Fibroblast Growth Factor 10-Fibroblast Growth Factor Receptor 2b Mediated Signaling Is Not Required for Adult Glandular Stomach Homeostasis

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Abstract

The signaling pathways that are essential for gastric organogenesis have been studied in some detail; however, those that regulate the maintenance of the gastric epithelium during adult homeostasis remain unclear. In this study, we investigated the role of Fibroblast growth factor 10 (FGF10) and its main receptor, Fibroblast growth factor receptor 2b (FGFR2b), in adult glandular stomach homeostasis. We first showed that mouse adult glandular stomach expressed Fgf10, its receptors, Fgfr1b and Fgfr2b, and most of the other FGFR2b ligands (Fgf1, Fgf7, Fgf22) except for Fgf3 and Fgf20. Fgf10 expression was mesenchymal whereas FGFR1 and FGFR2 expression were mostly epithelial. Studying double transgenic mice that allow inducible overexpression of Fgf10 in adult mice, we showed that Fgf10 overexpression in normal adult glandular stomach increased epithelial proliferation, drove mucus neck cell differentiation, and reduced parietal and chief cell differentiation. Although a similar phenotype can be associated with the development of metaplasia, we found that Fgf10 overexpression for a short duration does not cause metaplasia. Finally, investigating double transgenic mice that allow the expression of a soluble form of Fgfr2b, FGF10’s main receptor, which acts as a dominant negative, we found no significant changes in gastric epithelial proliferation or differentiation in the mutants. Our work provides evidence, for the first time, that the FGF10-FGFR2b signaling pathway is not required for epithelial proliferation and differentiation during adult glandular stomach homeostasis.

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Introduction

Gastric adenocarcinoma is the fourth most common cancer and the second leading cause of cancer-related death worldwide [1] with an overall 5-year relative survival rate in most countries around 20% [2]. Gastric carcinogenesis is most commonly associated with H. pylori infection, but other risk factors include nutritional consumption (high salt intake and/or low fruit and vegetable intake) as well as African-American ethnicity and low socioeconomic status [3]. Parietal cell loss, or oxyntic atrophy, is the most reliable preneoplastic correlate in humans. The loss of parietal cells, regardless of the cause (Helicobacter infection or pharmacologic agents), leads to the subsequent development of metaplasia that can be accelerated by gastrin or histamine deficiency [4,5]. In humans, two types of mucous cell metaplasia can arise as a result of oxyntic atrophy: goblet cell intestinal metaplasia (IM) or spasmolytic polypeptide-expressing metaplasia (SPEM) [4,6]. The Fibroblast growth factor (FGF), Hedgehog, Transforming growth factor beta (TGFB)/Bone morphogenetic protein (BMP), and Wnt signaling pathways are important and related morphogenetic networks that regulate stem cells, particularly in the gastrointestinal tract [7,8]. These signaling pathways are crucial during embryonic development, adult homeostasis, tissue repair and regeneration, and carcinogenesis. Defining the role of the FGF10-FGFR2b signaling pathway during adult glandular stomach homeostasis is a first step to delineating the cellular mechanisms of gastric epithelial repair and regeneration after injury.

Fibroblast growth factors (FGFs) play key roles in cellular proliferation, differentiation, migration, and inflammation in several organs [8]. FGFs bind to one or more tyrosine kinase transmembrane FGF receptors (FGFRs) [9]. As with several other highly conserved signaling pathways, FGF signaling tends to occur in a paracrine fashion between the epithelium and mesenchyme, with the FGF ligand expressed in the tissue adjacent to its corresponding FGFR(s) [10]. For example, during gastric organ-
FGF10-FGFR2b Mediated Signaling in Adult Stomach

Expression of Fgf10, its receptors, Fgrfr1b and Fgrfr2b, and the other FGFR2b ligands in adult mouse glandular stomach

In order to study the expression of Fgf10, its receptors, Fgrfr1b and Fgrfr2b, and the other FGFR2b ligands in adult glandular stomach, we performed RT-PCR on wild type adult glandular stomach (n = 3). Both receptors were expressed in adult glandular stomach and in the positive control, wild type E14.5 mouse whole embryo (Figure 1A). The genes encoding all of the ligands binding FGFR2b (Fgf1, Fgf7, Fgf10, Fgf22) were expressed in adult glandular stomach except for Fgf3 and Fgf20, whereas as all six were expressed in the positive control (Figure 1A). RT negative controls for both adult glandular stomach and the positive control were negative for Fgrfr1b, Fgrfr2b, all FGFR2b ligands, and ß actin (Figure 1A).

