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FGF10 signaling controls stomach morphogenesis

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Abstract

Maintenance of progenitor cell properties in development is required for proper organogenesis of most organs, including those derived from the endoderm. FGF10 has been shown to play a role in both lung and pancreatic development. Here we find that FGF10 signaling controls stomach progenitor maintenance, morphogenesis and cellular differentiation. Through a characterization of the initiation of terminal differentiation of the three major gastric regions in the mouse, forestomach, corpus and antrum, we first describe the existence of a "secondary transition" event occurring in mouse stomach between E15.5-E16.5. This includes the formation of terminally differentiated squamous cells, parietal, chief a nd gastric endocrine cells from a pre-patterned gastric progenitor epithelium. Expression analysis of both FGF and Notch signaling components suggested a role of these networks in such progenitors, which was tested through ectopically expressing FGF10 in the developing posterior stomach. These data provide evidence that gastric gland specification and progenitor cell maintenance is controlled by FGF10. The glandular proliferative niche was disrupted in pPDX-FGF10^{FLAG} mice leading to aberrant gland formation, and endocrine and parietal cell differentiation was attenuated. These effects were paralleled by changes in Hes1, Shh, and Wnt6 expression, suggesting that FGF10 acts in concert with multiple morphogenetic signaling systems during gastric development.

Keywords

endoderm; stomach; FGF10; FGFR2; Notch; Hes1; differentiation; Sox2; chief cells; parietal cells; SPEM

Introduction

Development of the mammalian gut is a highly orchestrated process involving a multitude of morphogenetic processes, tissue interactions, and cell differentiation events. Of the tissues derived from the gut endoderm, development of the budding organs such as the lung, liver and pancreas is best characterized, and several gene products and signaling systems have been described securing regional identity and morphogenetic development of these organs (Bellusci et al., 1997; Costa et al., 2001; Jensen, 2004; Lemaigre and Zaret, 2004). In contrast, the morphogenesis of the main gut tract, composed of esophagus, stomach and intestinal system is less well characterized, and most studies related to cell differentiation within these regions have focused on the behavior of the gut endoderm in the normal adult, or transformed state, through investigations of stomach and intestinal cancer.

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Gastric mesenchyme is essential for survival of the epithelium in vitro and tissue-grafting experiments have shown that a diffusible factor from the mesenchyme is critical for stomach epithelial development in the chicken (Koike and Yasugi, 1999). Recombination with forestomach mesenchyme did not change the fate of glandular epithelium and vice versa after E11.5 in the mouse (Fukamachi et al., 1979), suggesting that the primary specification of the gastric epithelium occurs before E11.5. Factors that influence this primary specification include the mesenchymal-expressed Hoxa5 (Aubin et al., 2002) and Barx1, a homeobox gene that act upstream of Wnt-antagonists (Kim et al., 2005). In addition to this primary specification of the stomach, a secondary cell specification and glandular formation is required to form the mature stomach. Differentiation of specific cell lineages and invaginations of epithelium to form glands begins already in the embryo, but mature glands are not formed until after birth. Sonic hedgehog expressed in the epithelium has been shown to regulate gland development in the adult mouse stomach (van den Brink et al., 2001), and BMP (Narita et al., 2000) and Notch signaling (Matsuda et al., 2005) is critical for gland formation in the chicken proventricular stomach. Yet, we do not know the identity of any mesenchymal expressed morphogen(s) being responsible for regulating epithelial cell differentiation in the embryonic stomach.

Several studies have revealed significant sharing of gene function along the gut. We and others previously showed that the Notch signaling system, via *Hes1*, acts as a unified regulatory mechanism controlling the development of endodermal endocrine cells in the lung (Ito et al., 2000), stomach (Jensen et al., 2000), pancreas (Jensen et al., 2000), small intestine (Jensen et al., 2000; Milano et al., 2004; van Es et al., 2005) and colon (Jensen et al., 2000). Furthermore, this signaling system might be critical for proper differentiation from several, if not all, of the endodermal stem-cell populations in the gut throughout life (van Es et al., 2005; Milano et al., 2004; Zecchini et al., 2005; Collins et al., 2004; Apelqvist et al., 1999). Also, Ngn3 has been shown to be necessary for endocrine development in both the pancreas (Gradwohl et al., 2000), intestine (Jenny et al., 2002), and stomach (Jenny et al., 2002; Lee et al., 2002).

We and others recently described the effects of over-expressing the FGFR2- IIIb ligand FGF10 in pancreatic epithelium (Norgaard et al., 2003; Hart et al., 2003). FGF10 is normally expressed by the distal-most pancreatic mesenchyme, and this mesenchymal/epithelial signaling is critical for pancreatic epithelial growth (Bhushan et al., 2001). However, FGF10 also plays a role in coordinating epithelial-derived cell differentiation, as increased FGF10 signaling leads to an almost complete abrogation of all differentiated pancreatic cell types: endocrine, exocrine and ductal (Norgaard et al., 2003; Hart et al., 2003). As this effect is comparable to that observed during increased Notch-signaling (Hald et al., 2003; Murtaugh et al., 2003) and given that pancreatic expression of notch signaling components was increased in the pPDX-FGF10 mouse (Norgaard et al., 2003), it is plausible that FGF10 may exert control of differentiation via the Notch pathway. The requirement of Notch signaling in FGF10-mediated suppression of pancreatic differentiation was recently demonstrated by Miralles et al. using an explant culture model (Miralles et al., 2006).

In the present study we questioned if such a genetic link would be conserved in stomach development. We first define the temporal onset of the gastric secondary transition and demonstrate the spatial expression of FGF10/FGFR2 and Notch components at this time. These results suggest a role in mesenchymal-to-epithelial signaling of the FGF10/FGFR2 functional pair and a role of downstream Notch signaling in epithelial progenitor maintenance. Taking advantage of the expansion of the Pdx1-promoter domain into the stomach and duodenal regions, we have here analyzed the specific effects on stomach development in the presence of increased levels of FGF10 signaling as provided by Pdx1-FGF10 transgenic mice. We find that the stomach endodermal regions behave with an overall similar mechanism to the pancreatic epithelium in that cell differentiation is suppressed, although with selective effects on specific cell lineages. Consequently, stomach morphological development is severely

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disrupted. We also conclude that similar to the pancreas, FGF10 induces expression of the Notch target gene *Hes1*, indicating a conserved regulatory network.

Materials and Methods

Transgenic mouse derivation and embryo isolation

FVB mice with transient ectopic expression of FGF10 controlled by the Pdx1-promoter were generated as described in Norgaard et al (2003) by oocyte injection of a pPdx1- $FGF10^{FLAG}$ fragment (Norgaard et al., 2003). Date of transfer was set at E0.5 and the embryonic gut was harvested in ice-cold PBS under stereomicroscope at day E12.5, E14.5 or E18.5 for tissue or RNA. Genotyping was performed by PCR using primers specific for the transgenic construct (Supp. table 1). A total of n=3 E12.5 transgenic (TG) embryos, n=3 E14.5 TG embryos, and n=6 E18.5 TG embryos were analyzed in this study. Non-transgenic littermates (WT) were used as controls throughout.

Histology

Tissues were fixed in 4% PFA o.n, transferred to 30% glucose for 2 hours and 15% sucrose in 50% OCT for 1 hour followed by 1 hour of 100% OCT before being quick frozen in OCT. Immunohistochemistry was performed on 6 μm frozen slides. Slides were dried at 37C, microwaved for 2x 5 min. in citrate buffer pH 6 and washed in PBS. Slides were then treated with 3% hydrogen peroxide for 5 min, washed in PBS and blocked with TSA blocking buffer (Perkin Elmer) for 1 hour. Primary antibodies were applied o.n. The next morning, slides were washed in PBS and incubated with either appropriate secondary antibody conjugated to Texas red/Cy2/AMCA (Jackson ImmunoResearch) for 1 hour or biotinylated secondary antibody from Zymed (Histostain) for 30 min. After washes in PBS, slides incubated with biotinylated secondary antibody were incubated with streptavidin peroxidase conjugate (Zymed) for 15 min, washed in PBS and inc ubated with 1:50 FITC-conjugated tyramide signal amplification (TSA) reagent (Perkin Elmer) for 10 min. All slides were mounted in 30% glycerol in PBS.

Primary antibodies and dilutions used in this study includes: Goat α-FGF10, 1:200, Abcam, USA; Mouse HRP-conjugated α-FLAG, M2, 1:1000 (TSA), Sigma, MO, USA; Rabbit α-IDX1 (PDX1), Dr. Joel Habener, Boston, MA, USA, 1:2000; rat α-E-cadherin, ECCD2, 1:200, Zymed, CA, USA; rabbit α-β-catenin Ab-1, 1:100, Neomarkers/Labvision, CA, USA; mouse α-human smooth muscle actin, clone1A4, 1:500, DAKO, Denmark; rabbit α-chromogranin A, 18-0094, 1:200, Zymed, CA, USA; Sheep α-human Pepsinogen II, K90132S, 1:200, Biodesign, ME, USA; rabbit α-H,K-ATPase α-unit (C terminal), AB1674, 1:100, Chemicon, USA; rabbit α-secretin, H-067-04, 1:300, Phoenix, CA, USA; mouse-α-glucagon, K79BB10, 1:200 Sigma, St. Louis, MO; rabbit-α-somatostatin, 1:100, DAKO, Denmark; goat-α-ghrelin, sc-10368, 1:1000, Santa Cruz Biotech, CA, USA; rabbit-α-CCK, 1:200, Immunostar, USA; rabbit-α-gastrin 1:200, Neomarkers-Labvision, CA, USA; rabbit α-p-Histone-H3, 06-570, 1:100, Upstate, VA, USA; rabbit α-PCNA, FL-261, 1:150, Santa Cruz Biotech., CA, USA; rabbit α-Bek/FGFR2 (C17, recognizes both splice forms), 1:800 (TSA), Santa Cruz Biotech., CA, USA; rabbit α-Hes1 (Lee et al., 2005), 1:500 (TSA); rabbit-α-Sox2, 1:800 (TSA), Abcam, USA; Nkx-6.1, 1:500 (Jensen et al., 1996), Denmark; Rhodamine-Dolichos Biflorus Agglutin (DBA), 1:200, Vector Laboratories, CA, USA ; Fluorescein-Ulex Euopaeus Agglutin I (UEA), 1:200, Vector Laboratories, CA, USA. Nuclei were stained for 5 min with 1:2000 Hoechst where needed for morphometric studies.

In-situ hybridization was performed on 6μm frozen slides using digoxygenin-labeled RNA probes. The RNA probes were generated by *in-vitro* transcription from linearized template DNA as described in (Norgaard et al., 2003). DNA templates were derived from the following clones: FGF10 in pCR4 (own); Hes1 in pBS-SKII (Dr. R. Kageyama, Uni. Kyoto, Japan);

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Notch1 in pBK-CMV (Dr. J. Hald, Denmark); Notch2 in pBK-CMV (Dr. J. Hald, Denmark); Jagged1 in pCR4 (own); Jagged2 in pSKII (Dr. G. Weinmaster, UCLA); CDX2 in pT7T3D-pac (IMAGE clone 437757, Invitrogen); IFABP in bluntII-topo (Dr. L. Sussel, UCHSC, USA); TFF2 in pSPORT1 (IMAGE clone 6431145, Open Biosystems); Wnt6 in pCMV-SPORT6 (IMAGE clone 6511061, Open Biosystems); Shh in Bluescript II-SK (Dr. A.P. McMahon, Harvard, USA).

Hybridized probe was visualized using anti-Dig-AP-conjugated antibodies (Roche) as previously described (Norgaard et al., 2003) followed by NBT-BCIP substrate color reaction (Roche).

Slides were analyzed using an Olympus BX51 microscope station equipped with Nomarski interference and images where obtained through a Pixera CL600 camera. At least 3 E12.5, 3 E14.5 and 3 E18.5 TG embryos from different litters were characterized by histology. 3 WT littermates were included per time point for pair wise comparisons. The transgenic phenotypes of the three replicates were similar although not identical.

Morphometry

Morphometric analysis was done using ImagePro v. 4.5 (Media Cybernetics) software. Digital images were saved from 5–10 non-overlapping FOVs at 10x or 20x for each slide, and at least 3 slides distributed across the stomach were counted for each animal. Epithelial area was quantified by area integration based on E cadherin staining. For mean epithelial area per slide, the following number of slides distributed across the stomach were measured: E18.5: 4 slides/80 total sto mach slides, E14.5: 3 slides/35 total, E12.5: 3 slides/15 total. Cell numbers were counted automatically based on Hoechst staining, where a limited watershed algorithm was applied to separate overlapping nuclei. Specialized cell types were counted manually.

