Introduction

In adult mammals, the epithelium of the small intestine comprises two structures: finger-like villi and pocket-like crypts of Lieberkuhn. Intestinal stem cells are located at the bottom of the crypt. Crypts also contain transit amplifying progenitor cells. These proliferating cells differentiate, then migrate to villi and are removed at the top of the villi by apoptosis. There are four cell lineages that derive from intestinal stem cells: the non-secretory absorptive enterocytes, and the secretory cells, which include mucous-secreting goblet cells, regulatory peptide-secreting enteroendocrine cells and antimicrobial peptide-secreting Paneth cells. Although fibroblast growth factor (Fgf) signaling is important for cell proliferation and differentiation in various tissues, its role in intestinal differentiation is less well understood.

Abstract

Background: There are four cell lineages derived from intestinal stem cells that are located at the crypt and villus in the mammalian intestine the non-secretory absorptive enterocytes, and the secretory cells, which include mucous-secreting goblet cells, regulatory peptide-secreting enteroendocrine cells and antimicrobial peptide-secreting Paneth cells. Although fibroblast growth factor (Fgf) signaling is important for cell proliferation and differentiation in various tissues, its role in intestinal differentiation is less well understood.

Methodology/Principal Findings: We used a loss of function approach to investigate the importance of Fgf signaling in intestinal cell differentiation in zebrafish; abnormal differentiation of goblet cells was observed when Fgf signaling was inhibited using SU5402 or in the Tg(hsp70ldnfgfr1-EGFP) transgenic line. We identified Fgfr2c as an important receptor for cell differentiation. The number of goblet cells and enteroendocrine cells was reduced in fgfr2c morphants. In addition to secretory cells, enterocyte differentiation was also disrupted in fgfr2c morphants. Furthermore, proliferating cells were increased in the morphants. Interestingly, the loss of fgfr2c expression repressed secretory cell differentiation and increased cell proliferation in the mib^{a52b} mutant that had defective Notch signaling.

Conclusions/Significance: In conclusion, we found that Fgfr2c signaling derived from mesenchymal cells is important for regulating the differentiation of zebrafish intestine epithelial cells by promoting cell cycle exit. The results of Fgfr2c knockdown in mib^{a52b} mutants indicated that Fgfr2c signaling is required for intestinal cell differentiation. These findings provide new evidences that Fgf signaling is required for the differentiation of intestinal cells in the zebrafish developing gut.
proliferated cells and increase differentiation into goblet cells in Apcmin mice [10].

Fibroblast growth factor (Fgf) signaling is involved in intestinal development and cell differentiation. There are 22 Fgf6 and 4 Fgfs in mice [19,20]. Fgf1~3 has two isoforms, a and c, which result from alternative splicing. These two isoforms have different ligand-binding specificities [21]. Fgft10 signaling is required, in a dose-dependent manner for the survival and proliferation of colonic epithelia progenitor cells [22]. Overexpression of Fgf10 can attenuate stomach and duodenum cell differentiation [23,24]. Goblet cells, but not Paneth cells or enteroendocrine cells, were increased in recombinant FGF7 protein treated rats [25]. Furthermore, the depth of the crypt and the numbers of proliferating cells were increased in Fgf3 deficient mice but villi length and the distribution of differentiated intestinal cells were unaffected [26]. Nevertheless, a recent report indicated that Paneth cell differentiation is reduced in Fgf3 deficient mice [27]. These evidences suggest that the Fgf signaling pathway has a regulatory role in cell differentiation in the gastrointestinal tract. However, few reports address how Fgf signaling controls intestinal cell differentiation.

Zebrafish offer many advantages for studying intestinal cell differentiation, they have rapid development and transparent embryos, and techniques for the manipulation their gene expression are well-established. Furthermore, the early-stage development of the zebrafish gastrointestinal tract has been well described [5,28,29]. Using Tg(hsp70:dnfgfr1-EGFP) transgenic fish to inhibit Fgf signaling [30], we found that heat-shock treatments inhibited the differentiation of secretory cells. We further found that Fgfr2c signaling controls the differentiation of secretory and absorptive cells by regulation of progenitor cell proliferation. These findings provide new evidence that Fgf signaling is required for the differentiation of intestinal cells in the zebrafish developing gut.