To determine the spatial expression pattern of Fgf10, we performed ß-galactosidase staining on sections of adult glandular stomach from Fgfl0+/- reporter mice (n = 3), which have been previously validated [20,22]. We found that Fgf10 expression was present in the mesenchyme just beneath the gastric glands of the epithelium (Figure 1B). Negative controls (wild type Fgfl0+/-, n = 3) showed no LacZ staining (Figure 1E). To confirm the expression of FGF10 receptors in adult stomach, we performed immunohistochemistry staining for FGFR1 and FGFR2 on wild type adult mouse glandular stomach (n = 3). Since specific antibodies for the ßIIb isoform of these receptors are not available, antibodies that react with both the ßIIb and ßIIc isoforms of each receptor were used. The ßIIb isoform is usually expressed in the epithelium whereas the ßIIc isoform is typically expressed in the mesenchyme. Both FGFR1 and FGFR2 (Figure 1C and 1D, respectively) were identified with strong immunostaining in the gastric epithelium and weaker staining in the mesenchyme. Our negative controls did not show any specific staining (Figure 1F, 1G). The presence of Fgf10 and its receptors in the adult mouse stomach suggests a role for FGF10 in stomach homeostasis.

Results

In this study, we investigate the role of FGF10-FGFR2b signaling during adult glandular stomach homeostasis. We first demonstrated the presence of Fgf10 and its receptors, Fgrfr1b and Fgrfr2b, in adult glandular stomach, along with all the genes encoding the other FGFR2b ligands (Fgf1, -3, -7, -20, -22). Investigating double transgenic mice allowing Fgf10 overexpression, we showed that Fgf10 overexpression increases epithelial proliferation, drives mucus neck cell differentiation and reduces parietal and chief cell differentiation during adult glandular stomach homeostasis. Although the loss of parietal cells and increase in mucus-producing cells can be associated with the development of metaplasia, we did not identify any metaplasia in gastritis from our FGF10's receptor mice (n = 3). This study demonstrates that FGF10-FGFR2b signaling is not required for adult glandular stomach homeostasis.

FGF10 overexpression during homeostasis changes gastric gland histology and increases epithelial proliferation in glandular stomach

In order to investigate the role of FGF10 during adult glandular stomach homeostasis, we generated inducible double transgenic heterozygous mice that ubiquitously overexpressed Fgf10 (R26+;tet(O)Fgf10+/-; R26rtTA/+; tet(O)Fgf10+/+ hereafter). Overexpression of Fgf10 was induced by feeding doxycycline to adult (4 week old) mutant mice and control littersmates for 10 days prior to sacrifice. The overexpression of Fgf10 was confirmed by qRT-PCR and the mutants showed a significant increase in the expression of Fgf10 when compared to control littersmates (Figure 2C). Mutant mice typically develop a wet-hair appearance and an obvious proliferation of cutaneous epithelium, including swelling of the eyelids and an abnormally enlarged tongue.

Hematoxylin and cosin staining of sections of control littermate adult glandular stomach showed a simple columnar epithelium organized into gastric glands containing numerous parietal cells with large eosinophilic cytoplasm, chief cells at the base of the glands with basophilic cytoplasm, basally located nuclei, and apical secretory granules, and mucus neck cells with mucus seen in white at the apical portion of the cells in the neck of the glands (Figure 2A). This histology was significantly altered in the adult glandular stomach of the Fgf10 overexpressing mutant mice (Figure 2B). There was a visible decrease in the parietal cell population, with an increase in mucus-secreting cells and a clustering of these cells closer to the base of the gland (black arrowheads).

As it is well established that FGF10 promotes proliferation in a number of organs including the gastrointestinal tract [13,19,20,23,24], we analyzed the proliferation of the gastric epithelium by PCNA immunostaining in the control littersmates and mutant mice. Compared to littermate controls, the mutant glandular stomachs showed a significant increase in the proliferation of the epithelium (14.7 ± 1.8% PCNA-positive epithelial cells vs. 22.7 ± 2.6% in the mutants, p = 0.017, n = 5 for each genotype) (Figure 2D-F). Our data demonstrated that overexpression of FGF10 promotes cell proliferation in the epithelium of adult mouse glandular stomach.

Gastric epithelial differentiation is significantly altered by Fgf10 overexpression during homeostasis

In order to further define the role of FGF10 in epithelial differentiation during adult glandular stomach homeostasis, we...
performed immunostaining for differentiated gastric epithelial cell markers in mutant and control stomachs. Mucous neck cells in glandular stomach were identified by lectin GSI-II, which has been previously established as a mucous neck cell marker [25,26,27].

\( Fgf10 \) overexpression resulted in an 85% increase in the percentage of mucous neck cells in the gastric epithelium of the mutant mice (Figure 3B) compared to control littermates (Figure 3A) (14.4 ± 1.7% vs. 7.8 ± 0.5%, \( p = 0.003 \), \( n = 5 \) for each genotype) (Figure 3C). Furthermore, the GS-II positive cells were located closer to the base of the glands in the mutant mice compared to the controls. This corroborates our histological data, and indicates that FGF10 may promote differentiation of the mucous neck cell lineage. Intrinsic factor (IF) immunostained the chief cells at the base of the gastric glands. Although IF is produced and released by parietal cells in humans, it is an established marker of chief cells in rodents [21,28,29]. Our results showed a significant decrease of chief cells in the gastric epithelium of the mutants (Figure 3E) compared to control littermates (Figure 3D) (5.2 ± 1.4% vs. 12.4 ± 1.7%, \( p = 0.0044 \), \( n = 5 \) for each genotype). These data suggest that FGF10 plays a role in the differentiation of gastric epithelial mucous neck cell, chief cell, and parietal cell lineages during adult glandular stomach homeostasis. Fgf10 overexpression not only significantly alters the number of these differentiated epithelial cell types as described above, but also changes the location of the mucous neck cells from the neck to the base of the gastric gland.

