal–distal axis of the GI tract during development is poorly understood at this time since most of the studies on GI tract have used adult intestine as a model system. Identification of the growth factors controlling these processes is crucial since the molecular mechanisms regulating organogenesis are often the same as those necessary for repair following...
injury (Warburton et al., 2001). Furthermore, mis-regulations of embryonic signaling pathways are often associated with neoplastic diseases (Clark et al., 2001).

**Fibroblast growth factor (Fgf)** genes encode a large family of secreted molecules, which consists of 22 members in both mouse and human. These growth factors act through tyrosine kinase transmembrane receptors encoded by at least four separate genes (McKeenan et al., 1998). Each Fibroblast Growth Factor receptor (FGFR) comprises an extracellular region composed of two or three immunoglobulin-like (Ig) domains, a transmembrane segment and an intracellular tyrosine kinase domain (Johnson and Williams, 1993; McKeenan et al., 1998). Alternative splicing of the exons that encode the C-terminal half of the third Ig domain in Fgfrs-1, -2 and -3 results in receptor isoforms termed ‘IIIb’ or ‘IIc’, each with respectively distinct ligand-binding specificity and tissue distributions (Ornitz et al., 1996). The Fgfr2 gene splice variant containing the IIIb exon (Fgfr2-IIIb, hereafter Fgfr2b) is expressed mainly in epithelia, and is activated by four known ligands, FGFs-1, 3, 7 and 10, which are synthesized predominantly within the mesenchyme. In contrast, FGFR2-IIIc (FGFR2c) is located primarily in the mesenchyme (Peters et al., 1992; Orr-Urtreger et al., 1993; Mason et al., 1994; Ornitz et al., 1996; Yamasaki et al., 1996; Bellusci et al., 1997). FGF signaling plays important roles in controlling tissue patterning, proliferation and differentiation in multiple developing organs. In particular, stimulation of FGFR2b has been shown to trigger proliferation of the intestinal epithelium in the adult (Housley et al., 1994). We have also previously described that Fgfr2b plays also a critical role in embryonic GI tract development (Fairbanks et al., 2006). However, the specific roles of FGFR2b known ligands in controlling survival, proliferation, differentiation and morphogenesis during colonic development are still unclear.

We previously reported that **Fibroblast growth factor 10** (Fgf10) is critical for the proper development of the stomach (Spencer-Dene et al., 2006), the duodenum (Kennard et al., 2005) and the cecum in the mouse (Burns et al., 2004). We have also reported that the gut abnormalities observed in the Fgf10 null embryos are not the consequence of mesenteric vasculature occlusion (Fairbanks et al., 2005). In this study, we expand our analysis of Fgf10 expression and the corresponding null phenotype to more distal regions of the GI tract. Using a mouse reporter line where LacZ is expressed under the control of Fgf10 regulatory sequences, we describe the precise temporal–spatial expression of Fgf10 during colon development. Using immunohistochemistry with FGFR1 and FGFR2 antibodies, we describe the expression of FGF10 receptors along the proximal–distal axis of the developing colon. The colon phenotype of Fgf10 null embryos at different developmental stages is also analyzed. We demonstrate that FGF10 plays a critical role in the survival and proliferation of the colonic epithelial progenitor cells during embryonic development. Analysis of differentiation in the Fgf10 hypomorph using immunostaining suggests that FGF10 is not critical for the differentiation of the epithelium and mesenchyme but plays an important role in crypt formation. Thus our work demonstrates that FGF10 is necessary for survival and proliferation of the epithelium, biological activities essential for crypt formation. In contrast, FGF10 is not required for the differentiation of the epithelium and mesenchyme located in the distal region of the colon.