Multiplex RT-PCR

RNA was isolated from the stomachs of each of two transgenic E18.5 embryos from different litters and two littermate WT controls. A time series of RNA from E14.5–E18.5 stomachs was isolated from CD1 embryos by careful dissection of the stomach into glandular stomach and forestomach. Stomachs from 4 embryos were pooled for E14.5 samples, 3 embryos were pooled for E15.5 samples, and 2 embryos were pooled for each of the samples at E16.5, E17.5 and E18.5. Tissue was homogenized immediately in TriZol reagent (Invitrogen), and RNA was isolated according to manufacturers' protocol. cDNA was synthesized from 2 μg RNA in a 50μl reaction with 7μg random primer (Invitrogen), 40 μM dNTPs, 1x Promega opti buffer, 0.1M DTT, 40 units RNAsin (Fisher) and 200 units M-MLV RT enzyme (Fisher) at 37C for 60 min.

Semi-quantitative multiplex RT-PCR analysis was done essentially as described in (Jensen et al., 1996). PCR reactions with app. 0.05 μg cDNA in a 22 μl reaction with 10x buffer incl. MgCl (Promega), 8mM dATP, dGTP and dTTP, 4mM dCTP, 20μM of each primer (up to 6 primers in each reaction), 0.5U Taq polymerase (Promega) and 4 mM α-^{32}P-dCTP were run for 19 or 24 cycles depending on target abundance. The reactions were analyzed by polyacrylamide gel electrophoresis, and the intensity of the individual bands was quantified using ImageQuant software version 6.0 (Molecular Dynamics). All values are expressed as mean +/− SEM relative to the internal standard. Primers were designed according to Genebank reports and the sizes of the amplicons were 150–300 bp. Primer sequences are included in supplementary table 1.

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Results

A secondary transition of the murine stomach occurs at E15.5–E16.5

The murine stomach develops from a homogenous pseudo-stratified epithelium, but differentiates into several different regions. At E17.5 the stomach consists of a forestomach region with squamous epithelium and a glandular region with stratified epithelium. These two regions can be easily distinguished from the exterior morphology of the stomach (Fig. 1A). The glandular stomach can be further divided into the corpus and the antrum based on differentiated cell lineages. In order to determine the exact onset of terminal cell differentiation in the developing glandular stomach, we performed multiplex RT-PCR for markers of differentiated stomach cell lineages. Analyzing for expression of maturing endocrine cells (Chromogranin A), zymogenic chief cells (pepsinogen C), parietal cells (H^+/K^+-ATPase) and pre-pit cells (trefoil factor 1 and 2) we found complete absence of expression at embryonic day 14.5, whereas all are highly expressed at E16.5 (Fig. 1C). The pan-endocrine marker chromogranin A is present at low levels at E15.5 but expression doub les on E16.5 and remains constant for the remainder of embryonic development. The parietal cell marker H^+/K^+-ATPase is likewise undetectable until E16.5, peaking at E17.5. Pepsinogen expressed by the zymogenic chief cell is weakly detectable at E15.5 but increases dramatically on E16.5. Finally, trefoil factor (TFF) 1 is strongly expressed from E16.5 onwards, while TFF2 is weakly expressed at E15.5, increases dramatically on E16.5 and remains more or less constant during embryonic development. These results were confirmed by immunohistochemistry for Pepsinogen C, H^+/K^+-ATPase and Chromogranin A (Fig. 1B and D). All three markers were completely absent in E15.5 stomach, while abundant positive cells were found in E16.5 stomach. The A-P patterning into forestomach, corpus and antrum is already evident at the histological level at this age as a region-specific distribution of the three cell types was apparent: Chromogranin A positive endocrine cells were distributed throughout the stomach glandular epithelium, Pepsinogen C positive zymogenic cells were only present in the antrum, while H^+/K^+-ATPase positive parietal cells were only found in the corpus (Fig. 1B and 1D). Only one immature gland noticed in this study in the corpus-antrum transition region contained both pepsinogen and H^+/K^+-ATPase positive cells (Fig. 1D inset).

We note that for both antrum and corpus, morphogenetic development of the gastric pit-gland unit is only rudimentary at E15.5–E16.5, with shallow epithelial invaginations (Fig 1B and 1D). Consequently, gastric cytodifferentiation is initially independent of a complete pit-gland unit morphogenesis. In contrast to the glandular regions, the mouse forestomach appears to generate only a single differentiated cell type, easily distinguishable through the presence of a stratified squamous epithelium, not found elsewhere in the stomach. Inspecting the developmental profile of the mouse forestomach between E14.5–E18.5 we conclude that squamous cell development occurs concomitantly with onset of terminal differentiation in the glandular regions, between E15.5–E16.5, as the forestomach epithelium changes from pseudostratified to squamous during this time (data not shown). Thus, the stomach epithelium goes through a secondary transition in a temporally and spatially coordinated fashion in the mouse.

FGF10 and FGFR2 are expressed complementarily in the gastric mesenchyme and epithelium during embryonic development

The temporal and spatial specificity of cell differentiation in the stomach suggests that cell differentiation is tightly regulated here. Given the role of FGF10 in pancreatic progenitor maintenance, we speculated that FGF signaling might play a role in regulating the secondary transition of the stomach as well. First, we identified members of the FGF-family expressed during the late embryonic development of the stomach (E14.5–E18.5) using a multiplex RT-PCR screen of all known FGF-family genes in the mouse (FGF1 to FGF23). Here we find that

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FGF1-3, FGF7, FGF9 and FGF10 were expressed at or above 20% of our control gene, TATA box binding protein (TBP), at one data point (Fig. 2A), but FGF10 was the only FGF expressed at high levels before the secondary transition. *In-situ* hybridization for FGF10 confirmed the multiplex analysis and showed the confinement of FGF10 expression to the gastric mesoderm with strongest expression posterior (Fig. 3A, and Supp. Figure 1A and 2). FGF10 is an FGFR2-IIIb ligand, and was previously found to be expressed in the pre-differentiated mouse stomach at E11.5 well before cytodifferentiation (Bhushan et al., 2001). *Fgfr2-IIIb* mRNA was expressed at a constant level throughout the stages analyzed, except for E14.5 expression that was higher in glandular regions (where the *Fgfr2-IIIb*/TBP volume ratio of 2.5 compared to ratios of 1–1.7, Fig. 2B). Using an antibody that detects both splice forms we found expression of FGFR2 protein in the pre-forestomach and pre-antrum epithelium at E15.5 (Fig. 4A). Only negligible staining could be discerned in the mesenchyme or in the epithelium of the pre-corpus region. Expression data based on *in-situ* hybridization at E14.5 showed epithelial localization of *Fgfr2IIIb* mRNA, as well as *Fgfr3* and *Fgfr4* (Supp. Fig. 1B), confirming the immunohistochemical analysis and establishing that the FGFR2IIb isoform is expressed here. At E18.5 FGFR2 protein expression in the forestomach epithelium was confined to the basal stem cell layer. Expression of FGFR2 had moved into the corpus region where it is localized to the forming glands, and expression was persisting in the antrum, with strongest expression in the forming glands (Fig. 4B). The region-specific expression of FGFR2 makes it likely that FGF signaling is involved in spatial patterning as well as temporal regulation of stomach development. In order to validate the gastric patterning of FGFR2, we used the gastric HMG-box containing transcription factor Sox2, as this is only expressed within the anterior-most endoderm with a posterior boundary at the pyloric sphincter (Tsukamoto et al., 2004). Immunohistochemistry for Sox2 on a consecutive slide confirmed the gastric identity of the FGFR2 expressing epithelium. Also, Sox2 expression was reduced in the corpus mirroring FGFR2 expression (Fig. 4A). We conclude that similar to the pancreas and lung, the FGF10/FGFR2 pair is expressed in the stomach in a manner consistent with mesenchymal-to-epithelial signaling.

Notch signaling components are expressed in embryonic stomach

Notch signaling is downstream of FGF-signaling in the embryonic pancreas (Norgaard et al., 2003) and has been shown to control gastric enteroendocrine development, through activation of the bHLH factor Hes1. Expression of Notch signaling components have been addressed during chicken stomach development, where both *cNotch1* and *cNotch2*, *cHairy* 1 (orthologous to *Hes1*) and *cHairy2* (orthologous to *Hes2*) are predominantly confined to the epithelial compartment (Matsuda et al., 2005). Mouse embryonic stomach has not been similarly analyzed, although RT-PCR analysis of *Hes1*, *Hes3*, *Notch1*, *Jagged1* and *Jagged2* has demonstrated their expression in the embryonic mouse stomach at E18.5 (Jensen et al., 2000). We here demonstrate expression of *Hes1* mRNA in the embryonic stomach throughout development (Fig. 2B) and confinement of *Hes1* mRNA to the embryonic gastric epithelium at E18.5 (Fig. 3B). We also find that abundant expression of the transcriptional regulator *Hes3* occur throughout stomach development (Fig. 2B) and that *Notch1* is expressed in the embryonic stomach epithelial progenitors in the mouse (Fig. 2B and 3B). Given that Norgaard et al. suggested a role for Jagged-type ligands during FGF10 stimulated notch signaling in the pancreas, we were particularly interested in the spatial pattern of *Jagged1* and *Jagged2* expression. *Jagged1* was not expressed by stomach epithelium, but instead confined to vessels (Fig. 3B). In contrast, *Jagged2* was expressed in a completely overlapping manner with *Notch1*/*Hes1* in the gastric epithelium in antrum and corpus, and basal population of the forestomach epithelium (Fig. 2B and 3B).

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Generation of pPdx1-FGF10^{FLAG} transgenic mice

To address the functional role of FGF10 signaling in gastric development we generated transient transgenic embryos through injection of the full length, C-terminally FLAG-tagged FGF10 coding region under control of 4.5 kb upstream *Pdx1* proximal promoter (Norgaard et al., 2003; Stoffers et al., 1999; Apelqvist et al., 1997; Li and Edlund, 2001) (Fig. 5A). The Pdx1 promoter is abundantly expressed in the dorsal part of the primitive gut from E8.5 (Ohlsson et al 1993) and at lower levels in the distal foregut region, including the posterior stomach and duodenum E10 and onwards (Madsen et al., 2001; Miller et al., 1994). Importantly, this non-pancreatic expression is functionally relevant, as a hypermorphic allelic series of Pdx1 promoter mutants gradually deleting Pdx1 activity provided evidence of a role of Pdx1 in both pyloric gland and Brunners gland formation (Fujitani et al., 2006). The pPdx1-FGF10^{Flag} construct used here was previously shown to disrupt pancreatic morphogenesis by blocking terminal differentiation of all pancreatic cell types (Norgaard et al., 2003). Previous studies of the exogenous Pdx1 promoter (using a 4.5 kb promoter fragment activity) have revealed expression in posterior stomach and duodenal endoderm, in addition to the pancreas. We confirmed the expression of endogenous Pdx1 in the anterior duodenal epithelium, and posterior stomach epithelium at E12.5, E14.5 and E18.5 (Fig. 5C), which was mimicked by the 4.5kb Pdx1 promoter used here (Fig. 6 and data not shown). We analyzed expression of the FGF10^{FLAG} transgene by virtue of the FLAG-tag present as well as by ectopic expression of FGF10 protein and mRNA. At E12.5 FGF10 was only expressed in a few cells in the most posterior regions of the stomach epithelium (Fig 6A), co-responding with a very limited PDX1 domain here (Fig 5C). No expression was found in Wt littermates. In E14.5 TG stomach, a much stronger expression of FGF10^{FLAG} protein was found (Fig. 6B) in stomach epithelial cells. Similar to the pancreas, strongest staining was observed in the plasma membrane rather than in the cytoplasm. No FLAG or FGF10 staining was observed in WT stomach (data not shown), and no FLAG staining was observed in TG stomach mesenchyme (Fig. 6B). Compared to E14.5, reduced expression was observed in E18.5 stomach – a time point where expression of FGF10^{FLAG} persists in pancreas (Fig 6C). We performed *in-situ* hybridization for *fgf10*, which revealed transgenic expression in a few antral epithelial cells at E12.5 (Fig. 6A). In contrast, *in-situ* hybridization revealed a strong expression at E14.5, overlapping with Fgf10 and FLAG staining (Fig. 6B). At E18.5 the *fgf10* mRNA level was reduced, and it was only expressed in cells in forming glands (Fig. 6C). No positive epithelial cells were found in WT littermates. Corroborating the histological results, RT-PCR revealed the presence of exogenous FGF10-Flag mRNA in transgenic stomach at E18.5 (Fig. 6D). Given the transient transgenic nature of the e mbryo generation process, individual TG embryos represent individual lines and therefore displayed degrees of variation of phenotype penetrance; the two transgenic embryos analyzed by RT-PCR were from independent litters and differed considerably in the amount of FGF10-Flag mRNA expressed. We accredit this to mosaicism, TG integration site, copy number insertion, or a mix thereof. Notwithstanding, all TG embryos analyzed displayed similar alterations of stomach development.

