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Fibroblast growth factor 10 represses premature cell differentiation during establishment of the intestinal progenitor niche

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ABSTRACT

Spatio-temporal regulation of the balance between cell renewal and cell differentiation is of vital importance for embryonic development and adult homeostasis. Fibroblast growth factor signaling relayed from the mesenchyme to the epithelium is necessary for progenitor maintenance during organogenesis of most endoderm-derived organs, but it is still ambiguous whether the signal is exclusively mitogenic. Furthermore, the downstream mechanisms are largely unknown. In order to elucidate these questions we performed a complementary analysis of fibroblast growth factor 10 (*Fgf10*), gain-of-function and loss-of-function in the embryonic mouse duodenum, where the progenitor niche is clearly defined and differentiation proceeds in a spatially organized manner. In agreement with a role in progenitor maintenance, FGF10 is expressed in the duodenal mesenchyme during early development while the cognate receptor FGFR2b is expressed in the epithelial progenitor niche. *Fgf10* gain-of-function in the epithelium leads to spatial expansion of the progenitor niche and repression of cell differentiation, while loss-of-function results in premature cell differentiation and subsequent epithelial hypoplasia. We conclude that FGF10 mediated mesenchymal-to-epithelial signaling maintains the progenitor niche in the embryonic duodenum primarily by repressing cell differentiation, rather than through mitogenic signaling. Furthermore, we demonstrate that FGF10-signaling targets include ETS-family transcription factors, which have previously been shown to regulate epithelial maturation and tumor progression.

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Introduction

Organogenesis is a fascinating developmental process involving a series of integrated events leading to the fully functional organ. Expansion of the committed progenitor cell population is an early event in organogenesis. This phase has received increasing attention during the past few years, due to the promising prospect of stem cell therapy, which has led to a need for knowledge on how to expand progenitor cells as a source for replacement therapy for multiple diseases. Likewise, balancing of progenitor cell expansion and differentiation features prominently in adult homeostasis of renewable organs, such as the intestine, and in the mutated state, also during cancer progression.

It is well known that reciprocal signaling between the epithelium and the surrounding mesenchyme via soluble and/or membrane-tethered signals is necessary for regulating growth and differentiation of the gut epithelium (Haffen et al., 1987;Kedinger et al., 1987), and several signaling pathways, including WNT (Korinek et al., 1998;Pinto et al., 2003), BMP (He et al., 2004), Notch (Fre et al., 2005;Jensen et al., 2000;van Es et al., 2005;Wong et al., 2004), and Hedgehog (Madison et al., 2005;Ramalho-Santos et al., 2000) have been implicated in regulating gastric and enteric development. Members of the fibroblast growth factor (FGF) family are mediators of mesenchymal-to-epithelial signaling in several organs, and are included as a component in many stem cell differentiation protocols. FGF10 in particular, has been shown to be necessary for progenitor maintenance during organogenesis of endoderm-derived organs, but it is still ambiguous whether the signal is exclusively mitogenic, or whether it also regulates cell differentiation. Furthermore, the downstream mechanisms are largely unknown.

Fgf10 is expressed in the mesenchyme of the embryonic duodenum (Bhushan et al., 2001;Kanard et al., 2005;Nyeng et al., 2007), as well as in the mesenchyme of the lung, stomach, pancreas, colon, and cecum (Bellusci et al., 1997;Burns et al., 2004;Fairbanks et al., 2004;Nyeng et al., 2007;Sala et al., 2006). Loss-of-function mutation of *Fgf10* or its cognate receptor *Fgfr2b* results in lung agenesis (Arman et al., 1999;Sekine et al., 1999), and severely affects the development of the glandular stomach (Spencer-Dene et al., 2006) and the pancreas (Bhushan et al., 2001; Pulkkinen et al., 2003). In the intestines, loss of *Fgf10* has been analyzed thoroughly in the colon (Sala et al., 2006) and the cecum (Burns et al.,

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2004), where attenuated proliferation and increased apoptosis led to colonic atresia and failure of cecal budding. Studies of clinical relevance have implicated *Fgf10* in intestinal pathobiology, as *Fgf10*−/− mice have duodenal (Kanard et al., 2005) and colonic (Fairbanks et al., 2005) atresia. Unfortunately, the duodenal phenotype was not analyzed further. So far, clinical studies have however failed to identify any association of mutations in *Fgf10* and duodenal atresia or anorectal malformations (Kruger et al., 2008;Tatekawa et al., 2007). The severe phenotype of loss of *Fgf10* prompted an analysis of *Fgf10* gain-of-function in order to understand the mechanism in more detail. Our studies of the stomach and pancreas have demonstrated that ectopic *Fgf10* overexpression in the epithelium results in inhibition of cell differentiation (Norgaard et al., 2003;Nyeng et al., 2007), a result that was corroborated by Hart et al in the pancreas (Hart et al., 2003). Quite likely, a central function of FGF-signaling in enteric progenitor maintenance has been overlooked, as previous studies focused mostly on the time after terminal differentiation. We therefore decided to study whether ectopic expression of *Fgf10* in the duodenal epithelium, disrupting the endogenous FGF10 gradient emanating from the mesenchyme, would suppress differentiation outside the progenitor niche, leading to an expansion of the niche. Reciprocally, we wished to test whether loss of *Fgf10* in the duodenum is sufficient to release progenitor cells and cause premature differentiation.

In the present work, we demonstrate that FGF10 mediated mesenchymal-to-epithelial signaling maintains the progenitor niche in the embryonic small intestine by repressing differentiation. Furthermore, we demonstrate that FGF10-signaling targets in the intestine include ETS-family transcription factors Etv4 and Etv5.