**Materials and methods**

**Mutant embryos**

The Mlc1v-LacZ-Z/2 (hereafter Fgf10<sup>+/−</sup>) mouse line is bred on a mixed background and has been previously described (Kelly et al., 2001). The transgene containing an nLacZ reporter gene is integrated upstream of the Fgf10 gene. Fgf10<sup>+/−</sup> embryos (Mailuns et al., 2005) were generated by crossing Fgf10<sup>+/−</sup> on a C57Bl/6 background (Sekine et al., 1999) with Fgf10<sup>+/−</sup> mice. Offspring were genotyped as previously described (Mailuns et al., 2005). The total number of mutant embryos use in this study are: Fgf10<sup>−/−</sup> (E10.5, n=5; E11.5, n=5; E12.5, n=4; E13.5, n=6; E14.5, n=5; E15.5, n=10; E18.5, n=8; P0, n=3), Fgf10<sup>+/−</sup> (E10.5, n=10) and Fgf10<sup>+/+</sup> (E12.5, n=8; E14.5, n=7; E15.5, n=10; E18.5, n=8; P0, n=3).

**Whole-mount in situ hybridization and LacZ staining**

All samples for in situ hybridization were fixed in 4% paraformaldehyde (PFA) in PBS at 4°C. The fixation time was adjusted for the age of gestation as follows: E10.5, 30 min; E11.5, 1 h; E12.5, 2 h; E15.5, overnight. The samples were washed twice in PBS for 5 min, washed in 70% ethanol over night and stored in 100% ethanol until needed. The murine cDNA used as templates for riboprobes was a 584 bFgf10 cDNA (described by Bellusci et al. (1997), a 1 kb Hinf-30 cDNA, a 642 bShh cDNA, a 1.8 kb Ihh cDNA and a 1.5 kb full-length Bmp1 cDNA. The whole-mount in situ hybridization protocol has been described by Winner et al. (1995). LacZ expression on whole-mount colon was monitored by β-galactosidase activity as described by Kelly et al. (1995).

**Proliferation analysis**

Intraperitoneal injection of 0.2 ml Bromodeoxyuridine (BrdU, Amersham Biosciences) was given to pregnant female mice at E10.5. The mice were sacrificed after 15 min. The colon was dissected from the embryo, preserved in 4% PFA solution as described above and processed for paraffin sectioning. Dissection and orientation of tissue were standardized to facilitate identification of structures. The embedded specimens were sectioned at 5 μm. The sections were then incubated with monoclonal anti-bromodeoxyuridine (Clone BU-1) RPN 202 as recommended by the manufacturer (Amersham Biosciences). Cy3 labeled anti-mouse secondary antibodies were used (Jackson). Vectorshiel with DAPI was used as a mounting medium. The sections were photographed using fluorescence microscopy.

**Cell death analysis**

The “In Situ Cell Death Detection, Fluorescein” kit (TUNEL technology, Roche Applied Science) for detection and quantification of apoptosis was used per manufacturer protocol on 5 μm paraffin sections of E10.5 Fgf10<sup>+/−</sup>, Fgf10<sup>−/−</sup> and Fgf10<sup>+/+</sup> colonos.

**Data analysis**

For analysis of cell proliferation and cell death, we compared samples from the same litter. The statistics presented correspond to an experiment including 2 wild type, 2 Fgf10<sup>+/−</sup>and 2 Fgf10<sup>−/−</sup> embryos harvested from the same litter. The experiments were repeated with two other litters (3 Fgf10<sup>+/−</sup> embryo and 3 wild type embryos) and a drastic reduction in epithelial proliferation and increase in cell death between Fgf10<sup>−/−</sup> and wild type colons was noted. The presented data are representative of the results obtained with the other litters.

After staining for BrdU incorporation or TUNEL, the total number of epithelial cells per section are counted (average 200 cells/section), as well as the

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number of cells positive for BrdU or TUNEL. The ratio of positive epithelial cells is determined. The data are expressed as a mean of the ratio±standard deviation.

167 Immunostainings

168 The colon was dissected from embryos at different stages of development to separate the colon from the rest of the intestine. At this stage, the colon has elongated significantly (Fig. 3C). In the distal half of the colon, FGFR1 and FGFR2 immunostainings, antigen retrieval was performed by boiling the samples for 10 min in Na-Citrate buffer (10 mM pH 6.0). Incubation of antibodies directed against FGFR1 (1:100, Novocastra) and FGFR2 (1:50, Santa Cruz Inc.) was performed in TBS with 3% bovine serum albumin and 0.5% Triton overnight at 4°C. A Cy3 conjugated goat anti-rabbit was used as secondary antibody for both antibodies; vectashield with DAPI was used as a mounting medium. For FGFR1 and FGFR2 immunostainings, antigen retrieval was performed by boiling the samples for 10 min in Tris–EDTA (pH 9.0). FGFR1 antibody (Fig. 1:200, Santa Cruz Inc.) and FGFR2 antibody (Bek, 1:200, Santa Cruz Inc.) were incubated for 1 h at room temperature. The signals were visualized with Envision+ Dual Link Kit (Invitrogen) as recommended by the manufacturer.