Disrupted stomach morphogenesis in pPdx1-FGF10^{FLAG} mice

Visual inspection of E12.5 TG embryos (n=3) revealed minor disruptions of stomach morphogenesis. In contrast, at E14.5 (n=3), we detected a major disruption of normal stomach morphogenesis. The size of the stomach appeared slightly reduced, and the ventricular lumen of the stomach appeared filled with tissue. Furthermore, we could not identify the presence of a pyloric sphincter, leaving the attachment zone of the pancreas to the gastrointestinal tract undecided. We first performed a histological analysis of the developing stomach using markers of the epithelium (β-catenin), and the developing circular and longitudinal muscles (smooth muscle actin) (Fig. 7A). In the normal E14.5 stomach, the ventricular space is lined by a homogeneous pseudo-stratified epithelium, with little folding. At a fixed radial distance, smooth muscle (SM) cells differentiate, aligning accordingly to the future position of the

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contractile muscles of the muscularis (Fig. 7A). In transgenic littermates, almost the entire luminal area was populated by an increasingly folded endodermal epithelium. SM cell differentiation occurred at a similar radial distance to the hyperplastic epithelium, properly following the epithelial folding pattern, suggesting that the irregular muscle condensation is secondary to the morphogenesis of the epithelium (Fig. 7A). Consequently, we interpret these data to signify that the epithelial/mesenchymal signaling regulating SM differentiation is not disturbed. At E18.5 the phenotype was even more striking, with excess folding of the epithelium in the corpus and antral regions, which branched into complex tree-like structures never observed in the WT littermates (Fig. 7A, 7B and Supp. Fig. 3 and 4). The forestomach region developed a stratified squamous epithelium indistinguishable from the WT forestomach region, although in some regions we noted a disturbed differentiation process, which included failure in development of the stratified epithelium (Supp. Fig. 3, Supp. Fig 4C–D). In those regions we also observed that the normal confinement of DNA-incorporating nuclei to the basal cells seen in WT, was disrupted (Supp. Fig. 4E–F). The smooth muscle development appeared normal in the forestomach.

Conservation of distal foregut patterning in pPdx1-FGF10^{FLAG} mice

Due to the difficulty in defining the stomach/duodenal junction morphologically in both E14.5 and E18.5 transgenic embryos, we decided to investigate whether this region had been properly specified using a combination of stomach, pancreatic, and intestine-specific markers. Using the region-specific lectins DBA and UEA which marks the prospective duodenal and gastric regions at E14.5, respectively, we were able to define the attachment zone of the Nkx6.1 -expressing hyperplastic pancreas cells within the prospective duodenal region (Fig. 8A–D), as it normally occurs. However, the regionalization of the pancreas/duodenum connection was not strict as Nkx6.1-expressing pancreatic cells were clearly observed within the duodenal area at E14.5. Given the complete absence of a main pancreatic duct, and the absence of the normal narrowing of the evaginating pancreatic epithelium, this is not surprising. We noted that UEA-positive gastric cells were clearly observed in a more anterior location than the pancreatic/duodenal zone, indicating the conservation of the distal foregut patterning in the FGF10 transgenic embryo. It therefore seems that the distal foregut patterning mechanisms operate even in the absence of a pyloric sphincter and a normal pancreas. These results were corroborated at E18.5, where both pancreatic evaginations, dorsally and ventrally, entered the adjacent endoderm at the level of the *Cdx2*-expressing duodenum. (Fig. 8E).

Proliferation analysis of stomach epithelial cells

The increased folding of the epithelium might be suggestive of an increased cellular proliferative rate, or a result of FGF10-mediated morphogenetic effects upon branch-point selection. To analyze for total epithelia area changes, we measured gastric glandular epithelial area at E12.5, E14.5 and E18.5. We also compared cell proliferation and cell death rates within the glandular region (antrum/corpus, forestomach excluded). We did not find any evidence suggesting an increase in total epithelial area (Fig. 9B), nor did we find a reduction in cell death using a combination of Hoechst and TUNEL-staining, as this parameter was negligible in the normal embryonic stomach as well as in the TG stomach (data not shown). To account for any change in the proliferative index, we analyzed for M-phase-activity (using phosphorylated histone H3, pHH3), specifically in the stomach epithelium by co-staining with E-cadherin. Analysis of E12.5, E14.5 and E18.5 TG mice and WT littermates did not reveal a significant increase in the proliferative activity in stomach glandular epithelium (Fig. 9A). Also, the number of cells (counted as Hoechst nuclei) per area was unchanged, signifying that cell size was not affected by ectopic FGF10 (results not shown). We can therefore exclude that the increased folding is caused by an overall increase in epithelial area. Instead, it seems likely that FGF10 is specifically promoting epithelial folding as a chemotaxic factor similar to what is seen in the lung (Park et al., 1998). Therefore, to elucidate whether FGF10 changes the

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pattern of proliferating cells rather than the overall proliferative index, we analyzed the distribution of cycling cells with PCNA staining. Cycling PCNA positive cells are confined to the glandular compartment in normal E18.5 antrum epithelium, but was distributed throughout the luminal and glandular compartments in the TG antrum (Fig. 9C). This disruption of the proliferative niche was not absolute, as patches of PCNA negative luminal cells were found (Fig 9C, upper right panel). To further pursue the effect of FGF10 on luminal and glandular compartments we next analyzed the distribution of gland and luminal markers.

Differential effects of FGF10 on development of stomach secretory cells

Pepsinogen C and H+/K+ ATPase is expressed by chief and parietal cells respectively in the glands of the E18.5 stomach, while Trefoil factor 1 (TFF1, formerly pS2) and Trefoil factor 2 (TFF2, formerly spasmolytic polypeptide, SP) are expressed by mucus producing luminal cells (Otto and Patel, 1999). Immunostaining for Pepsinogen C showed a 68% increase in the number of chief cells ($P<0.05$) (Fig. 10B and 10C) and an expansion of these cells to nodes of branching epithelium not located within the mesenchyme (Fig. 10B). Interestingly, immunostaining for the C terminal end of the H^+,K^+-ATPase α-unit showed a 78% decrease in parietal cells ($P<0.05$). This decrease was corroborated by multiplex-RT-PCR. mRNA expression of the mucin associated Trefoil Factor 2 was unchanged (Fig. 10A), but *in-situ* hybridization revealed a shift in localization from luminal cells in the WT corpus to glandular cells in the TG (Fig. 10B). It thus seems that the *Tff2* expressing mucus cells form unperturbed by FGF10, but that their localization has been shifted. Trefoil factor 1 expression and localization were unchanged (Fig. 10A and data not shown). *In-situ* hybridization for intestinal fatty acid binding protein (*Ifabp*), that is expressed in the stomach of Ngn3 null mice (Lee et al., 2002) and in intestinal metaplasia of the stomach, did not show any expression in the stomach of pPDX-FGF10 mice (Fig. 10C and data not shown), and neither did the intestinally expressed TFF3 (data not shown). The analysis of exocrine cells showed that not only are the luminal and glandular cell types displaced by FGF10, certain cell fates are also suppressed while others are accelerated. Previously published results show suppression of terminal endocrine cell fates by FGF10 during ectopic expression in the pancreas (Norgaard et al., 2003). We therefore continued to analyze differentiation of endocrine cell types.

FGF10 attenuates stomach endocrine terminal differentiation

We described the onset of gastric endocrine differentiation at E15.5–E16.5 using the pan-endocrine marker Chromogranin A. More specifically, the gastric endocrine cell complement includes secretin, glucagon, somatostatin, ghrelin, and gastrin producing cells, predominantly. The majority of the endocrine cells are formed based on the activity of bHLH components, including the *Ngn3* gene (Jenny et al., 2002; Jensen et al., 2000; Lee et al., 2002), and are repressed by the Notch system, acting through Hes1 (Jensen et al., 2000). We compared E18.5 WT and TG littermates for effects on stomach cell differentiation by semi-quantitative multiplex RT-PCR (Fig. 10A) and immunohistochemical evaluation of markers for differentiated stomach cell types (Fig. 10B and 10C). Multiplex RT-PCR showed a down-regulation of mRNA for Chromogranin A (with relative ratios of Chgr.A/TBP of TG1: 0.07, TG2: 0.02, WT1: 0.21, WT2: 0.24; Fig 10A). This effect was most pronounced in the TG embryo with the highest expression of ectopic FGF10. Immunohistochemistry for Chromogranin A positive cells likewise showed a significant reduction (81% reduced compared to WT ($P<0.05$)) However, a few Chromogranin A positive cells were still present and we went on to discern the identity of those cells. Glucagon staining revealed an almost complete loss of A cells (92% reduced compared to WT ($P<0.05$)) and staining against secretin showed a significant decrease in enterochromaffin cells (89% reduced compared to WT ($P<0.05$)), while analyzing for somatostatin producing D cells showed no significant change. This is a surprising result, since a lack of somatostatin expression was reported in the *Ngn3*−/− stomach (Lee et al., 2002). Quantitation of Grelin cells did not reveal a statistically significant

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change, although ghrelin cells were significantly reduced in two out of three transgenic mice. Stomach gastrin expressing cells (G-cells) where not detected at this time in development in WT or TG embryos.

FGF10 leads to nuclear translocation of FGFR2

As shown by multiplex-RT-PCR, mRNA for FGFR2b is present in the embryonic stomach, and based on immunohistochemistry the protein is present in the cytoplasm/membrane of the stomach epithelium at E14.5 and E18.5. Upon expression of FGF10 in the TG mice the levels of FGFR2b are unchanged (Fig. 12), but immunohistochemical detections reveals a change in FGFR2 localization from the cytoplasm/membrane to the nucleus in the forming glands (Fig. 11B). This localization event was never detected in stomach epithelium of wildtype littermates (Fig. 11B and 4A and B). Given the observed increase in pepsinogen-expressing cells, we speculated whether nuclear localization of FGFR2 was associated with chief cell differentiation. Co-staining with pepsinogen and FGFR2 showed no co-localization (Fig. 11B). In contrast, the widespread nuclear presence of FGFR2 in E-cadherin expressing and terminal marker negative stomach epithelial cells suggests that FGFR2 nuclear entry is a result of ongoing FGF10 signaling in the arrested progenitors.

FGF10 induces Hes1 expression in embryonic stomach

We next analyzed expression of notch pathway components in the pPdx-FGF10^{FLAG} stomach by multiplex-RT-PCR (Fig. 12). *Hes1* expression is very low in wildtype mice, but it was considerably increased in the TG mice, while levels of *Jagged1* and *2* were unchanged (Fig. 12). Immunohistochemistry for Hes1 using an antibody (Lee et al., 2005) that in our hands only allows detection of high Hes1 levels, showed upregulation of Hes1 in the epithelium of the TG mouse (Fig. 11A). Hes1 was detected in the forming glands in the antrum, consistent with the area where we find the strongest expression of FGFR2 and ectopic FGF10 at E18.5.

Effects of FGF10 on gastric morphogenetic signaling networks

Sonic Hedgehog (Shh) has been shown to be important for stomach gland development in both chicken and mouse (Fukuda et al., 2003; van den Brink et al., 2001) and is downstream of FGFR2b in limb development (Revest et al., 2001). *Shh* is expressed in gastric epithelium and has been localized to parietal cells in human adult stomach and in parietal and zymogenic cells in murine adult stomach (van den Brink et al., 2001). Our expression ana lysis showed a 2-fold downregulation of Shh in E18.5 TG stomach (Fig. 12). We also analyzed the expression of BMP4, as this mesenchymal expressed signaling molecule has been shown to be a downstream target of Shh in mouse adult stomach (van den Brink et al., 2001). No change in BMP4 expression was noted. Similarly, BMP2 expressed by gastric mesenchymal cells (Kaestner et al., 1997), was unaltered (Fig. 12). Wnt-signaling is critical for intestinal progenitor/stem cell maintenance, and multiple studies indicate a role in both normal and transformed growth of gastric cells (Perreault et al., 2005; Tsukamoto et al., 2003; Kim et al., 2003). Wnt5A is highly expressed in the gastric and intestinal mesenchyme (Lickert et al., 2001), but we did not observe any changes in overall gastric expression in the Pdx1-FGF10 transgenic model. In contrast, Wnt6 is expressed in the normal embryonic stomach, but we were unable to detect any expression of Wnt6 in transgenic stomach (Fig. 12). In-situ hybridization for Wnt6 in gastric development showed that Wnt6 is exclusively expressed by the gastric epithelium (Supp. Fig. 5). The transcription factor Sox2 is expressed in gastric and esophageal epithelium with a more anterior expression domain early in gut development, that expands into the posterior stomach just prior to the secondary transition (Fig 4 and results not shown) and is also one of a few transcription factors expressed in embryonic stem cells that is lost upon differentiation (Carlin et al., 2006). Sox2 expression was lowered in the pPDX-FGF10 mouse in a manner correlating with levels of ectopic FGF10 (Fig. 12).