Fgf10 overexpression during homeostasis does not cause metaplasia of the gastric epithelium

Intestinal metaplasia (IM) and spasmolytic polypeptide-expressing metaplasia (SPEM) are both metaplasias of the gastric epithelium that typically develop after acute oxyntic atrophy and result in an increase in mucus-secreting cells: intestinal goblet cells in IM or mucous neck cells in SPEM [32]. It is currently thought that the loss of parietal cells results in transdifferentiation of mature chief cells in addition to inhibition of the normal mucous neck cell to chief cell differentiation [5,21,33]. Since the mutant mice demonstrated a similar phenotype with a significant loss of parietal cells, increase in mucous neck cells, and reduction in chief cells, we sought to investigate if Fgf10 overexpression could cause metaplasia. In order to confirm if metaplasia was present in our

Figure 1. Normal expression pattern of Fgf10, its receptors, all other FGFR2b ligands in adult glandular stomach. (A) PCR of adult glandular stomach and E14.5 wildtype whole embryo (positive control). RT negative controls for both are all negative. (B,E) β-galactosidase staining of adult glandular stomach in Fgf10+/- (B) and wildtype Fgf10+/+ (E) mice. Fgf10 expression occurs only in the mesenchyme. Immunohistochemical analysis of wildtype adult glandular stomach sections stained with anti-FGFR1 (C) or anti-FGFR2 (D). (F,G) Negative control stainings, in which the primary antibody was absent, show minimal to no signal. FGFR1 and FGFR2 staining is strong throughout the gastric epithelium whereas the mesenchymal staining is much weaker. The scale bar is 50 μm. M = mesenchyme, GE = gastric epithelium, L = lumen.
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mutant mice, we performed immunostaining for two well-established markers of metaplasia in both mice and humans: CDX2 (IM) [21] and HE4 (IM and SPEM) [6]. Both the mutant (Figure 4C) and the littermate control stomachs (Figure 4B) demonstrated no appreciable CDX2 staining in the gastric epithelium (n = 3 for each genotype). Colon served as a positive control and showed appropriate nuclear staining for CDX2 (Figure 4A). Similarly, no detectable HE4 staining was observed in the gastric epithelium of the mutants (Figure 4F) and littermate controls (Figure 4E) (n = 3 for each genotype). Human epididymis served as a positive control and had visible cytoplasmic staining for HE4 (Figure 4D). These results suggest that although Fgf10 overexpression may result in a SPEM-like phenotype, the gastric epithelium is not metaplastic.

FGF10-FGFR2b mediated signaling is not required for gastric epithelial proliferation and differentiation during homeostasis

In order to determine the importance of the FGF10-FGFR2b signaling axis during adult glandular stomach homeostasis, we generated inducible double transgenic heterozygous mice that ubiquitously express a dominant-negative soluble form of Fgfr2b (R26rtTA/+; tet(O)sFgfr2b/+ hereafter). Expression of sFgfr2b was induced by feeding doxycycline to adult (4 week old) mutant mice and control littermates for 1 month prior to sacrifice. The expression of sFgfr2b in the mutant mice was confirmed by qRT-PCR. The controls had nearly undetectable amounts of sFgfr2b, while the mutants had a variable but robust expression (Figure 5C). Expression of sFgfr2b acts in a dominant negative fashion by binding all FGFR2b ligands (FGFs-1, 2, 3, 4, 7, 10, 20, 22) and preventing their action. This has been previously validated in our laboratory where we demonstrated that inducible expression of sFgfr2b during embryonic development phenocopied Fgfr2b−/− embryos [34], whereas single transgenic embryos exposed to dox and double transgenic embryos not exposed to dox were identical to wild type embryos [35]. In contrast to the severe phenotype when FGFR2b is inactivated during embryogenesis, the postnatal expression of sFgfr2b results only in minor defects including defective incisors, longer claws, and reduced white adipose tissue [36].

Hematoxylin and eosin staining of sections of control littermate (Figure 5A) and mutant Fgf10 overexpressing mice (Figure 5B) compared to control littermates (Figure 5D) was given for 10 days. The scale bar is 50 µm. M = mesenchyme, GE = gastric epithelium, L = lumen.