169 Histological stainings

170 5 μm cross-sections were obtained as described above. The sections were stained with hematoxylin and eosin according to standard procedures or with alcian blue as described in http://www.thehworld.com/protocols/special stains/alcian_blue.htm.

171 Morphometric analysis of the wild type and mutant colons

172 Wild type (n=4), Fgf10 hypomorphic (n=4) and Fgf10 null (n=4) colons at E18.5 were photographed at the same magnification. The length (in arbitrary units or a.u.) of each colon was measured using the software ImageJ from the NIH. For the wild type colons, we measured the length from the cecum to the rectum. For the mutant colons, we measured the length form the cecum to the site of atresia. The average length for wild type, Fgf10 hypomorphic and Fgf10 null colons was calculated and compared. The data are expressed as a mean±standard deviation. We also calculated the ratios of the average length of the mutants colons compared to the average length of the wild type colon. The significance of the differences between length was evaluated by ANOVA. P values less than 0.05 were considered statistically significant.

173 Results

174 Fgf10 is expressed in the embryonic colon

175 We investigated the expression pattern of Fgf10 in the colon between embryonic day 10.5 (E10.5) and E18.5 using Fgf10 mice (Kelly et al., 2001). Previous studies suggest that this mouse strain is a useful reporter for the colon. FGFR1 and FGFR2 are expressed throughout the GI tract epithelium (Fig. 2K, inset). The presence of FGF10 and FGFR1 antibody (Flg, 1:200, Santa Cruz Inc.) was performed in TBS with 3% bovine serum albumin and antibodies directed against FGFR1 (1:200, Santa Cruz Inc.) were incubated for 1 h at room temperature. The signals were visualized with Envision+ Dual Link Kit (Invitrogen) as recommended by the manufacturer.

176 Immunohistochemistry at different developmental stages was carried out to determine the expression of FGFR1 and FGFR2. These antibodies recognize both the “b” isoform, generally expressed in epithelia, and the “c” isoform usually expressed in mesenchyme. FGF10 binds to the “b” isoform of both receptors. At E12.5, FGFR1 is expressed at low levels in the epithelium (Fig. 2F). By E14.5, FGFR1 expression extends into the mesenchyme (Fig. 2G). At E18.5, FGFR1 staining is strongly localized to the muscular layer and is expressed at lower levels in the remaining mesenchyme and the epithelium throughout the entire colon (Figs. 2H–J). FGFR2 is strongly expressed in both the epithelium and the mesenchyme at E12.5 and E14.5 (Figs. 2K and L, respectively). At E18.5, FGFR2 is expressed in the proximal region that does not express Fgf10 (Fig. 2M), as well as in the mid and distal regions expressing Fgf10 (Figs. 2N and O, respectively). FGFR2 is mainly expressed in the epithelium but also at lower levels in the muscular layers and the serosal lining. Because the antibodies used for this study do not discriminate between the “b” and “c” isoforms of FGFR2, we performed a WM-ISH at E12.5 for Fgf2b. The staining shows uniform expression of this receptor isoform throughout the entire GI tract epithelium (Fig. 2K, inset). The presence of Fgf10 and the receptors FGFR1 and FGFR2 in the colon suggest a function for FGF10 in colon development. Moreover, the specific expression of the Fgf2b isoform in the epithelium makes the epithelium a likely target for FGF10.