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Discussion

Although the stomach is amongst the largest organs, relatively little is known of its morphogenesis and development, particularly in relation to which genetic networks may coordinate the process of gastric cell differentiation. To better characterize the temporal onset, and local patterning of gastric cell differentiation, we employed staining techniques for the terminal gastric products allowing the visualization of chief, parietal, mucous and gastric endocrine cells, combined with an RT-PCR procedure. We conclude that each of the three major gastric regions; forestomach, corpus and antrum, initiates terminal differentiation concomitantly at E15.5. We define this strict temporal onset of terminal differentiation as the "secondary transition", reserving the temporal specification of pro-gastric endodermal cells towards the gastric fate at early somite stages as the "primary transition" – a process involving early splanchnic mesoderm signaling controlled by Hox-gene family patterning (Pitera et al., 1999; Aubin et al., 2002) and the homeobox gene Barx1 (Kim et al., 2005). No terminal differentiation events appear associated with the gastric primary transition, in contrast to that of the pancreas. The gastric secondary transition is characterized by the initial presence of terminal cell types, and coincides with the time when the caudal gastric epithelium is first starting to form glandular structures. At E15.5 the invagination of epithelium and subsequent glandular formation is initiated, but is far from completed when the terminal cellular fates are induced (E16.5). Thus, we can conclude that gastric cytodifferentiation during the embryonic development, operates irrespectively of the presence of a mature gastric pit-gland unit.

In comparison to the pancreas, where the "pancreatic secondary transition" occurs at E13.5–E14.5 and signifies the development of mature exocrine, ductal, and insulin-producing cells, the stomach is two days delayed. This delay argues against sharing of any systemic factors that might help trigger the secondary transition in these adjacent organs. It is currently believed that organ-intrinsic factors acting in mesenchymal-to-epithelial signaling are the major determinants in orchestrating the onset of the secondary transition in the pancreas. FGF10 is a critical part in this and helps to maintain the undifferentiated pancreatic progenitor state prior to the secondary transition, in part through maintenance of Notch signaling (Norgaard et al., 2003). These observations motivated us to address if FGF signaling similarly guide stomach progenitor cell maintenance and if this may be similarly involving the control of Notch signaling. To address the identity of FGF-family proteins expressed in gastric development, we screened all members by RT-PCR performed on a kinetic series of embryonic stomach cDNA. These results highlighted FGF10 as a plausible FGF-family member playing a role prior to the secondary transition in the stomach. FGF10 is expressed abundantly in the adjacent gastric mesenchyme, in a decreasing gradient from the distal mesenchymal cells in the posterior stomach (Fig. 3 and Supp. Fig. 1A and 2). It is thus positioned as a component that might influence gastric epithelial cells, prior to the secondary transition. The predominant receptor for FGF10, FGFR2IIIb, was in turn expressed mainly in the gastric epithelium, arguing for a mesenchymal/epithelial interaction, similar to what is observed in lung and pancreas. We noted that FGFR2 expression was also not uniform, as epithelium in the corpus expressed only minor amounts of FGFR2 prior to the secondary transition, which could indicate that corpus might differentiate between FGF10 availability as compared to the antrum. As FGF10 and FGFR2IIIb expression is maintained after the secondary transition, FGF-signaling appears to have a later role in the more mature gastric epithelium as well. We find expression of multiple Notch signaling components in gastric progenitor cells. Notch1, Notch2, the downstream target Hes1, and the ligand Jagged2, are all expressed at high levels in the stomach prior to the secondary transition. Following the transition, expression can still be observed in the basal population of the forestomach and in epithelial cells of the antrum and corpus, but not in differentiated squamous cells. It is thus likely that notch signaling also plays a role in murine gastric development.

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In order to investigate the role of FGF10/FGFR2 signaling in embryonic stomach, we forced epithelial expression of FGF10, disrupting the FGF10 gradient from the mesenchyme. The results provide evidence for a role of FGF10 in gastric gland formation and progenitor maintenance and the resulting suppression of differentiation bears some resemblance to that observed during pancreatic development (Norgaard et al., 2003).

The most apparent phenotypic disruption is a luminal branching of the epithelium, contrasting with the folding into the mesenchyme observed during gland formation in the normal stomach. We show that this luminal b ranching is not due to an increase in overall epithelial area or proliferation, but rather a change from gland niche restricted to diffuse proliferation. This phenotype reflects what has been seen in the lung, where FGF10 regulates directional outgrowth as a chemoattractant, but only acts as a modest proliferation factor (Park et al., 1998). Very recently, two studies describing the gastric phenotype of Fgf10 −/−, Ffgr2IIb −/− mice (Spencer-Dene et al., 2006), and virus-mediated overexpression of Fgf10 and a secreted FGFR2b in chicken (Shin et al., 2006) observed a similar role of Fgf10 to the one reported here in regulation of gland formation. Both knockout mouse models show a decrease in the formation of complex glands, as does the model of secreted FGFR2b in chicken, while the virally mediated fgf10-overexpression model shows hypermorphic epithelial structures similar to what we report here. In contrast to our over-expression model, the chicken study ascribes the role of Fgf10 in gland formation to a direct regulation of cell proliferation. However, only a modest increase in the percentage of proliferating cells upon over-expression of fgf10 (Shin et al., 2006) is observed. This difference may be due to species differences in regulation of proliferation, also supported by the observation that the chicken gastric epithelium at late stage 36/day 10 does not have a defined proliferative zone (Shin et al., 2006). Also, the level of Fgf10 expression in the chicken study likely exceeds that obtained through the ectopic expression in the mouse, possibly leading to the observed difference in mitogenic activity. This argument would also help explain the difference in mitogenic FGF10 signaling in the pancreas as compared to the stomach in the pPDX-FGF10^{FLAG} model; Activity of the Pdx1-promoter in the pancreas is higher than in the gut (Fig. 5), and expression of TG fgf10 protein is indeed lower in the stomach (this paper and Norgaard et al). Thus, we believe it is entirely possible that fgf10 operates both as a chemotaxic factor in gland development at lower levels, and at higher levels through induction of epithelial growth.

We hypothesized that the increased branching would associate with exogenous gland formation, and indeed expression of the glandular marker pepsinogen C was increased and chief cells had spread to branching nodes in the lumen. In contrast, the parietal cell type was almost absent, while the luminal marker TFF1 was preferentially expressed in forming glandular structures, suggesting that luminal cells ectopically differentiate in the glandular domain. This reversal of localization of pepsinogen and TFF1 is similar to what is seen in the chicken Fgf10 overexpression model (Shin et al., 2006). We are curious as to why the chief cell fate was promoted while parietal cell fate was repressed. Both cell types are glandular, altho ugh chief cells are located deeper in the gland. We speculate that early gastric A-P patterning might have been affected. We show that at the initial time of their development, a spatial separation of parietal and chief cells in the corpus and a ntrum exist. Therefore, the observed loss of parietal cells and gain of chief cells in the transgenic model would reflect that the corpus acquires antral identity, as we also found no data to support expansion of the antrum by increased proliferation. The hypothesis that Fgf10 regulates AP-patterning is reinforced by the fact that Shh, which is normally expressed mostly in the anterior stomach (Aubin et al., 2002), is downregulated by overexpression of FGF10 (this study) and upregulated in the fgf10 knockout (Spencer-Dene et al., 2006). It is worthwhile to note that for both the fgf10 and Fgfr2IIIb knockout mice a reduced antral stomach epithelium is observed (Spencer-Dene et al., 2006), consistent with a role of FGF-signaling in promoting antral regionalization. Interestingly, “antralization” is observed in certain gastric metaplasias, also known as

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"SPEM" (spasmolytic peptide expressing metaplasia), characterized by oxyntic atrophy and a shift in localization of TFF2 mRNA from the mucous neck cells in the normal adult fundus to the lower two thirds of the glands in the H. felis–infected fundus and in SPEM (Nomura et al., 2004; Wang et al., 1998), similar to what we see. Obviously the two phenotypes cannot be directly compared, as the embryonic stomach described here does not posses the mature glands described in SPEM, but these results nonetheless suggest that ectopic expression of FGF10 in the embryonic stomach results in a SPEM-like embryonic phenotype and consequently indicates a role of FGF signaling in gastric metaplastic development (Schmidt et al., 1999). We speculate that the normal gradient of FGF10 and FGFR2-expression in the stomach may help specify antrum versus corpus/fundus fate choices. The lower levels of FGF10 and FGFR2 in corpus (this study and (Aubin et al., 2002)) suggest that progenitors here normally experience lower FGFR2 signaling as compared to antrum. In TG embryos, on the other hand, the FGF10 availability is much increased, possibly reaching up to, or above, normal antral levels, and presumably causing the antralization of the corpus. Further experiments are needed to validate if this gradient of FGF signaling helps specify stomach region identity.

Similar to what is seen in the pancreas of pPDX1-FGF10^{FLAG} mice (Norgaard et al., 2003), we saw almost complete elimination of endocrine cell fates. Taken together with the attenuation of parietal cell differentiation, we conclude that Fgf10 inhibits differentiation of the gastric progenitors towards the majority of cell fates, thus maintaining the progenitor cells. In the pancreas, this Fgf10 effect is mediated through Notch signaling (Norgaard et al., 2003). When we analyzed downstream changes in transcription of notch signaling components, we noted that Fgf10 elicits an upregulation of Hes1 expression; supporting our hypothesis that Fgf-signaling is upstream of Notch signaling in the stomach. This does not seem to occur through increased levels of jagged-ligand production, as we previously suggested for the pancreas. It has previously been shown that Hes1 regulates endocrine differentiation in the murine stomach (Jensen et al., 2000), and active Notch signaling inhibit terminal cell fates in the stomach of the chicken (Matsuda et al., 2005). Interestingly, activated Notch also promote glandular specification in the chicken (Matsuda et al., 2005), suggesting that not only the progenitor maintenance effect of FGF10, but also the increased formation of glandular structures seen, could be mediated through notch signaling. Further research is needed to elucidate this aspect in the mouse.

Fgfr signaling affects several downstream signaling pathways, and so we also analyzed changes in alternative pathways. An immediate molecular effect of ectopic expression of FGF10 was the occurrence of nuclear localization of FGFR2. The biological relevance of nuclear localization of FGFR2 is unknown, but in this case it seems to be a response to hyper-stimulation of the receptor, as it was never observed in wildtype stomach epithelium. However, nuclear localization of FGFR2 as a response to FGF can not be dismissed as just an artifact of over-expression of ligand in all cases, as it has previously been described in normal Sertoli cells, where it disappears in FGF9 null mice (Schmahl et al., 2004). Nuclear localization of FGFR2 also occurs in osteoblasts in association with prostaglandin induction (Sabbieti et al., 2005), and thus seems to have functional relevance. Interestingly, nuclear FGFR1 acts as a transcriptional regulator that stimulates growth and differentiation responses (reviewed in (Stachowiak et al., 2003)), and nuclear FGFR2 has been associated with differentiation of Sertoli cells (Schmahl et al., 2004). A direct association with terminal differentiation in the gastric epithelium is not evident, as nuclear FGFR2 did not co-localize with pepsinogen in the pPdx1-FGF10 TG mice.

Stomach development and homeostasis has been shown to be regulated in part by Wnt, Shh and BMP–signaling and we therefore analyzed whether ectopic FGF10 expression had any influence on the transcription of components within these pathways. Our data suggest negative effects of FGF10 on Wnt6 and Shh expression, but not on BMP2, BMP4 and Wnt5A. It has

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previously been shown that Shh helps to induce BMP4 expression in adjacent submucosal cells of the hindgut in chicken, but this induction was not found in midgut (Roberts et al., 1995). Our results indicate that in the mouse, gastric BMP4 expression is likewise independent of Shh in the embryonic stomach. We also analyzed the expression level of the transcription factor Sox2, which is selectively expressed in the stomach and esophagus regions of the endoderm, and found a marked downregulation. This was surprising, given that Sox2 is induced in FGF treated osteoblasts (Mansukhani et al., 2005), but reinforces the hypothesis that Fgf10 regulates AP-patterning. It is noteworthy that Hes1, Wnt6, Shh and Sox2 all are expressed by gastric epithelium, whereas the BMP2, 4 and Wnt5a are mesenchymal. This supports the fact that we did not see any changes in morphology of the mesenchyme, and shows that FGF10 ectopically expressed by epithelial cells predominantly influences the epithelium itself.