Materials and Methods

Ethics Statement

All embryos were handled according to protocols approved by the Institutional Animal Care and Use Committee of Tzu Chi University, Hualien, Taiwan (approval ID: 99056).

Zebrafish

Zebrafish (Danio rerio) were raised as described in the Zebrafish Book [31]. The AB wild type (WT) strain was used for morpholino injection and other experiments. The transgenic and mutant fish lines used in this study were Tg(hsp70:dnfgfr1-EGFP) [30], provided by the Taiwan Zebrafish Core Facility at Academia Sinica, TzCAS, and mib (mib25), respectively.

SU5402 Treatment and Heat Induction Experiments

SU5402 (Calbiochem, UK) was dissolved into DMSO. Three days post fertilization (dpf) embryos were placed in a 6-well plate and SU5402 solution (2.5 or 3.4 μM) was added. We used 0.25% DMSO solution as control. At 4 dpf, the solution was replaced with fresh solution of the same concentration. All treated embryos were harvested at 5 dpf.

Water was pre-warmed to 37°C. Zebrafish embryos received a single 37°C heat-shock treatment for one hour in an air incubator, and the water then left to cool to 28°C.

Microinjection

All morpholinos (MOs) were obtained from Gene Tools, LLC. MOs targeting fgf2b, fgfr2c, fgfr4, and with a 5-base mismatch for fgf2c MO were used in this study. MOs sequences were as follows:

- fgf2b MO 5′-CGCTTCACGTCTTTACCTGATG-3′
- fgfr2c MO 5′-AAGCAGTGGGAAGTGTATACC-3′, 5-base mismatch for fgfr2c MO 5′-AAGCAGTGGGAAGTGTATACC-3′ [32];
- fgfr4-I1E2 MO 5′-ATATCTGTGGGATTAAAAATGGG-3′ [33]. MOs (4 ng) were injected into 1–2 cell stage embryos.

RT-PCR and qPCR Analyses

The whole gut was collected at 5 dpf using #5 forceps for RNA extractions. Total RNA was extracted using TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA). DNase-I treated total RNA (5 μg) was used to generate cDNA using a ImProm-II kit (Promega, Madison, WI, USA). PCR primers used in this study are listed in Table S1. For qPCR, reverse transcription reaction was performed using a GoScript kit (Promega, Madison, WI, USA). Expression level of pepT1 was detected according to the published primer sequences [34]. Expression of β-actin1 was detected as an internal control [35]. The primer sequence for pepT1 was F-AACACAAACATCAAAGCAAACC and R-AACTACCAACCTCTAACGCC. The primer sequence for β-actin1 was F-GCTGTTTTTCGCCCTCATTGGT and R-TCCCATGCGCAACATGACT. The Maxima SYBR Green qPCR Master Mix (Fermentas International Inc., Ontario, Canada) system was used to quantify the transcripts.

Whole Mount in situ hybridization

The following in situ probes were used: ngn2, glucagon, and pepT1 [34]; fgfr1–4 [36], and ifabp. The ifabp fragment was amplified by PCR and subcloned into pGEM-T vector (Promega). The DIG-labeled probes were generated by in vitro transcription using a DIG RNA labeling kit (Roche). For whole mount in situ hybridization, DIG-labeled probes were used to hybridize the embryos overnight at 65°C, and were then washed under high stringency condition. Embryos were treated with blocking buffer (Roche) and incubated with AP-conjugated anti-DIG antibody overnight at 4°C (1:2000, Roche). Embryos were washed to remove excess antibody and then colored with NBT/BCIP (Roche).

Whole Mount Wheat Germ Agglutinin (WGA), Immunofluorescence Staining and BrdU Labeling

For WGA staining, embryos were fixed in 4% paraformaldehyde at 4°C overnight, and the yolks were removed before staining. Fixed embryos were washed three times for 5 min with PBST (containing 0.3% TritonX-100) and buffer B (10 mM HEPES and 0.15 M NaCl, pH = 7.5), and then incubated with rhodamine conjugated WGA (Vector Laboratories, 1:100, in buffer B) at 4°C overnight. After washing with PBST (four times for 30 min), embryos were mounted in SlowFade Gold anti-fade reagent with DAPI (Molecular Probes, S-36938). We counted the WGA positive cells for all the embryos using ImageJ software.