177 Fgf10 inactivation leads to colonic atresia

178 WM-ISH for Hepatocyte nuclear factor 3β (Hnf3β), a specific marker of the epithelium in endoderm-derived organs, was used to determine the status of the gut epithelium in wild type and Fgf10 null colons at different developmental stages. At E10.5, the wild type GI tract consists of a tube of uniform diameter with a slight narrowing of the diameter at about one third of the length from the proximal end of the hindgut (Fig. 3A). The Fgf10/− GI tract shows a greater reduction in diameter at that point in the hindgut than the wild type, while the midgut appears normal (Fig. 3B). At E11.5, the cecum starts to develop, separating the colon from the rest of the intestine. At this stage, the colon has elongated significantly (Fig. 3C). In Fgf10/−...
embryos, the epithelium of the small intestine appears normal, while the formation of the cecum is arrested as previously reported (Burns et al., 2004). The epithelium is absent in two discrete regions, one located in the proximal region of the colon, and one located in the distal region of the colon (Fig. 3D). At this stage, the colonic mesenchyme is still visible in the mutant colon. At E12.5, the wild type colon continues to enlarge and elongate and the epithelium strongly expresses Hnf3β (Fig. 3E). By contrast, the distal colonic epithelium of the Fgf10 null mutants shows a weak expression of Hnf3β compared to the wild type. There is also an interruption of the continuity of the mesenchyme (arrow “a” in Fig. 3F). At E14.5, the colon of the

Fig. 1. Fgf10 expression during colonic development. Comparison of X-gal staining of whole-mount Fgf10LacZ/’ GI tract (A, A’, C, E, G) and Fgf10 expression by WM-ISH with Fgf10 antisense probe (B and insets in C and E). (A–B) At E10.5, Fgf10 is expressed in the distal part of the hindgut (yellow arrow). (C) At E12.5, the expression of Fgf10 extends proximally (WM-ISH for Fgf10 in inset). (C’) Vibratome sections of the X-gal staining and (D) WM-ISH show that the entire mesenchyme expresses Fgf10. (E) At E14.5, Fgf10 is expressed in the entire colon with the exception of the very proximal region adjacent to the cecum (WM-ISH in inset). (E’) Corresponding sections of the X-gal staining and (F) WM-ISH show that the mesenchyme immediately adjacent to the epithelium does not express Fgf10. (G) At E18.5, Fgf10 is still strongly expressed in most of the colon except the proximal region. (G’) Section of the mid colon stained with X-gal shows LacZ expression in the mesenchyme, mostly in the muscular layer and the serosa. (G’’) High magnification of panel G’. Dashed lines indicate the border between the epithelium and the mesenchyme. m, mesenchyme; e, epithelium; mg, midgut; c, cecum; si, small intestine; m, mesenchyme; e, epithelium; gt, genital tubercle.
Fgf10−/− mutant is very short (Fig. 3H) compared to the wild type (Fig. 3G). At E18.5, the colon of the Fgf10−/− mutant is almost completely absent. A small pouch adjacent to the cecum is representative of the residual colon (Fig. 3J), while the wild type colon continues to elongate (Fig. 3I). We conclude that absence of Fgf10 signaling in the developing GI tract consistently leads to an atresia of the colon from very early developmental stages onwards.

FGF10 is required for survival and proliferation of epithelial progenitor cells in the colon