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Figure 2. Fgf10 overexpression during homeostasis changes gastric gland histology but not epithelial proliferation in glandular stomach. (A–B) Hematoxylin and eosin staining of sections of adult glandular stomach in control littermate (R26rtTA/+; tet(O)Fgf10/+ without dox) (A) and mutant Fgf10 overexpressing mice (R26rtTA/+; tet(O)Fgf10/+ with dox) (B). There is a visible decrease in the number of parietal cells and an obvious shift of the mucous neck cells from the luminal side towards the gastric gland base (black arrowheads). (C) qRT-PCR showing a significant increase in Fgf10 expression in the mutants vs controls (*p<0.05). (D–E) PCNA immunostaining of sections of adult glandular stomach in control littermate (R26rtTA/+; tet(O)Fgf10/+ without dox) (D) and mutant Fgf10 overexpressing mice (R26rtTA/+; tet(O)Fgf10/+ with dox) (E). (F) Quantification of the percentage of PCNA-positive epithelial cells showed a statistically significant increase in the percentage of PCNA positive epithelial cells indicating increased gastric epithelial proliferation in mutant Fgf10 overexpressing mice (R26rtTA/+; tet(O)Fgf10/+ with dox) compared to control littermates (R26rtTA/+; tet(O)Fgf10/+ without dox). Dox was given for 10 days. The scale bar is 50 µm. M = mesenchyme, GE = gastric epithelium, L = lumen.
As it has been previously demonstrated that FGF10-FGFR2b signaling is essential for epithelial differentiation during gastric organogenesis, particularly for the development of the parietal cell lineage [13], we sought to determine if FGF10-FGFR2b mediated signaling was required for gastric epithelial differentiation during homeostasis. Sections of glandular stomach from the R\textsuperscript{26}\textsuperscript{TA+/tet(O)Fgf10/+} mutant mice and control littermates were immunostained with markers for differentiated gastric epithelial cells. There was no difference in the percentage of mucous neck cells (7.5 ± 0.8\% vs. 8.0 ± 0.8\%, p = 0.35, n = 5 for each genotype) (Figures 6A–C), chief cells (11.3 ± 1.6\% vs. 12.2 ± 1.5\%, p = 0.35, n = 5 for each genotype) (Figures 6D–F), endocrine cells (Figures 6G–I), and parietal cells (Figures 6J–L) between the control and mutant mice.

Figure 3. Gastric epithelial differentiation is significantly altered by Fgf10 overexpression during homeostasis. Immunostaining for markers of terminally differentiated gastric epithelial cells of sections of adult glandular stomach in control littermate (R\textsuperscript{26}\textsuperscript{TA+/tet(O)Fgf10/+} without dox) (A,D,G,J) and mutant Fgf10 overexpressing mice (R\textsuperscript{26}\textsuperscript{TA+/tet(O)Fgf10/+ with dox}) (B,E,H,K). (A-B) Mucous neck cells are identified by GSII and (C) quantification reveals a significant increase in mutant Fgf10 overexpressing mice compared to control littermates (*p<0.05). (D,E) Chief cells are marked by intrinsic factor and (F) quantification shows a significant decrease in chief cell number (*p<0.05). (G,H) Endocrine cells are identified by chromogranin A and (I) quantification demonstrates no significant change in endocrine cell number. (J,K) Parietal cells are visualized with H/K ATPase immunostaining and (L) quantification demonstrates a significant reduction in mutant Fgf10 overexpressing mice compared to control littermates (*p<0.05). Dox was given for 10 days. The scale bar is 50 μm. GE = gastric epithelium, L = lumen. doi:10.1371/journal.pone.0049127.g003
(1.9±0.3% vs. 2.4±0.4%, p = 0.22, n = 5 for each genotype) (Figures 6G–I), or parietal cells in the gastric epithelium (36.4±2.7% vs. 37.7±3.5%, p = 0.38, n = 5 for each genotype) (Figures 6J-K), between the littermate control and the mutant stomachs. Taken together, these results suggest that FGFR2b signaling is not required for gastric epithelial proliferation and differentiation during homeostasis.

Since the lifespan of a parietal cell is 54 days [37], a longer-term study was required to confirm the dispensability of FGF10-FGFR2b mediated signaling for parietal cell differentiation during adult glandular stomach homeostasis. In order to accomplish this, we induced ubiquitous overexpression of a dominant-negative sFgfr2b by feeding doxycycline to adult (4 week old) mutant mice and control littermates for 3 months prior to sacrifice. The expression of sFgfr2b in the mutant mice was significantly higher than controls as shown by qRT-PCR (Figure 7C). Hematoxylin and eosin staining of sections of control littermate (Figure 7A) and mutant Fgf10 overexpressing mice (R26^{TA+}; tel(t(o)Fgf10+/+) (F). Dox was given for 10 days. The scale bar is 50 µm.