For WGA and 2F11 double labeling, embryos were blocked in PBST, containing 4% BSA, for one hour after WGA staining. Embryos were then incubated in mouse monoclonal 2F11 antibody (1:1000, a gift from Dr. Julian Lewis) at 4°C overnight. After washing with PBST as described above, embryos were incubated in goat anti-mouse IgG Alexa Fluor 488 (Molecular Probes, A-11029, 1:100) at 4°C overnight. After washing with PBST, embryos were rinsed with PBS three times, and the embryos were nuclear counterstained with TOPRO-3 (Molecular
We examined whether the differentiation of enteroendocrine and goblet cells was affected in Fgf signaling inhibited embryos, by using 2F11 antibody to detect secretory cells [17]. After whole mount immunostain using 2F11 antibody, WGA staining, and Topro-3 nuclear counter staining, embryos were analyzed by confocal microscopy. Scattered 2F11 positive cells were present in WT controls, particularly in the intestinal bulb (Fig. 1 M and 1 M'). As expected, goblet cells were present in the mid-intestine (Fig. 1 M and 1 M'). After heat treatment of embryos from Tg(hsp70:dnfgfr1-EGFP), scattered 2F11 positive cells were reduced in number (Fig. 1 N and 1 N'). Goblet cell numbers were greatly reduced (Fig. 1 N and 1 N'). We further quantified the proportion of 2F11 and WGA positive cells in total intestinal epithelia. In control embryos, 9.8±1.6% of cells were positive for 2F11. Fewer 2F11 positive cells were observed in Fgf signaling inhibited embryos (7.0±1.8%, P<0.001; Fig. 1 O). For goblet cells, 4.0±1.1% of WGA positive cells were present in Fgf signaling inactivated embryos, but were fewer than 6.4±1.4% in control embryos (P<0.001; Fig. 1 O). These observations indicate that Fgf signaling is important for intestinal secretory cell differentiation.

**Fgf2c Signaling is Required for Secretory Cell Differentiation**

There are five fgf receptor genes, fgf1a, fgf1b, fgf2, fgf3, and fgf4, in zebrafish [36,44]. To identify receptors important for intestinal cell differentiation, we used RT-PCR to analyze fgfs expression in the whole gut, including the esophagus, intestine, liver, and pancreas at 5 dpf. Except for fgf1b, four receptors were detected in gut cDNA (Fig. S1A). We used whole mount in situ hybridization (WISH) to analyze the temporal and spatial expression pattern of the four receptors. Expression of fgf1a, fgf2, and fgf4 were detected in the intestine at 3 dpf (Fig. 2 A', B' and C'). To analyze the cellular localization of these receptors, specimens were sectioned in paraffin. The fgf1a and fgf2 genes were expressed in the mesenchymal and epithelial layers of the intestinal bulb, mid-intestine, and posterior intestine at 5 dpf (Fig. 2 A'-2A''' and 2 B'-2B''''), although many signals were present in mesenchymal cells of the esophagus at 5 dpf (inset in Fig. 2 G'). Expression of fgf1a was no longer expressed in intestine but in pancreatic islet at 5 dpf (Fig. 2 E'-2H''', data not shown). Only fgf2 and fgf4 were expressed in the intestine at 5 dpf (Fig. 2 F' and 2 H'). The fgf2 was strongly expressed in intestinal mesenchymal cells, but not in epithelium cells at 5 dpf (Fig. 2 F'–2H''' , arrows). Expression of fgf4 was more intensive in the intestinal epithelium at 5 dpf than they were at 3 dpf (Fig. 2 F'–2H''').

Because fgf2 was the only receptor that was strongly expressed in the intestine at both 3 and 5 dpf, we analyzed whether intestinal cell differentiation was disrupted in Fgf2 signaling inactive embryos. Two fgf2 isoforms, fgf2b and fgf2c, have been identified [36]. We used morpholino antisense oligonucleotides to knockdown gene expression in zebrafish embryos. Two morpholinos, which respectively, target fgf2b and fgf2c, have been used by us to study zebrafish embryo development. One fgf2c-3 mm morpho, which contained 5 mismatch bases in fgf2c morpholino sequence, was used as a control [32]. We studied intestinal cell differentiation in the morpholino-injected morphants. The number of goblet cells was reduced in fgf2b morphants compared to WT (Fig. 3 A–3F). Moreover, goblet cell differentiation was almost completely absent from the fgf2c morphants (Fig. 3 G–3 I). Goblet cell was normally differentiated in fgf2c-5 mm morphants (Fig. 3 J–L).