In order to study the biological processes underlying the appearance of colonic atresias in the Fgf10 null embryos, we determined cell proliferation and apoptosis in wild type, Fgf10+/− and Fgf10−/− colons. Our analysis is done at E10.5, a stage where the atresia is not yet visible in the null mutant (Fig. 3B). Figs. 4A–C show BrdU staining of the colon in the epithelium and mesenchyme. Fig. 4D summarizes the proliferative activity of the epithelium in the different genotypes. We found a reduction in the percentage of BrdU positive cells in the epithelium of Fgf10 null embryos compared to wild type colon (5.8±1.3% vs. 32±0.8%, respectively, n=2 for each genotype). Interestingly, Fgf10+/− embryos show an intermediate number of BrdU positive cells (24.5±0.7%, n=2), suggesting that the colonic epithelium is sensitive to FGF10 dosage for its growth. Figs. 4E–G illustrate the results for TUNEL staining of the colon at E10.5. Fig. 4H summarizes the index of apoptosis in the epithelium for the different genotypes. The apoptotic index increases with decreased Fgf10 levels.
Fig. 3. Absence of FGF10 results in defective colon development. Morphologic comparison of the development of the colon in WT (A, C, E, G, I) and Fgf10−/− (B, D, F, H, J) embryos at E10.5, E11.5, E12.5, E14.5 and E18.5. (A–F) WH-ISH for Hnf-3β, a marker of the epithelium. (A) At E10.5, the midgut and hindgut of the wild type embryos elongates. (C, E) Regionalization along the proximal–distal axis of the GI tract forms the small intestine, the cecum and the colon at E11.5 and E12.5. (B) The Fgf10−/− embryos first show a reduction of the diameter of the colon at E10.5 followed by (D) interrupted expression of Hnf3β in the colonic epithelium at E11.5 (arrows a) and (F) complete atresia at E12.5. (G, I) Wild type colon at E14.5 and E18.5. (H, J) Fgf10−/− embryos show a normal small intestine, no cecal epithelium and an atresia of the colon compared to the respective wild type. si, small intestine; c, cecum; a, atresia.
Fgf10LacZ/− cases examined, there is more residual colonic tissue in Fgf10 null colon is 1959 a.u. ± 341, 1100 a.u. ± 221 and 392 a.u. ± 55, respectively. The difference between Fgf10 null and wild type, Fgf10 hypomorph and wild type, and between Fgf10 null and Fgf10 hypomorph is statistically significant (P < 0.001). In all cases examined, there is more residual colonic tissue in Fgf10LacZ/− colons (56.1%, of the length of the wild type colon) in comparison to the residual colonic tissue observed in the Fgf10−/− colons at the same stage (20% of the length of the wild type colon) (Fig. 5C).

In order to determine the consequences of reduced Fgf10 levels on epithelial morphogenesis, we carried out histological analysis of cross-sections in the proximal and mid regions of Fgf10LacZ/+, Fgf10 hypomorphic and Fgf10 null colons at different developmental stages. At E12.5, the epithelium is pseudostratified all along the proximal–distal axis of the colon (Kaufman et al., 1992). At this stage, the Fgf10 hypomorph and the Fgf10 null colons display epithelial hypocellularity compared to the wild type (Figs. 5D–H). At E14.5, the epithelium of the wild type colon changes to columnar epithelium and folds to form the future crypts (Calvert and Poitier, 1990; Mathan et al., 1976; Schmidt et al., 1988) (Figs. 5I, J). In the Fgf10LacZ/− mice, the epithelium of the proximal (Fig. 5J) and mid colon (Fig. 5M) fails to fold and remains in a pseudostratified state. In the Fgf10−/− embryo, the epithelium of the proximal colon shows hypocellularity and stays in a pseudostratified state (Fig. 5K). At E18.5, the wild type colon exhibits well-formed crypts in the proximal region (Fig. 5N) as well as the mid region (Fig. 5O). At this stage, the Fgf10LacZ/− colon shows defined but shortened crypts in the proximal region (Fig. 5O) while the mid region exhibits an enlarged lumen with rudimentary crypts and meconium accumulating in the lumen (Fig. 5R). The proximal region of the Fgf10−/− colon also displays shortened crypts (Fig. 5P). We conclude that FGF10 is involved in the proper formation of the crypts in the proximal and mid regions of the colon.

**Differentiation of colonic epithelium can occur with reduced levels of Fgf10 expression**

To investigate the role of FGF10 in the differentiation of the epithelium, we stained cross-sections of Fgf10 hypomorphic embryos with antibodies against TUNEL labeling of E10.5 embryonic sections (Fig. 4H). Quantification of the percentage of TUNEL positive epithelial cells showing a significant increase of apoptosis in Fgf10−/− and Fgf10+− colon compared to wild type counterpart (**P < 0.001). (E-G) TUNEL labeling of E10.5 Fgf10−/− (E), Fgf10−/− (F) and Fgf10−/− (G) colonic sections. (H) Quantification of the percentage of TUNEL positive epithelial cells showing a significant increase of apoptosis in Fgf10−/− and Fgf10+− colon compared to wild type counterpart (**P < 0.05).