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(Figure 4. Fgf10 overexpression during homeostasis does not cause metaplasia of the gastric epithelium. (A–C) CDX2, a marker of intestinal metaplasia (IM), immunostaining in adult colon (positive control) (A), control littermate (R26^{TA+}; tet(O)Fgf10/+ without dox) (B) and mutant Fgf10 overexpressing mice (R26^{TA+}; tet(O)Fgf10/+ with dox) (C). (D–F) HE4, a marker of spasmolytic polypeptide expressing metaplasia (SPEM), immunostaining in human epididymis (positive control) (D), control littermate (R26^{TA+}; tet(O)Fgf10/+ without dox) (E) and mutant Fgf10 overexpressing mice (R26^{TA+}; tet(O)Fgf10+/+) (F). doi:10.1371/journal.pone.0049127.g004

Discussion

FGF10-FGFR2b mediated signaling is essential for embryonic gastric development [11,13,23]. However, little is known about the role of FGF10-FGFR2b signaling during maintenance of mature gastric epithelium. We sought to understand FGF10-FGFR2b mediated signaling during adult glandular stomach homeostasis. Double transgenic mice that ubiquitously overexpress Fgf10, displayed increased epithelial proliferation, and considerably altered differentiation of three of the four epithelial cell lineages in the stomach. However, double transgenic mice overexpressing a soluble form of Fgfr2b, FGF10's main receptor, revealed that FGF10-FGFR2b signaling is not necessary for epithelial proliferation and differentiation.

We found that Fgf10, its receptors, Fgfr1b and Fgfr2b, and most of the other FGFR2b ligands (Fgf-1, 2, 7, 10, and 22) in adult stomach may allow for some intrinsic redundancy in FGF signaling. Certainly the expression of Fgf10 and its receptor, Fgfr2b, in adult stomach suggests that FGF10-FGFR2b signaling occurs postnatally.

Multiple studies have recognized FGF10 as a promoter of epithelial proliferation during tracheal [42], colonic [19,20], and gastric [13,23] development as well as postnatally during mammary gland [36] and incisor [35] homeostasis. Many of these characterize a loss of function approach, demonstrating decreased epithelial proliferation in Fgf10^{-/-} [13,20,42] and/or
Fgf2b mice as well as in transgenic mice that overexpress soluble Fgfr2b [13,19]. Only two previous studies report a gain of function approach similar to ours, investigating the effects of Fgf10 overexpression during gastric organogenesis, with contradictory results [11,23]. Shin et al. demonstrated a modest increase in glandular epithelial proliferation in chick embryonic stomach with viral-mediated overexpression of Fgf10 compared to uninfected controls [23]. Our results align with these results in organogenesis although we are studying a much later time point when we demonstrate that FGF10 has a mitogenic effect during adult stomach homeostasis.

In addition, FGF10 plays an important role in epithelial differentiation during the development of numerous organs [13,42,43,44]. In particular, during gastric organogenesis, the loss of FGF10-FGFR2b mediated signaling results in the complete absence of parietal cells [13]. This indicates that FGF10-FGFR2b mediated signaling is essential for parietal cell differentiation during stomach development, and yet, Fgf10 overexpression during this same time period also has a negative effect on parietal cells with a 78% reduction at E18.5 [11]. Interestingly, we found that FGF10-FGFR2b mediated signaling is not required for parietal cell differentiation during adult stomach homeostasis, but Fgf10 overexpression caused a similar reduction in the parietal cell lineage as occurs during organogenesis. Thus, FGF10 at high levels down-regulates parietal cell differentiation during both stomach development and homeostasis, but once gastric organogenesis is complete, FGF10-FGFR2b mediated signaling appears to be dispensable for parietal cell differentiation.

Endocrine cells represent a small, but heterogeneous population of cells that secrete a variety of hormones in the gastric epithelium. Terminal endocrine cell fate appears unaffected by the loss of FGF10-FGFR2b mediated signaling during development [13]; however, ectopic Fgf10 overexpression results in significant suppression of the endocrine cell lineage [11]. Unlike these developmental studies, we did not observe any change in endocrine cell differentiation upon overexpression of either Fgf10 or soluble Fgf2b during stomach homeostasis. FGF10-FGFR2b mediated signaling promotes chief cell differentiation during gastric organogenesis as evidenced by the reduced abundance of chief cells in Fgf10-/- and Fgf2b-/- E18.5 stomachs [13] and the increase in chief cells in stomachs with ectopic Fgf10 overexpression [11]. In contrast, we observed a significant decrease in chief cells upon Fgf10 overexpression in adult glandular stomach homeostasis. We identified chief cells by immunostaining for intrinsic factor and these previous studies stained for pepsinogen [11,13], but both are well-established markers of chief cells in mice [21,28,29,45] and variation in immunohistochemical staining alone seems unlikely to account for this observation. It is possible that during homeostasis Fgf10 levels have adverse effects on chief cells as opposed to developmental stages.