We quantified the proportion of secretory cell in fgf2b and fgf2c morphants after 2F11 immunostaining and WGA staining. Goblet cell numbers were slightly reduced in the fgf2b morphants, similar
to our WGA staining results (Fig. S1B–1D; 5.4 ± 1.45% in WT and 3.7 ± 1.05% in morphants, P < 0.001). The percentage of 2F11 positive cells was not reduced in fgfr2b morphants (Fig. S1B–1D; 10.9 ± 2.33% in WT and 11.4 ± 1.64% in morphants, P = 0.7995).
Figure 2. The expression pattern of zebrafish fgfr genes. The expression of fgfr1a-4 was analyzed by WISH: (A–D) at 3 dpf and (E–H) at 5 dpf. WISH embryos were sectioned and analyzed for expression of fgfr1a-4 in: (A′–H′) the intestinal bulb, (A″–H″) the mid-intestine, and (A‴–H‴) the posterior intestine. Black arrows indicated the fgfr2 expression in mesenchymal cells. Scale bars = 200 μm. doi:10.1371/journal.pone.0058310.g002
In comparison with fgfr2b morphants, secretory cell differentiation was more significantly inhibited in fgfr2c morphants. Goblet cells were greatly reduced in number in fgfr2c morphants (Fig. 3M–3P; 1.37 ± 1.21% in fgfr2c morphants, 5.72 ± 1.00% in fgfr2c-5 mm morphants, and 5.25 ± 1.06% in WT, P < 0.001). The amount of 2F11 positive cells was reduced in fgfr2c morphants compared with...
control embryos (Fig. 3M–3P; 8.32 ± 2.41% in fgfr2c morphants, 10.34 ± 1.97% in fgfr2c-5 mm morphants, and 12.08 ± 1.58% in WT, P < 0.001). Consistent with the results for Tg(hsp70:dnfgfr1-EGFP) embryos, these findings indicated that Fgfr2c signalling is important for secretory cell differentiation.

The loss of WGA signal might also be resulted from downregulation of mucin production. We used a goblet cell specific marker gene, anterior gradient 2 (agr2) to analyze goblet cell differentiation [45,46]. The WISH results showed that agr2 expressing cells were presented in mid-intestine of control embryos (Fig. 4A and 4C). In fgfr2c morphants, agr2 expressing cells were dramatically reduced in number (Fig. 4B). These results indicated that goblet cell differentiation was inhibited in fgfr2c morphants.

We further used glucagon probe to analyze the differentiation of enteroendocrine [5,34,47]. The signal could be detected in pancreatic alpha cells in fgfr2c morphants and control embryos (Fig. 4D–4F). In contrast, glucagon-expressing cells were greatly reduced in intestine of fgfr2c morphants compared with control embryos (Fig. 4D–4F, arrow). Taken together, the differentiation of both secretory cell types was disrupted in fgfr2c morphants.

**Enterocyte Differentiation was Disrupted in fgfr2c Morphants**

We wondered whether the differentiation of absorptive cells was normal in fgfr2c morphants. We analyzed the expression of the gene for intestinal fatty acid binding protein ifabp, and oligopeptide transporter pepT1. These two genes were expressed in the differentiated enterocytes [34]. According to the expression pattern in 5 dpf embryos, three different levels of pepT1 and ifabp gene expression were classified (Fig. 5A–5F): level 1; expression in the whole intestinal bulb (normal; Fig. 5A and 5D). Level 2; expression in the whole intestinal bulb, but at reduced levels (mild; Fig. 5B and 5E). Level 3; expression only in the anterior intestinal bulb or no expression (severe; Fig. 5C and 5F). The ifabp expression level was normal in all WT embryos (Fig. 5H). For pepT1 expression, 91.7% WT embryos were normal. However, 17.4% expressed mild and 0.9% expressed severe patterns (Fig. 5H). The expression of pepT1 and ifabp in fgfr2c-5 mm morphants was similar to that of WT embryos (Fig. 5H). However, severe reduction of the enterocyte population was found in most fgfr2c morphants. We observed 14.8% morphants with normal, 16.4% morphants with mild, and 68.8% morphants exhibiting severe ifabp expression pattern. Reduction in pepT1 expression was even more obvious. Only 1.1% morphants exhibited the normal pepT1 pattern. Mild and severe patterns were observed in 5.3% and 91.6% morphants, respectively (Fig. 5H). The results of qPCR also showed the expression level of pepT1 was reduced in the fgfr2c morphants compared with control embryos (Fig. 5A). Therefore, we conclude that the differentiation of absorptive enterocytes was also affected in fgfr2c morphants.