**Fgf10 hypomorphic embryos display a less severe phenotype compared to Fgf10 null embryos**

The absence of distal colon at the late developmental stages in the Fgf10 null embryos prevents the study of the role of FGF10 in the differentiation of the epithelium. To circumvent this limitation, we generated Fgf10 hypomorphic mutants (Fgf10LacZ/−) by crossing Fgf10+− mice with Fgf10LacZ/− mice as previously described (Mailleux et al., 2005; Veltmaat et al., in press). Figs. 5A and B show, respectively, the colons of an Fgf10LacZ/− embryo and an Fgf10LacZ/− embryo at E18.5. These are stained with X-gal to reveal the Fgf10 expression domain. In order to quantify the extent of colon left in mutant and wild type gut at E18.5, we measured the length of the colon in mutant embryos from the cecum to the site of atresia and compared this length to the length of the colon in wild type embryos measured from the cecum to the rectum (n = 4 for each genotype). The average length for wild type, Fgf10 hypomorphic and Fgf10 null colon is 1959 a.u. ± 341, 1100 a.u. ± 221 and 392 a.u. ± 55, respectively. The difference between Fgf10 null and wild type, Fgf10 hypomorph and wild type, and between Fgf10 null and Fgf10 hypomorph is statistically significant (P < 0.001). In all cases examined, there is more residual colonic tissue in Fgf10LacZ/− colons (56.1%, of the length of the wild type colon) in comparison to the residual colonic tissue observed in the Fgf10−/− colons at the same stage (20% of the length of the wild type colon) (Fig. 5C).

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Fig. 5. Less severe phenotype of the Fgf10 hypomorphic colon compared to the Fgf10⁻/⁻ colon. (A–C) Morphologic comparison of Fgf10LacZ/⁺ (A), Fgf10LacZ/− (B) and Fgf10⁻/⁻ (C) colons at E18.5. Fgf10LacZ/− colons have been stained with X-gal to visualize the Fgf10 expressing region. Colons of Fgf10LacZ/− embryos show an atresia in the Fgf10 expressing domain (B), located more distally than the atresia observed in the Fgf10⁻/⁻ colon. (D–R) Hematoxylin and eosin staining in the proximal region that does not express Fgf10 and in the mid region expressing Fgf10 of Fgf10LacZ/+, Fgf10LacZ/− and Fgf10⁻/⁻ colonic sections at E12.5, E14.5 and E18.5. (D) Hematoxylin and eosin stained section of Fgf10LacZ/− colons showing absence of multi-layering of the epithelium at E14.5 and progressive enlargement of the crypts with reduced depth in the proximal part of the colon that does not express Fgf10 at E14.5 and E18.5. (E) Hematoxylin and eosin stained section at E18.5 of Fgf10⁺/+ and E18.5, Fgf10⁻/⁻ embryos. Note that both Fgf10LacZ/− and Fgf10⁻/⁻ embryos show an atresia in the Fgf10 expressing domain. (F) Hematoxylin and eosin stained section at E18.5 of Fgf10LacZ/− and Fgf10⁻/⁻ embryos showing absence of multi-layering of the epithelium at E14.5 and progressive enlargement of the crypts with reduced depth in the proximal part of the colon that does not express Fgf10 at E14.5 and E18.5. (G) Hematoxylin and eosin stained section at E18.5 of Fgf10LacZ/− and Fgf10⁻/⁻ embryos showing an atresia in the Fgf10 expressing domain. Scale bar: D–H: 50 μm, I–M: 60 μm, N–R: 125 μm.
FGF10 is not required for proper development of the mesenchyme

Shh and Ihh are genes known to directly control the differentiation of the mesenchyme and affect crypt formation (Madison et al., 2005; Ramalho-Santos et al., 2000). Bmp4 also regulates crypt formation, but its mechanism of action on the epithelium or mesenchyme is still unclear (Haramis et al., 2004). We performed a WM-ISH to study the role of FGF10 in the regulation of these genes. Shh, Ihh and Bmp4 are expressed at various levels throughout the developing gut either in the epithelium (Shh and Ihh, Figs. 7A, C respectively) or in the mesenchyme (Bmp4, Fig. 7E). No difference was found in the level or the pattern of expression of these genes at E15.5 between the wild type and the Fgf10 hypomorphic mutants (Figs. 7A–F). To assess the differentiation of the mesenchyme in more detail, we used immunofluorescence at E18.5 to analyze alpha smooth muscle actin (α-SMA), a marker for the intestinal subepithelial myofibroblasts (ISEMFs), and desmin, a marker for smooth muscle cells. Expression of α-SMA (Figs. 7G, H) and desmin (Figs. 7I, J) appears unchanged between the Fgf10 hypomorphic and the wild type colons. We therefore conclude that reduced levels of Fgf10 expression do not affect the differentiation of the mesenchyme.

