Fibroblast growth factor 10 is required for survival and proliferation but not differentiation of intestinal epithelial progenitor cells during murine colon development

Frédéric G. Sala a,b, Jennifer L. Curtis b, Jacqueline M. Veltmaat b, Pierre-Marie Del Moral b, Lendy T. Le b, Timothy J. Fairbanks b, David Warburton b, Henri Ford b, Kasper Wang b, R. Cartland Burns c, Saverio Bellusci a,b,⁎

a UMR144-CNRS/Institut Curie, 75248 Paris cedex 05, France
b Developmental Biology Program, Saban Research Institute of Children’s Hospital Los Angeles, Los Angeles, CA 90027, USA
c Division of Pediatric General and Thoracic Surgery, Children’s Hospital of Pittsburgh, Pittsburgh, PA 15213, USA

Received for publication 23 February 2006; revised 31 July 2006; accepted 1 August 2006
Available online 9 August 2006

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.

© 2006 Elsevier Inc. All rights reserved.

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 Melton, 2000; Roberts, 2000). The mesoderm differentiates uniformly into smooth muscles and connective tissue along the proximal–distal axis of the gut, while the endoderm differentiates with regional specification (Kedinger et al., 1988; Traber, 1998). Morphologically and functionally, the developing gut tube is subdivided into 3 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 (Haffen 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

⁎ Corresponding author. Developmental Biology Program, Saban Research Institute of Children’s Hospital Los Angeles, Los Angeles, CA 90027, USA. E-mail address: sbellusci@chla.usc.edu (S. Bellusci).

0012-1606/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2006.08.001
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 (McKeehan 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; McKeehan 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 ‘IIIC’, each with respectively distinct ligand-binding specificity and tissue distributions (Omitz et al., 1996; Omitz and Itoh, 2001). The Fgfr2 gene splice variant containing the IIb 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, 1994; Omitz et al., 1996; Bellusci et al., 1997). FGFR 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 (Kanard 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 Mice-lacZ(C4) (hereafter Fgflod5) 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. Fgflod5 embryos (Mailleux et al., 2005) were generated by crossing Fgflod5+/− on a C57BI/6 background (Sekine et al., 1999) with Fgflod5+/− mice. Offspring were genotyped as previously described (Mailleux et al., 2005). The total number of mutant embryos use in this study are: $Fgf10^{+/−}$ (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), $Fgf10^{+/−}$ (E10.5, n = 10) and $Fgf10^{+/−}$: (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 b $Fgf10$ cDNA (described by Bellusci et al., 1997), a 1 kb Hnf-3β cDNA, a 642 b Shh cDNA, a 1.8 kb Ihh cDNA and a 1.5 kb full-length Bmp4 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 UK Limited) 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). Vectashield 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, from Roche Applied Science) for detection and quantification of apoptosis was used per manufacturer protocol on 5 μm paraffin sections of E10.5 $Fgf10^{+/−}$, $Fgf10^{−/−}$ and $Fgf10^{+/−}$ colons.

**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 $Fgf10^{−/−}$ and 2 $Fgf10^{−/−}$ embryos harvested from the same litter. The experiments were repeated with two other litters (3 $Fgf10^{−/−}$ embryo and 3 wild type embryos) and a drastic reduction in epithelial proliferation and increase in cell death between $Fgf10^{−/−}$ 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...
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.

Immunostainings

The colon was dissected from embryos at different stages of development (E12.5 to E18.5), fixed overnight in formalin and stored in 70% alcohol. The tissues were embedded in paraffin and 5 μm cross-sections were made. For Ki-67 and ephrin-B immunostaining, antigen retrieval was performed by boiling the samples for 20 min in Na-Citrate buffer (10 mM pH 6.0). Incubation of antibodies directed against Ki-67 (1:100, Novoceastra) and ephrin-B1 (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 20 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.

Histological stainings

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.ihcworld.com/_protocols/special_stains/alcian_blue.htm.

Morphometric analysis of the wild type and mutant colons

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 colon 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.