**Figure 4. The expression of agr2 and glucagon in fgfr2c morphants.** WISH of agr2 (A–C, lateral view) and glucagon (D–F, ventral view) were used to analyze goblet cell and enteroendocrine differentiation, respectively, in 5 dpf embryos. Black arrows indicated the goblet cells (gc) and red arrows indicated the enteroendocrine cells (ee). p: Pancreatic alpha cells. Scale bars = 50 μm.

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Proliferating Cells were Increased in \textit{fgfr2c} Morphants

Because differentiation of both secretory and absorptive cells in the intestine was disrupted, we wondered whether cell death was altered in \textit{fgfr2c} morphants. TUNEL assay indicated that only very few cell deaths occurred in 5 dpf WT embryos, and \textit{fgfr2c} and \textit{fgfr2c-5 mm} morphants (0.2±0.3%, 0.2±0.2%, and 0.3±0.3%, respectively; Fig. 6). Thus, the observed reduction in cell differentiation was not caused by cell death.

We analyzed the cell proliferation status in \textit{fgfr2c} morphants. BrdU incorporation was used to detect the proportion of S-phase cells, and H3P antibody was used to detect M-phase cells, in 3 and 5 dpf embryos. Normally proliferating cells are densely scattered throughout the intestine at 52–74 hours post fertilization (hpf) and reduced in number at 74–120 hpf [5]. We observed 45.9±5.3% S-phase, and 5.6±1.6% M-phase cells in the intestine of WT embryos at 3 dpf (Fig. 7A and 7G). The proportion of cycling cells present in \textit{fgfr2c}-5 mm morphants at 3 dpf was similar to that for WT (S phase: 46.9±5.1%, and M phase: 5.6±1.3%; Fig. 7C and 7I). The percentages of proliferating cells in \textit{fgfr2c} morphants were similar to those of the control embryos at 3 dpf (S phase: 47.6±5.8% and M phase: 4.9±1.6%; Fig. 7B, 7H, and 7M). The cycling cells of WT embryos were significantly reduced in number at 5 dpf (S phase: 4.8±3.4% and M phase: 1.9±0.8%; Fig. 7D and 7J). A similar reduction occurred in \textit{fgfr2c}-5 mm morphants at 5 dpf (S phase: 9.6±4.2% and M phase: 0.8±0.3%; Fig. 7F and 7L). Although cycling cells were reduced at 5 dpf compared to 3 dpf in \textit{fgfr2c} morphants, the proportion of proliferating cells was still much greater than that of control embryos (S phase: 26.2±10.3% and M phase: 4.1±1.3%, \(P<0.001\); Fig. 7E, 7K, and 7M). In summary, cycling cells were unchanged at 3 dpf but increased in number at 5 dpf after inhibition of Fgfr2c signaling.

Disruption of Cell Differentiation and Proliferation in \textit{mibta52b} Mutants with \textit{fgfr2c} Knockdown