Discussion

The restricted expression pattern of Fgf10 along the proximal–distal axis of the developing GI tract suggests that regionalization of the GI tract into distinct anatomical and

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Fig. 6. Normal levels of Fgf10 are not critical for differentiation of the colonic epithelium. (A–B’) Immunofluorescence (IF) staining for Ki67, a marker of proliferating cells. (C–D’) IF staining for ephrin-B, a marker of differentiated cells. (E–F’) Alcian blue staining of terminally differentiated goblet cells. P0 wild type (A, A’, C, C’, E, E’) and Fgf10LacZ/− (B, B’, D, D’, F, F’) mid colonic sections. Panels A’, B’, C’, D’, E’ and F’ are higher magnification views of the boxes in panels A, B, C, D, E and F, respectively. In the wild type, the proliferating cells are located at the base of the crypts (A, A’), and the differentiated cells expressing ephrin-B (C, C’) and goblet cells (E, E’) are located at the upper region of the crypts. The Fgf10LacZ/− colons show groups of proliferating cells (B, B’) and groups of differentiated cells (D, D’) and goblet cells (F, F’). Arrows indicate the base of the crypts in the wild type and the presumptive crypt regions in the hypomorphic mutant.

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FGF10 dosage is important for proper colonic development

Total inactivation of Fgf10 leads to a complete loss of the distal part of the colon from an early stage of embryonic development. Many forms of intestinal atresia are thought to be the consequence of a mesenteric vasculature occlusion. However, Fairbanks et al. (2005) demonstrated that the absence of Fgf10 does not affect the proper development of the mesenteric vasculature. Our work demonstrates that the observed colonic atresia in Fgf10 null embryos is indeed the consequence of significant reduction in proliferation and increased apoptosis in the Fgf10 mutant colonic epithelium compared to the heterozygous and wild type colon. Similar to the development of the bladder, urethral tube and prostate (Bagai et al., 2002; Donjacour et al., 2003; Petiot et al., 2005), the development of the bladder, urethral tube and prostate compared to the heterozygous and wild type colons. Similar to the consequence of significant reduction in proliferation and increased apoptosis in the Fgf10 mutant colonic epithelium compared to the heterozygous and wild type colon. Similar to the development of the bladder, urethral tube and prostate.

Distinct signaling pathways control the proliferation of the proximal and distal colon

Comparison of the histology of the proximal region of the Fgf10 hypomorphic and Fgf10 null colons demonstrates that crypts are forming even in absence of Fgf10. This result suggests that other signaling pathways, independent of the FGF10 signaling pathway, are critical for the control of epithelial progenitor cell survival and proliferation in the proximal region of the colon. An identical phenotype was observed in the Fgf12-/- embryos (Fairbanks et al., 2006) suggesting that neither FGF10 nor FGFR2b is critical for development of the proximal region of the colon.

The signaling pathways regulating proximal colonic proliferation remain to be identified. A potential pathway for controlling the survival and proliferation of the epithelium in the proximal region of the colon is the canonical Wnt signaling pathway. Components of the Wnt signaling pathway have been shown to be expressed in the embryonic G1 tract (Theodosiou and Tabin, 2003). In addition, the Wnt signaling pathway...
regulates epithelial proliferation of the adult murine intestine (Pinto et al., 2003; van de Wetering et al., 2002). More work will have to be done to test the role of Wnt signaling during colon embryogenesis.