Results

Fgf10 is expressed in the embryonic colon

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 Fgf10 expression in several organs (Kelly et al., 2001; Maileux et al., 2005; Veltmaat et al., 2006). In order to validate the usefulness of these mice in the developing colon, we compared the expression of β-galactosidase to Fgf10 mRNA expression using whole-mount in situ hybridization (WM-ISH) between E10.5 and E14.5. β-galactosidase activity is first detected only in the distal-most portion of the colonic mesenchyme at E10.5 (Figs. 1A, A’). A similar expression pattern is observed for Fgf10 (Fig. 1B). The β-galactosidase expression domain extends proximally to encompass the distal half of the colon at E12.5 (Fig. 1C). At E14.5 and E18.5, the β-galactosidase expression continues to extend proximally and is seen in the entire colon with the exception of the proximal-most region (Figs. 1E and G, respectively). A similar expression pattern is observed for Fgf10 WM-ISH (insets in Figs. 1C and E). Vibratome sections at the level of the distal colon show that β-gal and Fgf10 are expressed throughout the mesenchyme but not in the epithelium at E12.5 (Figs. 1C’, D). At E14.5, β-gal and Fgf10 expressions are absent from the mesenchyme immediately adjacent to the epithelium (Figs. 1E’, F). At E18.5, β-galactosidase is expressed in the muscular layer and the serosa (Figs. 1G’, G”). Our results therefore indicate that LacZ expression in Fgf10LacZ/– mice is a reporter for Fgf10 expression in the developing colon. We also show that Fgf10 starts to be expressed in the distal part of the colon and expands proximally at later stages.

FGFR1 and FGFR2 are expressed throughout the colon

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 Fgfr2b. 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 Fgfr2b isoform in the epithelium makes the epithelium a likely target for FGF10.

Fgf10 inactivation leads to colonic atresia

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 Fgf10^{lacZ/} 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 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.

*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.

---

**Fig. 2. FGFR1 and FGFR2 expression in the embryonic colon.** Immunohistochemical analysis of E12.5, E14.5 and E18.5 wild type colonic sections stained with anti-FGFR1 (F–J) or anti-FGFR2 (K–O) polyclonal antibodies. Control stainings (A–E), in which the primary antibody was absent, show no signal. (F–J) FGFR1 is expressed at low levels in the epithelium at E12.5. At E14.5 and E18.5, FGFR1 staining is present in the epithelium and the mesenchyme with stronger expression in the muscular layer throughout the colon. (K–O) FGFR2 is expressed in both mesenchyme and epithelium at all stages. At E18.5, FGFR2 staining is mainly expressed in the epithelium, the muscular layer and the serosa of the entire colon (inset in K). WM-ISH with a specific probe for *Fgfr2b* on E12.5 wild type GI tract shows a staining in the epithelium.
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.
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 (Fgf10\textsuperscript{lacZ/-}) by crossing Fgf10\textsuperscript{+/+} mice with Fgf10\textsuperscript{lacZ/+} mice as previously described (Mailloux et al., 2005; Veltmaat et al., 2006). Figs. 5A and B show, respectively, the colons of an Fgf10\textsuperscript{lacZ/-} embryo and an Fgf10\textsuperscript{lacZ/-} 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 Fgf10\textsuperscript{lacZ/-} colons (56.1%, of the length of the wild type colon) in comparison to the residual colonic tissue observed in the Fgf10\textsuperscript{-/-} 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 Fgf10\textsuperscript{lacZ/-}, 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, 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, L). In the Fgf10\textsuperscript{lacZ/-} 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\textsuperscript{-/-} 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 Fgf10\textsuperscript{lacZ/-} 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\textsuperscript{-/-} colon also displays shortened crypts (Fig. 5P). We conclude that FGF10 is involved in the proper formation of the crypts in the mid and distal 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
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/+ and 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/+ (D–G), Fgf10LacZ/− and Fgf10−/− colonic sections at E12.5, E14.5 and E18.5. In F gf10LacZ/− colons, the crypts begin to form at E14.5 (I, L) with a multi-layered epithelium. At later stages, the epithelium folds to form well-defined crypts (N, Q). (M) Fgf10 hypomorphic mid colon shows absence of multi-layering of the epithelium at E14.5 and a progressive enlargement of the lumen. (R) The Fgf10LacZ/− mid colon fails to develop crypts at E18.5. (O, P) Note that defined crypts with reduced depth are forming in the proximal part of the colon that does not express Fgf10 in both Fgf10LacZ/− and Fgf10−/− embryos. Scale bar: D–H: 50 μm, I–M: 60 μm, N–R: 125 μm.
colon and corresponding regions of wild type colon on the first day of life (P0) with markers for undifferentiated and differentiated intestinal epithelial cells. Figs. 6A–B' show Ki-67 staining, a marker of the proliferating cells (Batlle et al., 2002). In the wild type, cells at the base of the crypts are positive for Ki-67 (Figs. 6A, A'). In the Fgf10LacZ/− colon, clusters of cells labeled for Ki-67 alternate with clusters of cells negative for this marker (Figs. 6B, B'). Figs. 7C–D' show ephrin-B staining, a marker of terminally differentiated intestinal epithelial cells (Batlle et al., 2002). In the wild type colon, the apices of the crypts are stained, while the bases of the crypts are devoid of signal (Figs. 6C, C'). In the Fgf10LacZ/− colon, ephrin-B is expressed in discrete regions separated by clusters of cells without staining (Figs. 6D, D'). Figs. 6E–F' show alcian blue staining of goblet cells in wild type and Fgf10 hypomorphic colon. As in the wild type (Figs. 6E, E'), goblet cells differentiate properly in the Fgf10LacZ/− colon (Figs. 6F, F'). We therefore conclude that reduced levels of Fgf10 expression do not affect the differentiation of the epithelium.