It seemed likely that proliferating progenitor cells in \textit{fgfr2c} morphants could not exit from cycling progression to undergo terminal differentiation. To test this hypothesis, we used the \textit{mibta52b} mutant to investigate the cell differentiation and proliferation. It was reported that Delta-Notch signaling is inhibited in \textit{mibta52b} mutant fish and differentiation of almost all intestinal cells is toward the secretory cell lineage [17,47]. If Fgfr2c signaling were important for cell cycle exit and terminal cell differentiation, then 2F11 positive secretory cells should be reduced and M-phase cycling cells should be increased in \textit{mibta52b} mutants injected with \textit{fgfr2c} morpholino. We observed a large ratio of 2F11 positive cells in \textit{mibta52b} and \textit{mibta52b} mutants injected with \textit{fgfr2c} morpholino at 5 dpf (50.5±6.8% and 54.6±9.3%, respectively; Fig. 8A and 8C). Less than one percent of H3P positive cycling cells could be detected in \textit{mibta52b} and \textit{mibta52b} mutants injected with \textit{fgfr2c} morpholino at 5 dpf (0.5±0.5% and 0.3±0.5%, respectively; Fig. 8A and 8C). There was a large reduction in secretory cells and an increased number of cycling cells in \textit{mibta52b} mutants injected with \textit{fgfr2c} morpholino compared with control embryos at 5 dpf (2F11:20.7±12.5%, \(P<0.001\); H3P: 1.6±1.2%, \(P<0.001\); Fig. 8B and 8D). We noticed that these H3P positive cells were not co-localized with the 2F11 signal. This indicated...
that proliferating cells were not differentiated secretory cells, but that they might be progenitor cells. Therefore, it is likely that intestinal proliferating progenitor cells cannot exit from cell cycling and are unable to differentiate further, in embryos that lack Fgfr2c signaling.

**Discussion**

Intestinal progenitor cells are highly proliferative. It is important to understand what signaling factor(s) control these cycling cells’ differentiation to various mature intestinal epithelial cells, and how this is accomplished. In this study, we noted a novel role of Fgfr2c signaling in the gut epithelium. In the absence of Fgfr2c signaling, the differentiation of secretory and absorptive cells was inhibited, and cell proliferation was increased. Inhibition of Fgfr2c signaling in miz1a2b mutant embryos showed a similar effect. These results suggest that Fgfr2c signaling promotes progenitor cell’s exit from cell cycling, and their differentiation to various intestinal cell types.

In mice, Fgfr1–3 are expressed in the developing gastrointestinal tract, including the forestomach, small intestine, colon, and cecum [22,24,27,48,49,50]. Although few reports indicate that Fgfr4 is expressed in gut tissue during developmental stages in mice, we found that Fgfr4 in the stomach and intestine using the Genepaint database, which contains in situ hybridization data [51]. Fgfr2b−/− mice exhibit decreased differentiation gastric mucosa, and reduced numbers of proliferating epithelial cells [52]. Fgfr3−/− mice showed significantly reduced numbers of clonogenic stem cells and Paneth cells in the small intestine [27]. However, we found no reports that either Fgfr1 or Fgfr3 signaling regulates intestinal development and cell differentiation in mice.

We used zebrafish to study the function of Fgf signaling in the gut. Only fgfr2 and fgfr4 genes were strongly expressed in the intestine at 5 dpf, in which most of the observed cell differentiation occurred. In zebrafish, the fgfr1 gene is duplicated to fgfr1a and fgfr1b [44]. RT-PCR did not reveal any evidence for the expression of fgfr1b at 5 dpf. Although fgfr1a was expressed in intestinal cells at 5 dpf, we did not detect its expression in intestinal mesenchymal or epithelial cells at 5 dpf. The fgfr3 gene was not expressed in intestinal cells at 3 and 5 dpf. These expression patterns, and that for fgfr3 in particular, were different from those reported for mice [27,30].

In this study, we observed expression of fgfr4 in the intestinal epithelial cells at both 5 dpf and adult fish (Fig. 2 and Fig. S2B). We administered fgfr4 morpholino to verify whether or not Fgfr4 signaling affected cell differentiation. The results of fgfr4 WISH showed that loss of fgfr4 expression does not disrupt intestinal cell differentiation (data not shown). Enterocyte expression of Fgfr4 is important for bile acid homeostasis [53]. Thus, we suggest that Fgfr4 signaling might have the similar function in the zebrafish intestine.