Materials and methods

Mouse derivation

Generation of a pPdx1-*Fgf10*^{FLAG} construct and derivation of transgenic mouse (*Mus musculus*) embryos, as described earlier (Norgaard et al., 2003), were approved by the UCHSC Animal Care and Use Committee. A total of 3 embryonic day 12.5 (E12.5) and 6 E18.5 pPdx1-*Fgf10*^{FLAG} embryos and their wildtype (WT) littermates were obtained by oocyte injection using FVB mice. The day of oocyte transfer was counted as embryonic day 0.5 (E0.5). BrdU (20 mg/kg) was injected 2 h prior to isolation of embryos. Targeted disruption of *Fgf10* has already been described (Min et al., 1998). A total of 8 E14.5 and 8 E18.5 *Fgf10*−/− embryos were obtained by timed mating of heterozygotes which had been outbred on a CD1 background. The day of vaginal plug was counted as E0.5. Heterozygotic littermates were used as controls throughout, as they were indistinguishable from wildtype embryos.

All embryos were genotyped by PCR. *pPdx1-Fgf10*^{FLAG} embryos were genotyped using *Fgf10-Flag* primers (Table S1) and *Fgf10*−/− embryos were genotyped using *Fgf10* KO P1, P2, P3 and P4 primers as described (Sekine et al., 1999). Expression of the *pPdx1-Fgf10*^{FLAG} construct was confirmed by RT-PCR of three E18.5 embryos and WT littermates. *pPdx1-Fgf10*^{FLAG} embryos varied somewhat in their expression of the transgene, but although phenotypes were not identical, they were highly similar, and the phenotypic variation never exceeded that observed in *Fgf10*−/− mice.

Tissue and RNA isolation

Whole guts were dissected in ice-cold PBS and fixed in 4% PFA overnight. They were then immersed in 30% sucrose for at least 4 h, in 1:1 30% sucrose and OCT embedding medium for 1 h and finally in OCT for 1 h before embedding. Tissue blocks were freeze sectioned at a thickness of 6 μm using a cryotome. Embryos for RNA analysis were dissected in ice-cold PBS and the upper half of the duodenum including mesenchyme and epithelium was extracted using the RNeasy (Qiagen) kit including a DNAse treatment step. Endogenous *Fgf* expression was analyzed using RNA from embryos from time-mated CD1 mice. RNA from E12.5 whole small intestine was pooled from 5 embryos for each sample. Whole duodenal tissue was pooled from 4 E14.5 embryos, 3 E15.5 embryos or 2 embryos for each of the samples at E16.5, E17.5 and E18.5. At least three samples were analyzed per time point.

Multiplex RT-PCR analysis

Purified total RNA was analyzed by a semi-quantitative low-cycle radioactive multiplex RT-PCR (MPX-RT-PCR) method previously described (Jensen et al., 1996) with 2 μg of total RNA per sample. A primer set for the TATA box binding protein (Tbp) or Alpha-tubulin (α-tubulin) was included in all reactions as an internal control and used for standardization. Primers (Supplementary Material Table S1) were specifically designed for MPX-RT-PCR, optimized for exponential amplification within the cycle range and tested for interference. Six primers were included in each reaction and the reaction was carried out at 96 °C for 1 min, followed by 28 cycles of 96 °C for 30 s, 55 °C for 30 s and 73 °C for 30 s. Following the RT-PCR reaction, samples were run on a denaturing sequencing gel. The gel was subsequently exposed to phosphoimage storage screen for 3 days, after which the screen was scanned. Experiments were carried out in triplicate for each sample and values are expressed as mean ± SEM relative to *Tbp* or *α-tubulin*.

Immunohistochemistry

Immunohistochemistry (IHC) was carried out as already described (Norgaard et al., 2003). Primary antibodies are included in Table S1. 0.1% Triton X-100 was added to the blocking buffer. After overnight incubation with the primary antibodies, slides were washed and incubated with rabbit/goat/rat/mouse/guinea pig secondary antibody conjugated to Texas red/Cy2/AMCA (Jackson ImmunoResearch, PA, USA) for 1 h or taken through the Histostain kit protocol (Zymed/Invitrogen, CA, USA), followed by tyramide signal amplification (TSA) (Perkin Elmer, MA, USA) as indicated in Table S1. Nuclei were stained with Hoechst dye for morphometric studies. Mature goblet cells were stained for 1 h using rhodamine-conjugated Dolichos biflorus agglutinin (DBA), while immature goblet cells were stained with fluorescein conjugated Bauhinia purpurea lectin/agglutinin (BPA). Apoptotic cells were detected using a TUNEL kit (DeadEnd Fluorometric TUNEL System, Promega, WI, USA) according to protocol.

In situ hybridization

Digoxygenin labeled RNA in situ probes where synthesized and in situ hybridization (ISH) of tissue sections carried out as in (Norgaard et al., 2003) from purified restriction fragments obtained from sequence-validated plasmids listed in Supplementary Material Table S1.

Histochemistry

Harris hematoxylin and eosin Y staining was carried out according to standard protocols.

Goblet cells were stained for morphometry using Alcian blue/Periodic Acid Schiff (PAS). Frozen slides were thawed and stained 15 min with Alcian Blue (Newcomersupply, WI, USA). After washing, the slides were covered with periodic acid (Sigma-Aldrich, MO, USA) for 5 min, rinsed and stained with Schiffs reagent for 6 min (Sigma-Aldrich). Finally, slides were washed and nuclei stained with Gills hematoxylin (Sigma-Aldrich).

Image analysis

Images were obtained in ImagePro v. 4.5 (Media Cybernetics, MD, USA) using a Pixera CL600 camera, mounted on an Olympus BX51 microscope station equipped with differential interference contrast.

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