We here provide evidence to support a role of FGF10 in gland formation and gastric patterning, in addition to demonstrating a functional role for FGF10 upstream of Hes1 in gastric progenitor maintenance, extending the knowledge of FGF10 as a mesenchymal-derived factor involved in epithelial cell differentiation also to that of the stomach. FGF10-mediated mesenchymal-epithelial signaling thus appears to be conserved from anterior endoderm (lung) (Sekine et al., 1999), over distal foregut (stomach and pancreas (Bhushan et al., 2001)). The role of FGF10 in more posterior regions of the gut needs to be clarified, although data obtained in parallel with this study indicate a similarly important role for FGF10 in duodenal progenitor maintenance (will be described elsewhere). Therefore, similar to the conserved nature of epithelial Notch signaling along the gut tract, FGF10 signaling operates to help maintain the epithelial progenitor niche. Further studies should be aimed at defining how these signaling systems may interact, as such knowledge is likely to be important in understanding both the development of gastric cancer, as well as programs of stomach regeneration, and how these processes may be modulated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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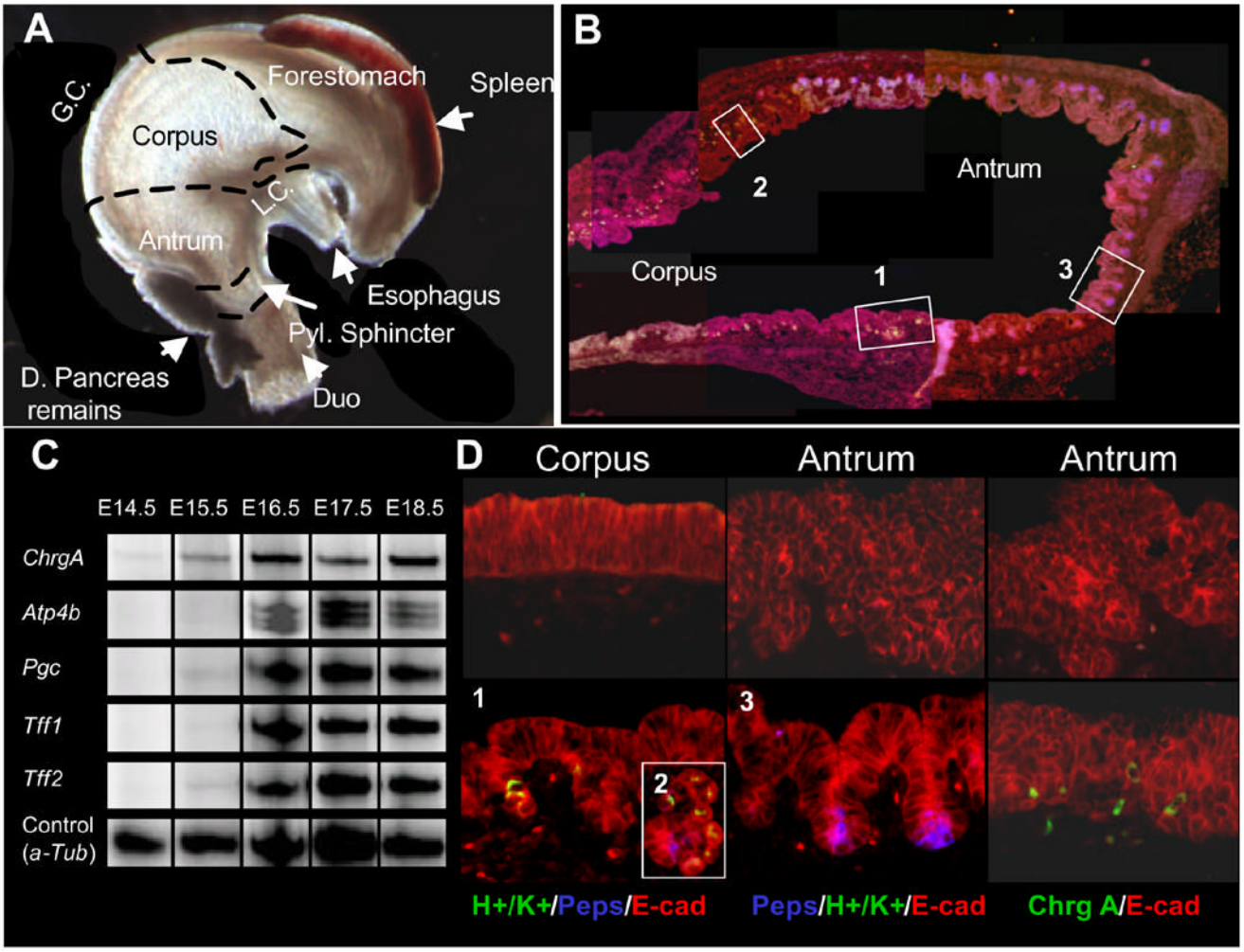

Figure 1. The secondary transition of the stomach

A: Regions of the stomach at E17.5. G.C: greater curvature, L.C. Lesser curvature. B: Co-staining of H^+/K^+-ATPase (green), Pepsinogen C (blue) and E-cadherin in E16.5 stomach. The numbers refer to pictures in D). C: Multiplex RT-PCR of expression of Chromogranin A (*Chrg A*), H^+/K^+ ATPase (*Atp4b*), pepsinogen C (*Pgc*) and Trefoil Factor 1 and 2 (*Tff1* and *Tff2*). a-tubulin (a-tub) was used as an internal control. The shown bands are representative examples from two experiments. Ages analyzed included E14.5, E15.5, E16.5 E17.5 and E18.5. (n=2/gestational time point). D: Immunohistochemistry for H^+/K^+ ATPase and Pepsinogen C or Chromogranin A co-stained with E-cadherin at E15.5 (top row) and E16.5 (bottom row). Numbers refer to positions in B). Insert 2 shows one forming gland with both pepsinogen and H^+/K^+ ATPase expressing cells. This was the only one found (n=3).

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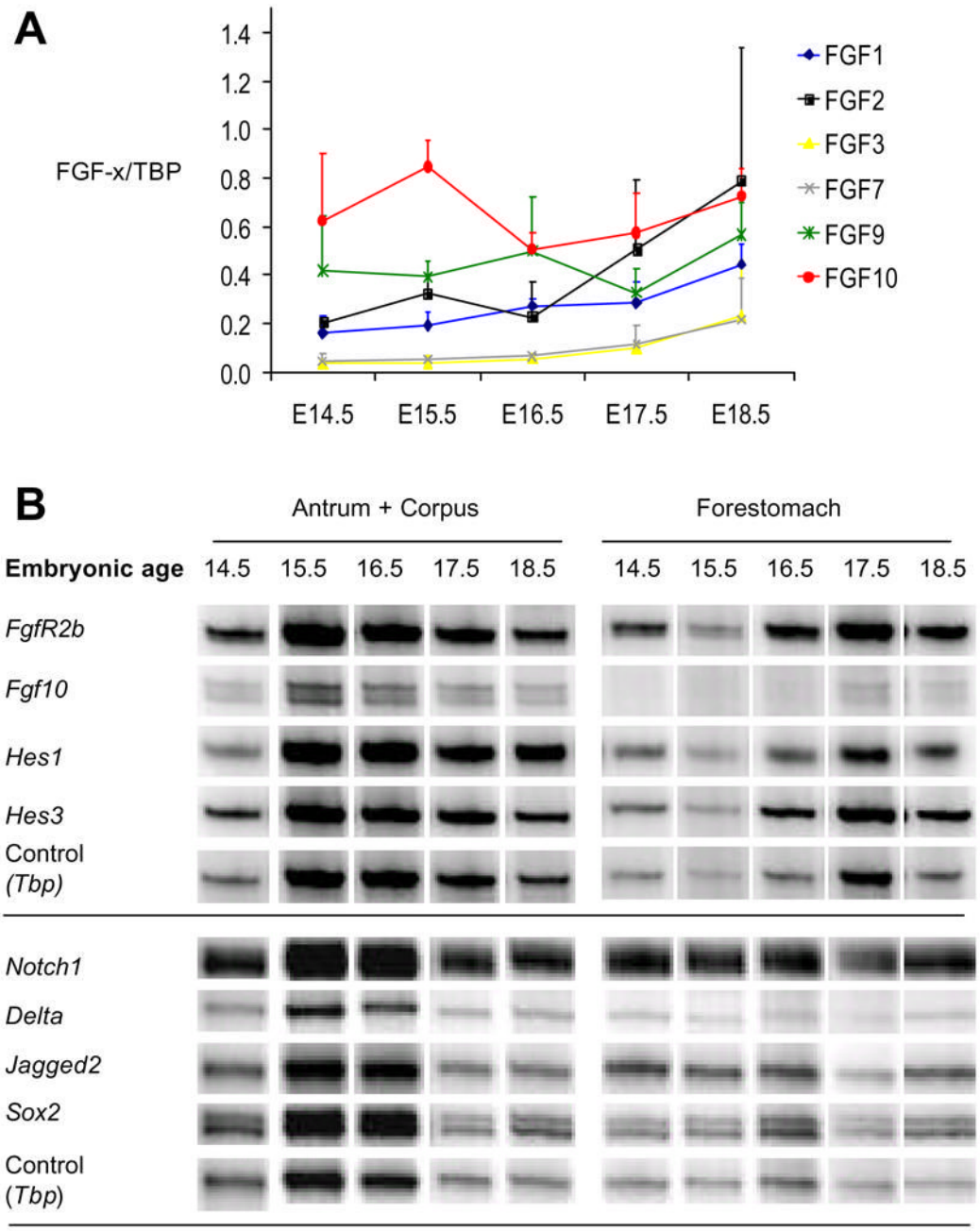

Figure 2. Cell signaling during normal stomach development

A: Semi quantitative multiplex RT-PCR of the expression of FGFs in the glandular stomach. Y-axis shows quantification of intensity of FGF-x bands relative to TBP +/− SEM. (n=3). B: Semi-quantitative multiplex RT-PCR of the expression of *Fgf10*, the FGF10 receptor *FgfR2b*, the Notch signaling components *Hes1*, *Hes3*, *Notch1*, *Delta* and *Jagged2* as well as the gastric transcription factor *Sox2*. The shown bands are representative examples from n=2 experiments with exception of *Fgf10* and *FgfR2b* RT-PCR that was performed four times. *Tbp* was used as internal control.

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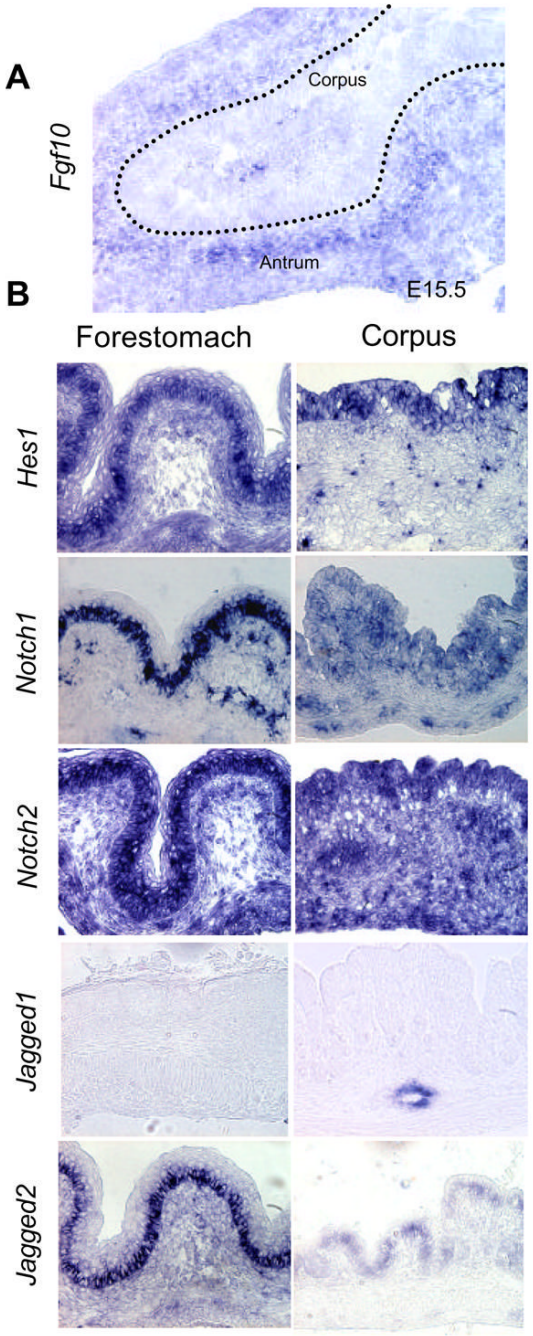