Reduced levels of Fgf10 are sufficient for the differentiation of intestinal epithelial cells

The FGF10 pathway has been shown to control epithelial differentiation in many organs, including the lung and the stomach. In the lung, FGF10 loaded heparin sepharose beads grafted at the level of the trachea can trigger formation of an ectopic bud expressing surfactant Protein C, a marker of type II pneumocytes (Hyatt et al., 2004). This result demonstrates that FGF10 is sufficient to reprogram the tracheal epithelium to become respiratory epithelium. We have also reported that Fgf10−/− and Fgfr2b−/− stomachs are smaller than the wild type stomachs, and that during mid to late fetal stages (E15.5–18.5) epithelial differentiation of mucous and chief cell lineages is rudimentary, with no expression of several early cytodifferentiation markers, including GATA4, GATA6, H/K-ATPase and abnormal expression of members of the Hedgehog family of signaling molecules (Spencer-Dene et al., 2006). Moreover, activation of FGFR2b signaling by FGF7 has previously been shown to induce epithelial differentiation into goblet cells in adult rat intestine (Housley et al., 1994).

By contrast, our results indicate that even with reduced levels of Fgf10 the epithelium differentiates normally. It is interesting to note that, in the Fgf10lacZ−/− colon, there is clustering of cells expressing ephrin-B alternating with clusters of ephrin-B negative cells, suggesting that even in the absence of normal crypt architecture, differentiated cells and undifferentiated cells are still regionalized. Our results indicate that FGF10 is not directly involved in controlling epithelial differentiation in the colon. A similar conclusion has been proposed concerning the role of Fgf10 in the developing prostate as prostatic rudiments from Fgfr2b−/− embryos transplanted into intact male hosts grew very little, but showed some signs of prostatic differentiation (Donjacour et al., 2003).

These data suggest that other pathways, such as the canonical Wnt pathway, which controls epithelial differentiation in the adult intestine (van de Wetering et al., 2002), may control differentiation during colonic development, while FGF10 controls epithelial survival and proliferation of the mid and distal colonic epithelium.

Fgf10 is not required for proper development of the mesenchyme

Shh, Ihh and Bmp4 have been reported to play important roles during gut development. For example, epithelial hedgehog signaling is involved in the patterning of the intestinal crypt–villus axis by controlling the differentiation of the intestinal subepithelial myofibroblasts that express α-SMA and the smooth muscle cells expressing desmin (Madison et al., 2004; Adegboyega et al., 2002; Ramalho-Santos et al., 2000; Powell et al., 1999). Inactivation of Ihh leads to a dilated colon with absence of crypts and an abnormally thin mesenchymal layer (Ramalho-Santos et al., 2000), similar to the histological findings in Fgf10lacZ−/− mice. Bmp4 expressed in the mesenchyme during intestinal development (Madison et al., 2004; Roberts et al., 1995, 1998; Sukegawa et al., 2000) also represses de novo crypt formation during adulthood (Haramis et al., 2004). These genes or the proteins encoded by these genes have been described to be downstream or upstream of FGF10 during the development of many organ systems, including the lung (Warburton et al., 2001). We initially hypothesized that the expression of these genes could therefore be altered as the result of reduced Fgf10 expression. Our data indicate that the expression patterns of Shh, Ihh and Bmp4 are unchanged between wild type and Fgf10 hypomorphic colons. In addition, we observe unchanged expression of α-SMA and desmin in the mesenchyme in the Fgf10 hypomorphic colons suggesting that the mesenchyme differentiates normally in spite of reduced levels of Fgf10 expression. Interestingly, the expression of Shh is also unchanged in Fgfr2b−/− developing urethra (Petiot et al., 2005), and the mesenchyme appears to differentiate normally in the mutant tissue. We therefore conclude that these developmental genes are not controlled by FGF10. Since the epithelium and the mesenchyme differentiate properly in the Fgf10lacZ−/− mice, we propose that defective survival and proliferation in the colonic epithelium is the underlying cause preventing normal crypt architecture formation.

In conclusion, our work demonstrates for the first time the crucial role played by FGF10 in colonic development. FGF10 controls survival and proliferation of the colonic epithelial progenitor cells. These two biological activities are essential for the proper formation of the crypts in the colon.

Uncited references

Ornitz and Itoh, 2001

Vidrich et al., 2004

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