It is known that FGF10-FGFR2b mediated signaling is not required for mucous cell differentiation during stomach development [13], and we found the same to be true during homeostasis. However, FGF10 has been shown in previous studies to either induce mucus-secreting cell number [24] or to shift cell location within the gastric gland from the lumen towards the gland base.
We observed both phenomena during adult glandular stomach homeostasis. This phenotype, combined with the loss of parietal cells, is often described as “antralization” and is observed in gastric metaplasias, such as IM and/or SPEM. The loss of parietal cells usually results in metaplasia [4,32,33]. During this process, chief cells transdifferentiate by losing expression of MIST1 and gaining expression of CDX2 in IM or TFF2 in SPEM [21,33]. Perhaps the reduction of chief cell number in our model is due to transdifferentiation, however, this would require further investigation to confirm.

Figure 6. FGF10-FGFR2b mediated signaling is not required for gastric epithelial differentiation during homeostasis. Mucous neck cells are marked by GSII immunostaining of sections of adult glandular stomach in control littermate (R26<sup>wt</sup>; tet(O)sFgfr2b+/+ with dox) (A) and mutant mice (R26<sup>rtTA</sup>; tet(O)sFgfr2b+/+ with dox) (B) and (C) quantification reveals no difference in mucous neck cell number. Chief cells are identified by intrinsic factor immunostaining of sections of adult glandular stomach in control littermate (R26<sup>wt</sup>; tet(O)sFgfr2b+/+ with dox) (D) and mutant mice (R26<sup>rtTA</sup>; tet(O)sFgfr2b+/+ with dox) (E) and (F) quantification reveals no significant change in chief cell number. (G,H) Endocrine cells are identified by chromogranin A and (I) quantification shows no significant change in mutant sFgfr2b overexpressing mice compared to control littermates. Parietal cells are identified by H/K ATPase immunostaining of sections of adult glandular stomach in control littermate (R26<sup>wt</sup>; tet(O)sFgfr2b+/+ with dox) (J) and mutant mice (R26<sup>rtTA</sup>; tet(O)sFgfr2b+/+ with dox) (K) and (L) quantification reveals no significant change in parietal cell number. Dox was given for 1 month. The scale bar is 50 μm. GE = gastric epithelium, L = lumen.

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We are not the first to observe a SPEM-like phenotype in association with FGF10 signaling. Spencer-Dene et al. demonstrated a disproportionately underdeveloped antrum with an enlarged simple unbranched gastric epithelium for both Fgf10$^{−/−}$ and Fgfr2b$^{−/−}$ embryos indicating a role of FGF10-FGFR2b mediated signaling in promoting antralization [13]. Furthermore, overexpression of Fg10 during stomach development resulted in a SPEM-like phenotype with a shift in localization of TFF2 mRNA in the mouse and cSP mRNA in the chick, in addition to a reduction in the number of parietal cells in the mouse [11][23]. cSP is a marker of luminal epithelial cells in the chick, the analog to mucous neck cells in the mouse [8]. Nyeng et al. speculated that the antralization of the corpus in their model could be explained by increased FGF10 availability in the corpus as compared to the normal gradient of Fgf10 expression, which is higher in the antrum and lower in the corpus [11]. This could explain the SPEM-like phenotype seen during homeostasis as well, since Fg10 was ubiquitously overexpressed in our model. Despite Fg10 overexpression resulting in a SPEM-like phenotype, well-established markers failed to confirm metaplasia during homeostasis. In spite of the evidence for a role of FGF10-FGFR2b mediated signaling during stomach homeostasis, FGFR2b signaling inhibition did not impair gastric epithelial proliferation or differentiation. FGF10 promotes mucous neck cell differentiation while suppressing parietal and chief cell differentiation, producing a SPEM-like phenotype; but does not appear to induce metaplasia. In the absence of stomach homeostasis, Fg10 overexpression affects both gastric epithelial proliferation and differentiation. FGF10-FGFR2b mediated signaling is not required during adult glandular stomach homeostasis.

**Materials and Methods**

**Ethics Statement**

All experimental protocols were in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee at Children’s Hospital Los Angeles (IACUC protocol .

Figure 7. FGF10-FGFR2b mediated signaling is not required for parietal cell differentiation during homeostasis. Hematoxylin and eosin staining of sections of adult glandular stomach in control littermate (R26$^{+/-}$; tet(O)sFgfr2b/+ with dox) (A) and mutant mice (R26$^{ITA/ITA}$; tet(O)sFgfr2b/+ with dox) (B) showing normal stomach histology. (C) qRT-PCR confirming a significant increase in the expression of sFgfr2b in the mutants as compared to controls (*p<.05). Parietal cells are identified by H/K ATPase immunostaining of sections of adult glandular stomach in control littermate (R26$^{+/-}$; tet(O)sFgfr2b/+ with dox) (D) and mutant mice (R26$^{ITA/ITA}$; tet(O)sFgfr2b/+ with dox) (E) and (F) quantification reveals no significant change in parietal cell number. Dox was given for 3 months. The scale bar is 50 μm. M = mesenchyme, GE = gastric epithelium, L = lumen. doi:10.1371/journal.pone.0049127.g007
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Mice
All animals were housed in the Saban Animal Care Facility of the Saban Research Institute at Children’s Hospital Los Angeles, Los Angeles, California. Animals were maintained in a temperature-regulated environment on a 12-hour light-dark cycle and given access to chow and water ad libitum. Wild type mice were C57Bl/6 mice from the Jackson Laboratory.