Figure 3. Notch components in stomach development

A: *In-situ* hybridization for FGF10 sho ws mesenchymal expression in an E15.5 stomach. B: *In situ* hybridization for *Hes1*, *Notch1*, *Notch2*, *Jagged1* and *Jagged2* in forestomach and fundus of E18.5 stomach

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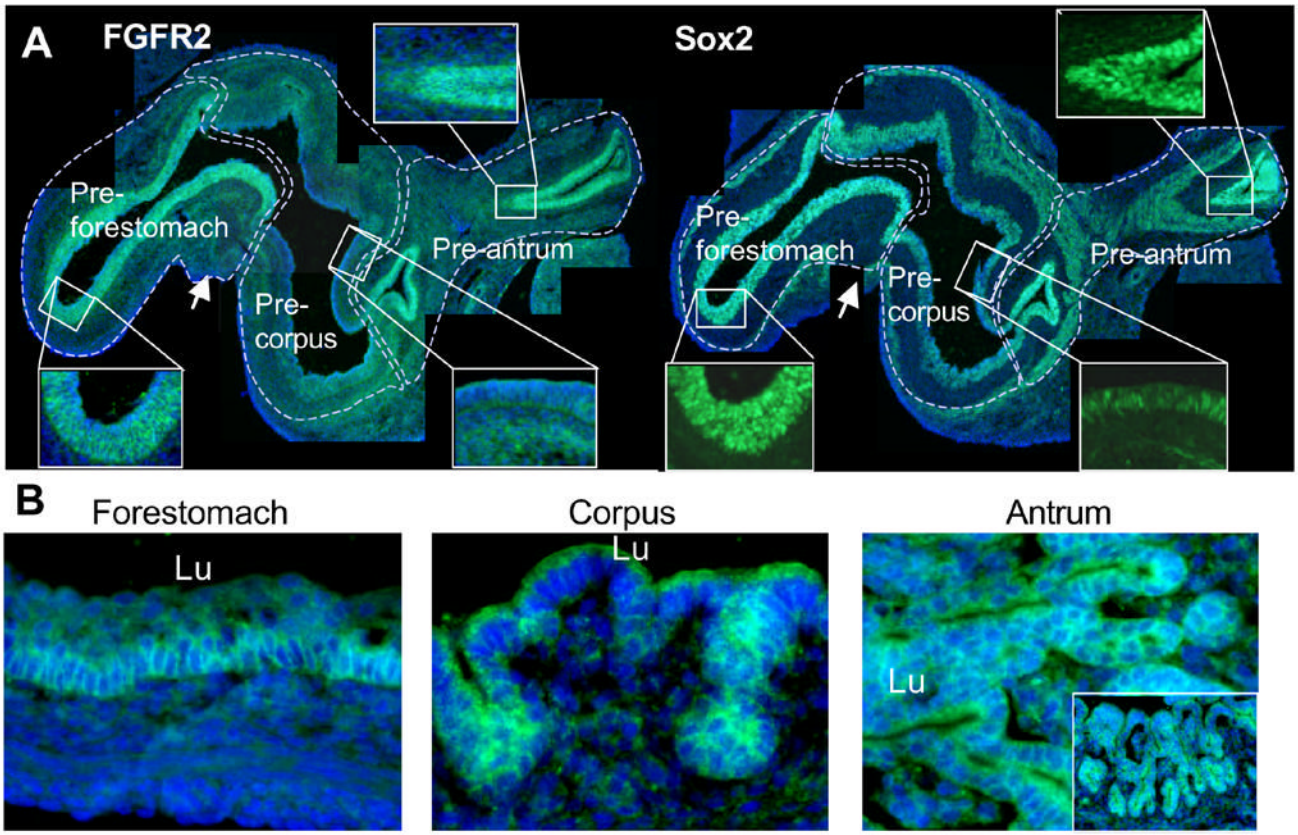

Figure 4. FGFR2 distribution in the stomach

A: Immunostaining for FGFR2 and Sox2 in E15.5 stomach FGFR2 is expressed in the epithelium in the pre-forestomach and pre-antrum regions at E15.5. Arrow points to the esophagus. The Sox2 antibody displays a specific nuclear staining in the gastric epithelium and a nonspecific cytoplasmic staining in mesenchymal cells. B: Immunostaining for FGFR2 in E18.5 stomach. FGFR2 is expressed in the basal layer of the forestomach epithelium, the forming glands in the corpus and in all epithelial cells in the antrum at E18.5. Picture showing antrum is rotated 90 degrees left and has an insert showing a lower magnification of the same region (not rotated). Nuclei are stained with Hoechst

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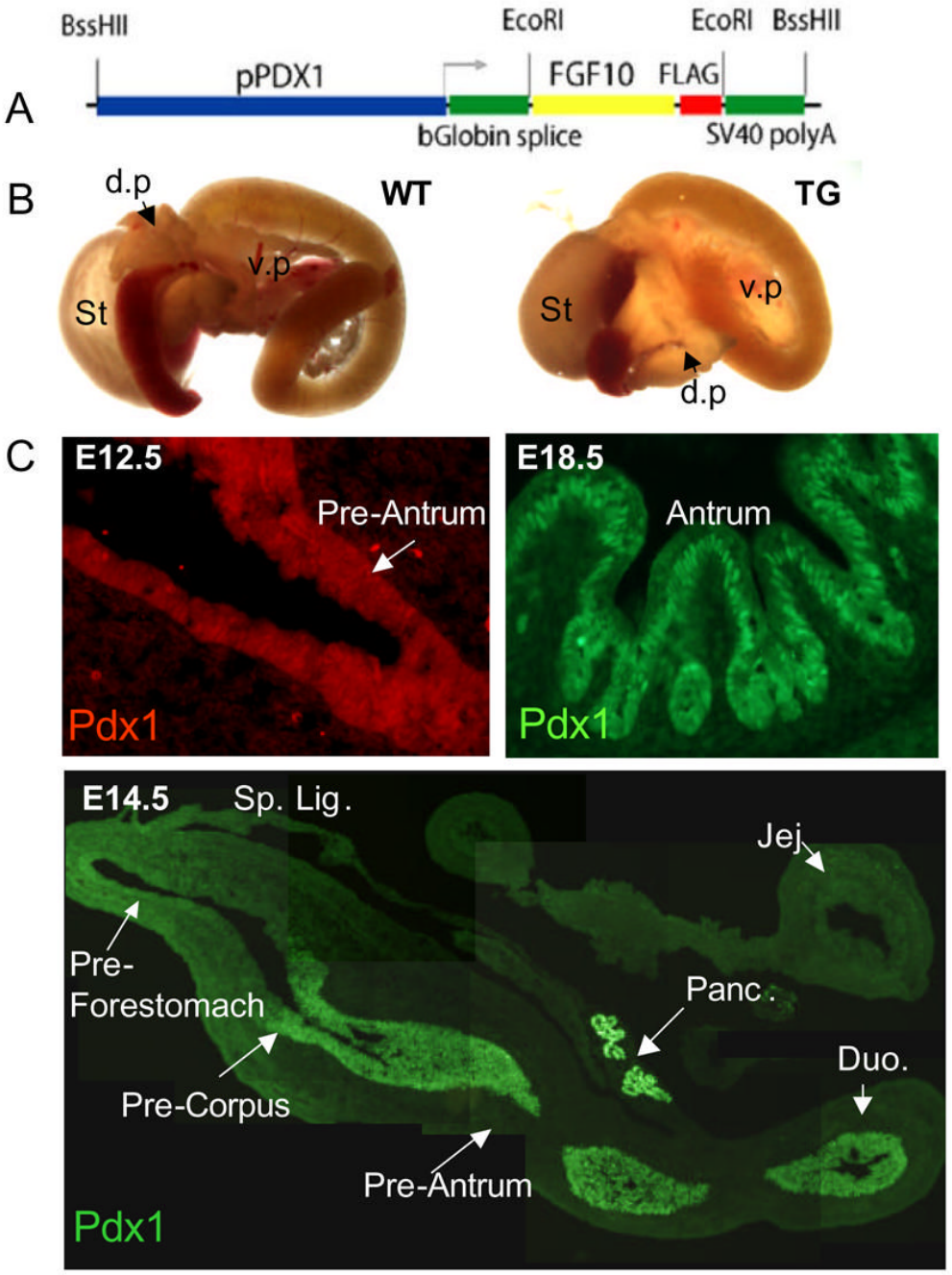

Figure 5.

A: pPDX-FGF10-Flag construct. B: Whole gut of E18.5 pPDX-Fgf10^{FLAG} embryo and wildtype littermate. The TG stomach is smaller and appears denser. C: Immunohistochemistry for PDX1 shows expression in E12.5 most posterior stomach, and E14.5, pancreas, anterior duodenum and posterior stomach including antrum and posterior corpus. PDX1 expression is still present in E18.5 antrum.

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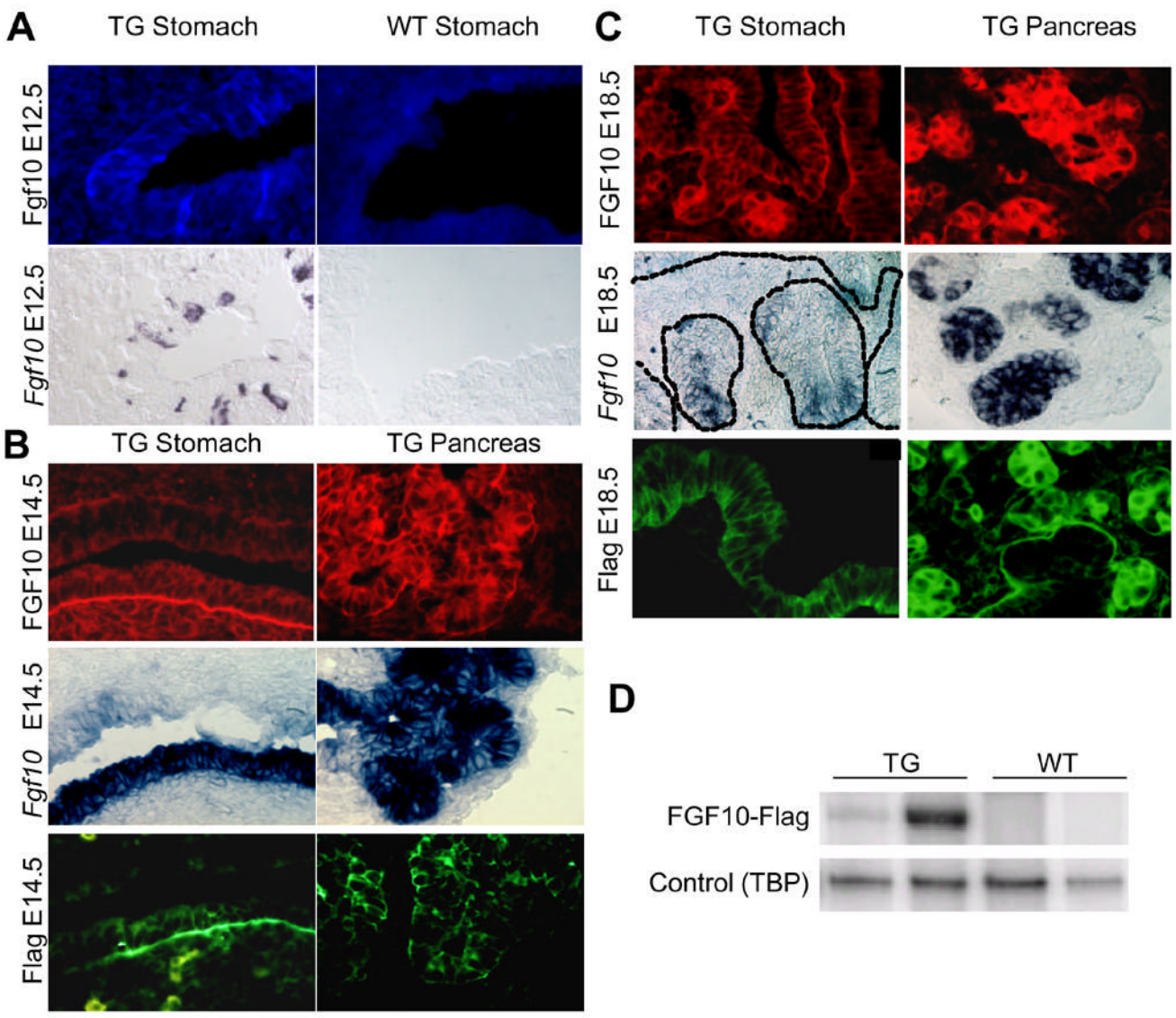