**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...
functional domains starts very early during development. Fgf10, expressed in most of the developing colon, plays a pivotal role in both survival and proliferation of intestinal epithelial progenitor cells, activities essential for crypt formation. In addition, using Fgf10 hypomorphic mice, we demonstrate for the first time that reduced levels of Fgf10 are sufficient for the differentiation of the colonic epithelium and mesenchyme.

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 colons. Similar to the development of the bladder, urethral tube and prostate (Bagai et al., 2002; Donjacour et al., 2003; Petiot et al., 2005), FGF10/FGFR2b signaling is critical for the maintenance and proliferation of the epithelial progenitor cells of the colon. Our results also indicate that the colon of Fgf10<sup>+/−</sup> mice, although phenotypically normal at birth (data not shown), displays lower proliferation and higher cell death in the epithelial layer at E10.5 than the corresponding wild type. At even lower Fgf10 levels, as demonstrated in the Fgf10 hypomorphic embryos, normal crypt architecture fails to develop. Evidence is accumulating that FGF10 dosage is critical for proper development of many organs, including the lung (Mailleux et al., 2005), the mammary glands (Veltmaat et al., 2006) and the limbs (Bellusci, unpublished results). Moreover, Entesarian and colleagues (2005) have recently shown the importance of FGF10 dosage for salivary and lacrimal gland development in humans. Individuals with autosomal dominant aplasia of lacrimal and salivary gland (ALSG) were found to be heterozygous for FGF10. Complementary studies in adult Fgf10<sup>+/−</sup> mice revealed that Fgf10 heterozygotes have absent parotid and smaller submandibular glands (Jaskoll et al., 2005). Our results demonstrate that the morphogenesis of the colon is indeed Fgf10 dose-dependent.

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 Fgrf2b<sup>−/−</sup> 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 GI tract (Theodosiou and Tabin, 2003). In addition, the Wnt signaling pathway

---

Fig. 7. Mesenchymal differentiation of the Fgf10<sup>lacZ−/−</sup> colon. Whole-mount in situ hybridization on E15.5 wild type or Fgf10<sup>lacZ−/−</sup> colons for Shh (A, B); Ihh (C, D) and Bmp4 (E, F). In the wild type, Shh and Ihh are expressed in the epithelium, and Bmp4 is expressed in the mesenchyme. In the Fgf10<sup>lacZ−/−</sup> colon, the expression of these genes is not affected by low levels of Fgf10. IF staining of E18.5 wild type and Fgf10<sup>lacZ−/−</sup> colonic sections for α-SMA (G, H) and desmin (I, J). Low levels of Fgf10 do not affect the expression of α-SMA and desmin. c, cecum.
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 FGFFR2b 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 Fgf10/lacZ−/− 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 Fgf10−/− 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., 2005; 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 (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.

Acknowledgments

We are grateful to Dr. Robert Kelly for the Fgf10lacZ−/− mice, and to Dr. Nobuyuki Itoh for the Fgf10−/− mice. This work was supported by an intramural grant from Children’s Hospital Los Angeles.

References