PCR
RNA was isolated from adult wild type (C57Bl/6) mouse glandular stomach (n = 3) and E1.5 wild type whole mouse embryo (n = 2) using the Qiagen RNA extraction kit (Qiagen, Valencia, CA). The iScript™cDNA Synthesis Kit (BioRad, Hercules, CA) was used to synthesize cDNA. All PCR reactions were performed following the manufacturer’s instructions using either the Taq PCR Master Mix (Qiagen, Valencia, CA) or 2x MyTaq Red mix (Bioline, Taunton, MA) in a C1000™ Thermal Cycler (BioRad, Hercules, CA). Primers were designed specifically to span an intron using the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). The following primer sequences were used: (forward 5'-3', reverse 5'-3'); Fgf10b CTTGACGTCTGAGACCATCT, CCAAGCTTCTGAGCTGA; Fgf26 ACGGGAGACAGGATTAAACG, GGAGATTGACCGAGGACG; Fgf1 ACCTTGGCAAGCCTGACGCA, GACGCCGCTTA-CAGCTCCCG; Fgfβ CATGAAAGAAGAGGGAGGCTGTATG, TCCTGACGACCCGACCACTTC; Fgf7 TCCTGCTCTAGATGTCG, TAGTTATGCTGCAATGAG; Fgf10 CACATTTGTGCGCTAGCCCTGTC, CCTGCCATTTGCTGCACACTTA; Fgf20 GGAGACTCAGAGATTACCCCA, TGGGACCCTGTCCTCCTAGTG; Fgf22 GCATCTGAGGGCGAGTG, CCACAGAGTACGACGCCGCC.

Analysis of LacZ expression
Fgf10b−/− mice (C57BL/6) [48] (hereafter called Fgf10+/−) were maintained on a mixed agouti background. Samples were briefly fixed in 4% PFA, laso were collected at the conclusion of dox administration (10 days for Fgf10 overexpression (n = 5 controls, n = 5 mutants) and 1 month (n = 5 controls, n = 5 mutants) and 3 months (n = 3 controls, n = 3 mutants) for Fgf2b overexpression). Approximately 30 mg of tissue was saved in RNA later® (Sigma-Aldrich, St. Louis, MO) for qRT-PCR. The remaining tissue was fixed in 10% formalin and embedded in paraffin. Adult glandular stomach from wild type (C57Bl/6) mice (n = 3) was also fixed in 10% formalin and embedded in paraffin. All paraffin embedded tissue was serially sectioned at 5 μm intervals and mounted on slides for staining analysis.

RNA extraction and Quantitative RT-PCR (qRT-PCR)
RNA was isolated from the glandular stomachs of control and mutant mice using a Qiagen RNaseasy kit (Qiagen, Valencia, CA) in accordance with the manufacturer’s directions. A Nano Drop ND-1000 was used to determine the concentration of the purified RNA. RNA (1 μg) was reverse-transcribed using the iScript™cDNA Synthesis Kit (BioRad, Hercules, CA) in accordance with the manufacturer’s directions. 2μl cDNA were used for each of the qRT-PCR reactions using the primers and probes designed by the online Roche software: Probe finder version 2.20, https://www.roche-applied%1Escience.com/six/rtcpr/upl/adcr.jsp. The following primer and corresponding probes were used (forward 5'-3', reverse; 5'-3', probe #: Fgf10 CGGGGACGAGAATGGAAGACT, AACAAGTCCGGATTTCCACGTA; probe 8b; Fgf2b GAAAGGATACCGGCGCTTCC, AGACAGATGCTTTTCCTTGG, probe 108. All qRT-PCR reactions were performed with Roche FastStart TaqMan® Probe Master kits, according to the manufacturer’s instructions in a Roche Light Cycler 1.5 Real-Time PCR machine. Each reaction was run in triplicate and expression levels of genes of interest were normalized to levels of Gapdh.

Immunofluorescence staining
Slides were deparaffinized and rehydrated through a successive ethanol concentration to water. An antigen retrieval step was performed by boiling the slides in a microwave for 12 min in 10 mM sodium-citrate buffer pH = 6.0 (PCNA, GSII, CGA, IF,
H/K ATPase, HE4). Slides were blocked with universal blocking solution with 2% goat or donkey serum for 30 minutes, followed by incubation with the primary antibody diluted in universal blocking solution with 2% goat or donkey serum overnight at 4°C. Primary antibodies included: mouse anti-proliferating cell nuclear antigen (PCNA) (1:100, Vector Laboratories), rabbit anti-chromogranin A (CGA) (1:100, Abcam), goat anti-intrinsic factor (IF) (1:2000, a gift from Jason Mills, Washington University, St. Louis, MO), mouse anti-H/K ATPase β (H/K ATPase) (1:100, Abcam), and rabbit anti-HE4 (1:50, Abcam). Slides were then incubated with secondary antibody diluted in universal blocking solution for one hour at room temperature. Secondary antibodies included: Cy3 conjugated goat anti-rabbit or mouse (1:200), Cy3 conjugated donkey anti-goat (1:200), or lectin GS-II from Griffonia simplicifolia, Alexa Fluor® 488 Conjugate (GS-II) (1:2000, Invitrogen). Slides were mounted using Vectashield with DAPI (Vector Laboratories). Images were acquired using a color camera attached to an upright fluorescent microscope (Leica DM5500).