Figure 6. Gastric expression of exogenous FGF10 mRNA and protein

A: E12.5 posterior stomach. Expression of FGF10 was detected with an antibody that detects only high levels of the protein. *Fgf10* mRNA was detected by *in situ* hybridization on an adjacent slide. A few cells in the most posterior stomach of the TG expressed FGF10. No staining was detected in WT littermates. B: E14.5 posterior stomach (lower epithelium). Flag and FGF10 protein detected by immunohistochemistry and *Fgf10* mRNA detected by *in situ* hybridization. No staining was detected in WT littermates (data not shown) n=3. ISH and IHC were performed on consecutive slides. C: E18.5 antrum. Flag and FGF10 protein detected by immunohistochemistry and *Fgf10* mRNA detected by *in situ* hybridization. No staining was detected in WT littermates (data not shown) n=3. *Fgf10* mRNA was only detected in glandular structures at this stage although the protein could also be detected in luminal cells. D: Semi-quantitative RT-PCR for the *Fgf10*-Flag construct in E18.5 stomach of two transgenic mice and their WT littermates. The shown bands are representative examples from three experiments

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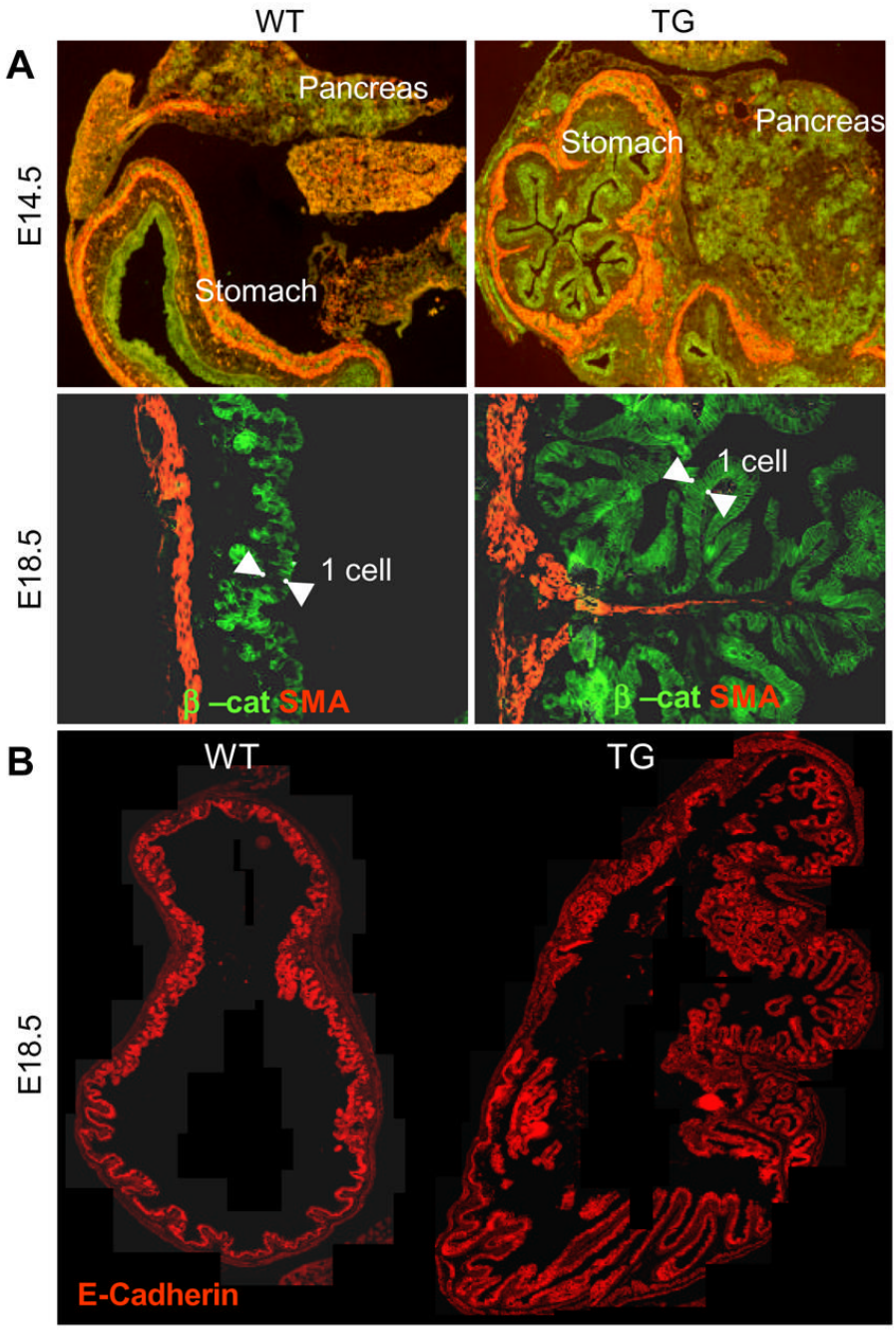

Figure 7. Disturbed gastric morphogenesis in Pdx1-FGF10 embryos

A: Immunohistochemistry for smooth muscle actin and the epithelial marker beta-catenin. B: Immunohistochemistry for E-cadherin on E18.5 TG and WT stomach.

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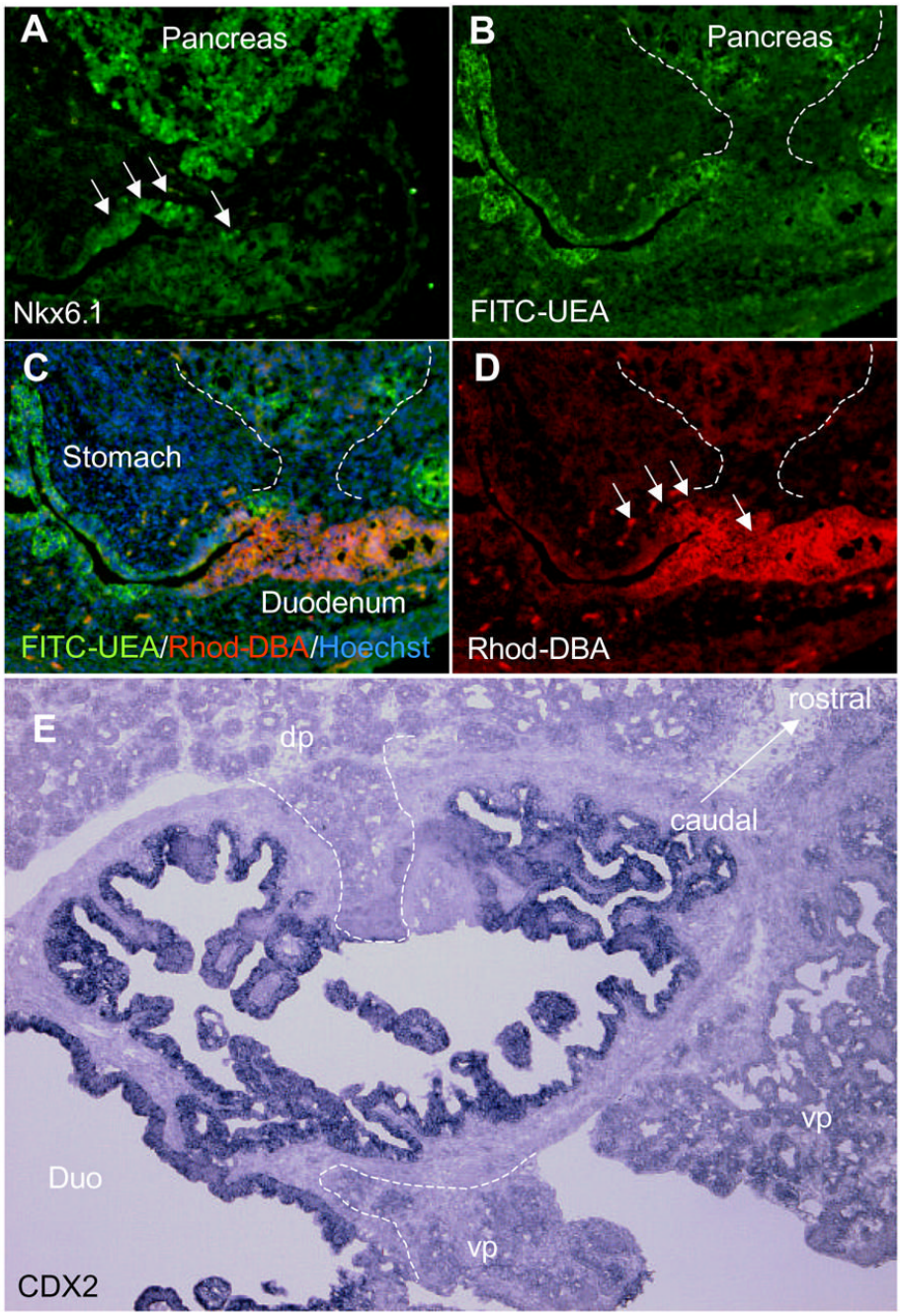

Figure 8. Distal foregut patterning

A: Nkx6.1 immunostaining showing the position of the pancreas at E14.5. B: FITC -UEA staining at E14.5. C: FITC-UEA, Rhodamine-DBA and Hoechst staining merge. D: Rhodamine-DBA staining showing the position of the duodenum at E14.5. E: *Cdx2* in situ of the duodenum at E18.5

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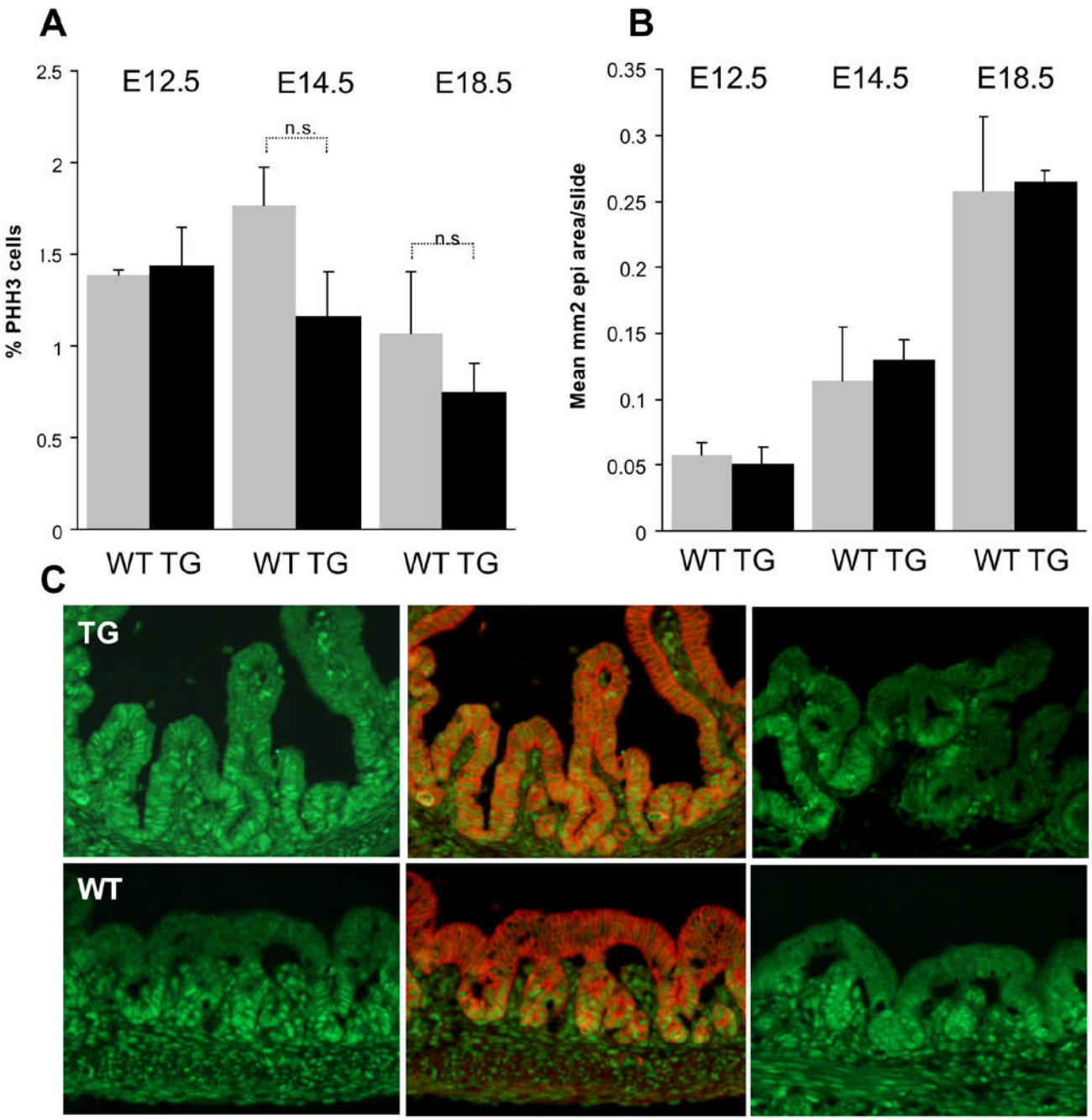

Figure 9. Analysis of cellular proliferation

A: Proliferation of epithelial cells in the glandular stomach as detected by pHH3, E-cadherin and Hoechst co-staining is not significantly altered by ectopic FGF10 at E12.5, E14.5 and E18.5 in the stomach (n=3 WT/TG embryos with each n=3 slides distributed across the stomach with no more than 160μm between slides analyzed at each time point.). B: Epithelial area per slide as measured by E-cadherin staining is unaltered by transgene expression at E12.5, E14.5 and E18.5. (n=3 WT/TG embryos with each n=4 (E18.5) or n=3 slides distributed across the stomach with no more than 120 μm between slides analyzed at each time point.) C: PCNA immunostaining shows disruption of the proliferative nice in E18.5 antral stomach. PCNA is shown in green and E-cadherin in red. The two PCNA stainings are from different embryos.

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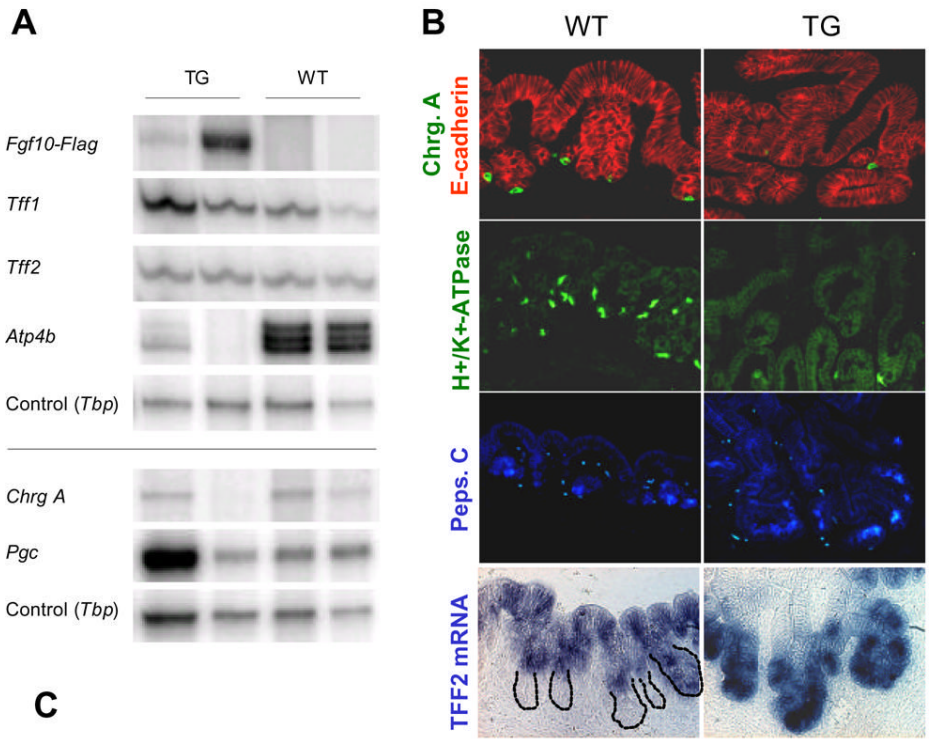

C

Cell Type	WT (cells per mm²)	TG (cells per mm²)	% change	P
All endocrine *(Chromogranin A)*	*234*	*44.0*	***-81%***	*< 0.05*
D cells (somatostatin)	13.8	19.0	n.a.	> 0.05
EC cells (secretin)	346	37.5	***-89%***	< 0.05
A cells (glucagon)	35.1	2.76	***-92%***	< 0.05
Ghrelin cells (ghrelin)	182	61	***-66%***	= 0.08
Parietal cells (HK ATPase)	762	168	***-78%***	< 0.05
Chief cells (Pepsinogen C)	278	732	***+163%***	< 0.05
IFABP	0	0	-	-

Figure 10. Analysis of Cell Differentiation

A: Semi-quantitative multiplex RT-PCR of the differentiation markers Trefoil Factor 1 (*Tff1*), Trefoil Factor 2 (*Tff2*), H^+/K^+-ATPase (*Atp4b*), Chromogranin A (*Chrg A*), and pepsinogen C (*Pgc*). The shown bands are representative examples from two experiments. B: Immunostainings of Chromogranin A with E-Cadherin, H^+/K^+-ATPase and pepsinogen and *in-situ* hybridization of *Tff2* in E18.5 pPDX-FGF10 and WT littermates (n=3). C: Quantification of immunostainings shown in B and immunostainings for somatostatin, secretin, glucagon and ghrelin. Positive cells per mm^2 epithelium as marked by E-cadherin was quantified on three slides distributed throughout the stomach for each of three different

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WT or TG mice and the mean calculated. Statistical significance was tested using the Student's T-test. No *Ifabp* was detected in the stomach by *in situ* hybridization of either WT or TG.

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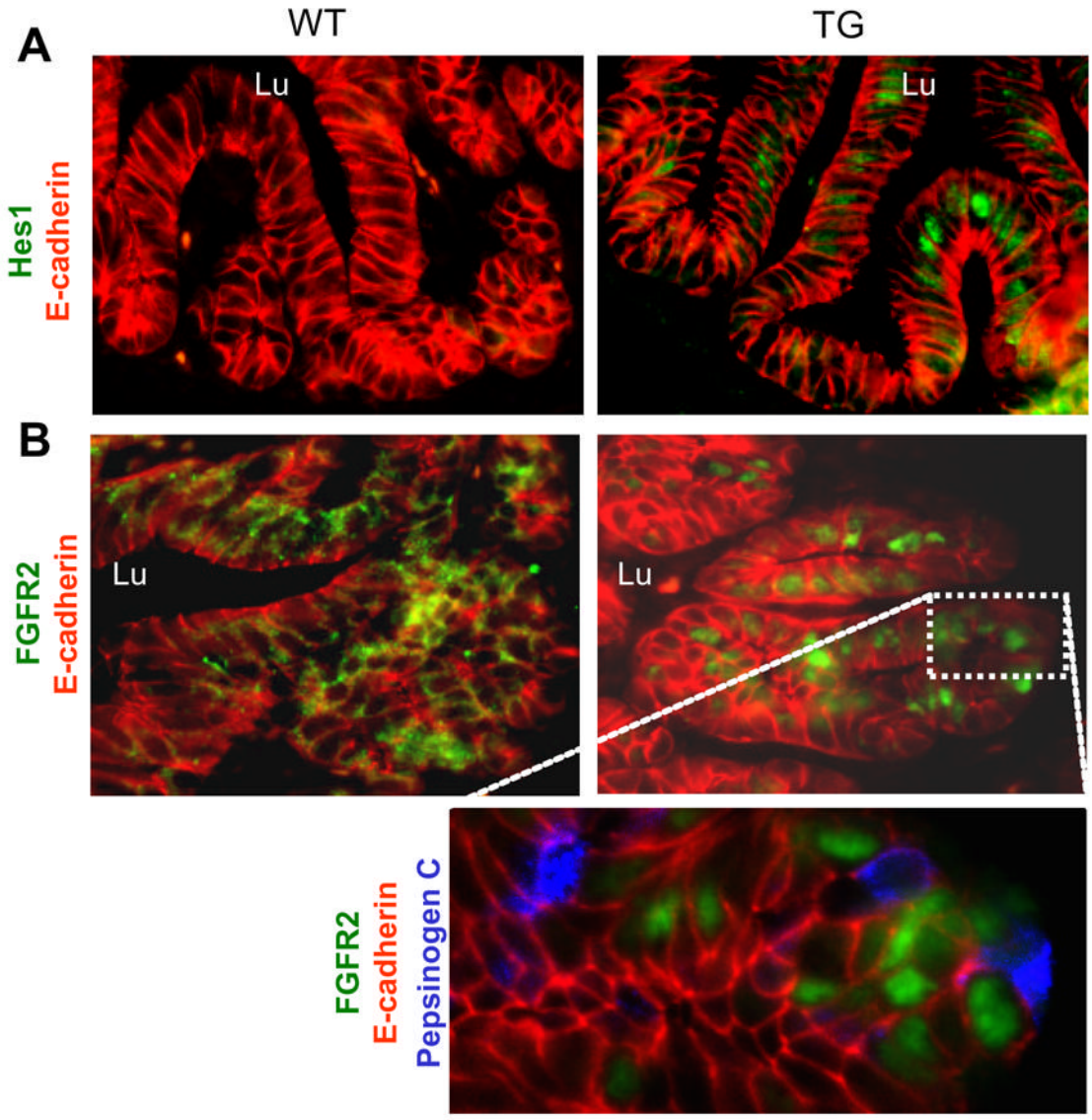

Figure 11. Changes in Hes expression and FGFR2 intracellular distribution

A: Immunohistochemistry of Hes1 and E-cadherin. B: Immunohistochemistry of FGFR2 and E-cadherin. Enlargement of area in the TG also shows pepsinogen staining. Lu: towards the lumen of the stomach.

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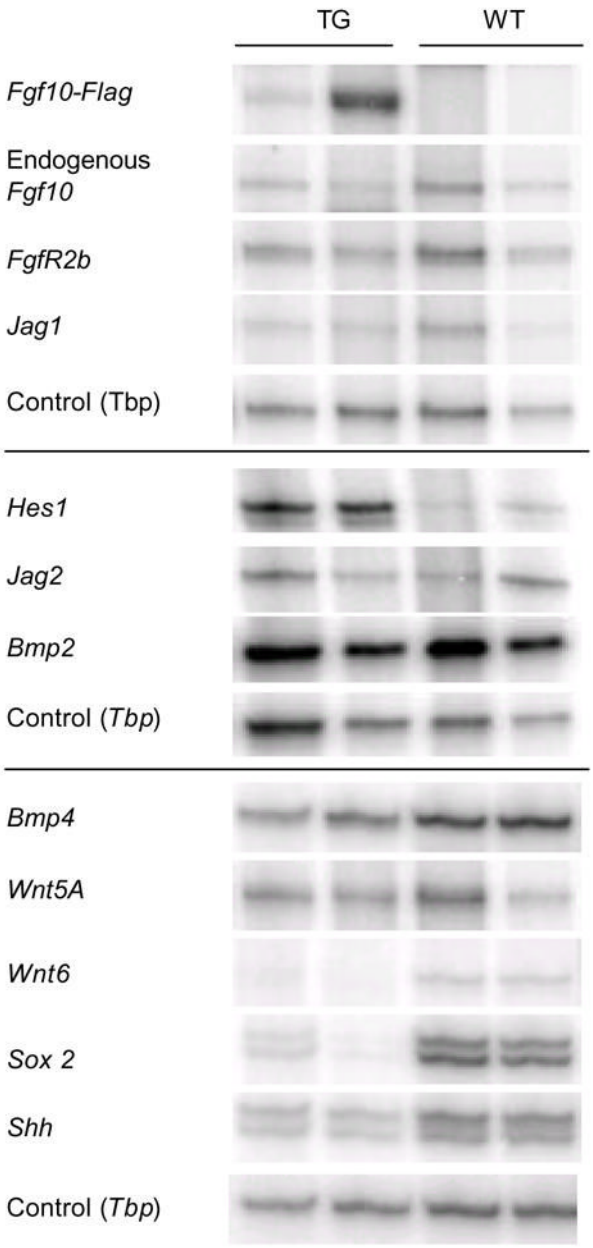

Figure 12. Changes in cell signaling in the pPDX-FGF10 stomach

Semi quantitative multiplex RT-PCR of the expression of ectopic FGF10-Flag, endogenous *Fgf10*, the notch targets *Hes1*, notch ligands *Jagged1* and *Jagged2*, *Bmp2* and *4*, *Wnt5a* and 6, *Sox2*, Sonic hedgehog (*Shh*), and the FGF10 receptor *FGFR2b*. *Tbp* was used as control. The shown bands are representative examples from two experiments