Immunohistochemistry

Slides were deparaffinized and rehydrated through a successive ethanol concentration to water. An antigen retrieval step was performed by boiling the slides in a microwave for 12 min in either Tris-EDTA (10 mM Tris Base, 1mM EDTA Solution, 0.05% Tween 20, pH = 9.0) (FGFR1 and FGFR2) or 10 mM sodium-citrate buffer pH = 6.0 (CDX2). Slides were then incubated with 15% H2O2 in 1x PBS for 15 minutes. Then slides were blocked with universal blocking solution with 2% goat serum for 30 minutes, followed by incubation with the primary antibody diluted in universal blocking solution with 2% goat serum overnight at 4°C. Primary antibodies used included: rabbit anti-FGFR1 (1:100, Flg (C-15) Santa Cruz), rabbit anti-FGFR2 (1:100, Bek (C-17) Santa Cruz) and rabbit anti-CDX2 (1:50, BioGenex, Fremont, CA). The Envision kit (Dako) was used to reveal the slides. Slides were incubated with labeled polymer-HRP for 30 minutes at room temperature. Slides were then revealed using DAB+ chromogen in substrate buffer for 5 minutes. Slides were then counter-stained with hematoxylin, dehydrated to 100% EtOH, cleared with xylene, and mounted with Permoun (Fisher Scientific, Fair Lawn, New Jersey). Images were acquired using a color camera attached to an upright fluorescent microscope (Leica DM5500).

Cell counting and quantification

Epithelial nuclei, PCNA-positive and H/K ATPase-positive stained cells were counted semi-automatically using MetaMorph 7.7.3.0 software (Molecular Devices, Sunnyvale, CA). First, the blue (DAPI) channel was processed as follows: background was subtracted by manual thresholding, intensity was normalized by dividing by a 20 x 20 low-pass filtered copy of the channel, and the normalized nuclei were smoothed with a 5 x 5 median filter to preserve edges. A binary image of the smoothed nuclei was created by thresholding automatically using the isodata histogram algorithm under visual inspection to manually adjust the threshold value if needed. Overlapping binary nuclei were separated by Watershed segmentation using FoveaPro 3.0 software (Reindeer Graphics, Asheville, NC). To count H/K ATPase-positive cells, the red (Cy3) channel was processed in the same manner as the blue channel except that a legacy heuristic algorithm was more appropriate for thresholding and an additional step to fill all dark holes was added just prior to Watershed segmentation to fill the unstained nuclei and prevent over-segmentation. To count PCNA-positive cells, the red (Cy3) channel was pre-processed in the same manner as for H/K ATPase-positive cells, except that the median filter kernel was 3 x 3. Binary objects were counted with the Integrated Morphometry Analysis function of MetaMorph. Object filters were set to exclude staining and processing artifacts from the counts, as well as mesenchymal nuclei: elliptical form factor (ratio of length to breadth) ≥ 3.0 and area ≥ 100 pixels for epithelial nuclei, ≥ 200 pixels for H/K ATPase-positive cells, and ≥ 40 pixels for PCNA-positive cells. GSII, CGA and IF-positive stained cells were counted by hand using ImageJ software [http://rsweb.nih.gov/ij/].

The total number of PCNA, GSII, CGA, IF, and H/K ATPase positive cells and the total number of epithelial cells were counted separately for 3 randomly selected high power fields (HPF = 20x magnification) in the corpus of a single mouse and averaged. Specifically, five mutant and five control mice were counted for all FGFR1 overexpression, and for FGFR2b overexpression induced for one month. For the FGFR2b overexpression induced for three months, we analyzed three mutants and three controls. The total number of cell-type positive cells per the total number of epithelial cells, or percent cell-type positive was calculated for each sample.

Statistical Analysis

Data are presented as the mean±standard error of the mean (SEM). Statistical analysis was performed using a one-tailed two-sample equal variance (homoscedastic) Student’s t-test (Excel, Microsoft). P-values <0.05 were considered significant.

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Author Contributions

Conceived and designed the experiments: ALS FGS DA HRF SB TG. Performed the experiments: ALS FGS DA. Analyzed the data: ALS FGS. Contributed reagents/materials/analysis tools: ALS FGS DA HRF SB TG. Performed the experiments: ALS FGS DA. Analyzed the data: ALS FGS. Contributed reagents/materials/analysis tools: ALS FGS DA HRF SB TG. Wrote the paper: ALS FGS DA HRF SB TG.
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