The expression of the two Fgfr2 isoforms is highly regional specific. Fgfr2b and Fgfr2c are limited to epithelial and mesenchymal cells, respectively [54]. The fgfr2 probe we used was full length over 4 kb cDNA for fgfr2 [36]. However, this probe was not specific to fgfr2c because there is only a 140 base difference between fgfr2b and fgfr2c in the full length region. We found that zebrafish fgfr2 was expressed in both epithelial and mesenchymal cells of the intestine at 3 dpf, but that it was only expressed in intestinal mesenchymal cells at 5 dpf. This indicated that fgfr2b might only express in epithelial cells at 3 dpf, and that expression of fgfr2c is sustained in mesenchymal cells from 3 to 5 dpf. The fgfr2 signal was restricted in mesenchymal cells of adult intestine tissue (Fig. S2A). We concluded that the major fgfr2 isoform expressed in zebrafish intestine was fgfr2c, and that this gene specifically expressed in mesenchymal cells. However, Fgfr2b is reported to be the major isoform expressed in the developing gastrointestinal tract in mice [24,48,49]. Thus, Fgfr2 signaling may...
regulate intestinal cell proliferation and cell differentiation in different ways. In mice, Fgfr2b signaling acted autonomously in intestinal epithelium cells. Because \textit{fgfr2c} was expressed in the mesenchymal cells of zebrafish intestine, other paracrine signal(s), regulated directly or indirectly by Fgfr2c may exist to control epithelial differentiation.

Mesenchymal-epithelial interactions are important for intestinal cell proliferation and differentiation. Many signaling factors are associated with these interactions, including Hedgehog (Hh) and Bmp factors [1]. The expression of \textit{Ihh} and \textit{Shh} could be detected in small intestinal epithelial cells and their receptors were observed in intestinal mesenchymal cells of mice; when Hh signaling was disrupted, the small intestinal epithelial cells were hyperproliferative, and these proliferating cells were negative for enterocyte differentiation [35]. Furthermore, with the exception of the proliferating zone, \textit{Bmp4} was expressed in the mesenchymal layer of the small intestine, and \textit{Bmpra} was expressed in the epithelial cells. In \textit{Bmpra} conditional knockout mice, proliferating cells were generally increased in number [14]. In zebrafish, mesenchymal-epithelial interactions also regulate gut epithelial development. \textit{MiR-145} is expressed in gut mesenchymal cells in zebrafish, while mesenchymal expressed \textit{miR-145} directly controls \textit{gata6} expression in the gut [56]. Recently reports suggest that \textit{miR-145} regulates paracrine signaling to control gut development [57]. We found that \textit{fgfr2c} is expressed in the mesenchymal layer, thus, some Fgf ligands must exist in the epithelial or mesenchymal layers of the zebrafish gut. Using RT-PCR, we identified putative Fgfr2c ligands (\textit{fgf1}, \textit{fgf2}, \textit{fgf4}, \textit{fgf17} and \textit{fgf24}) that were expressed in 5 dpf zebrafish gut (data not shown), but we still do not know which specific Fgf ligands interact with Fgfr2c.

Wnt signaling is critical for intestine development. When Wnt ligands are present, cytosolic $\beta$-catenin proteins are not degraded by the destruction complex. Stable $\beta$-catenin proteins then translocate into the nucleus and bind with \textit{T} cell factor (Tcf) transcription factors. This newly formed complex has transcriptional activity and can drive Wnt signaling target genes, including $\textit{c-Myc}$ and $\textit{cyclin D}$, to promote cell cycle progression [6,9]. In the \textit{fgfr2c} morphant intestine, there were more proliferating cells present than there were in control embryos. We used $\beta$-catenin antibody to verify whether Wnt signaling is activated in the

![Figure 7. Cell proliferation in \textit{fgfr2c} morphants.](https://www.plosone.org/figure/7/fgfr2c-morphants)
intestinal epithelial cells of \textit{fgfr2c} morphants; only membrane accumulated \( \beta \)-catenin was detected, and there were no differences in the \( \beta \)-catenin localization pattern between \textit{fgfr2c} morphants and the control embryos (data not shown). Thus, we still do not know whether Wnt signaling is activated in intestinal epithelial cells of \textit{fgfr2c} morphants.

In conclusion, we found that Fgfr2c signaling derived from mesenchymal cells is important for regulating the differentiation of zebrafish intestine epithelial cells by promoting cell cycle exit. The results of Fgfr2c knockdown in \textit{mibta52b} mutants indicated that Fgfr2c signaling is required for intestinal cell differentiation. However, the mechanism by which mesenchymal cells control the behaviors of epithelial cell needs investigation. Furthermore, although the expression of \textit{fgfr2} could be detected in the mesenchymal cells of adult zebrafish intestines, we do not know whether Fgfr2c signaling directs differentiation in adult fish.

Supporting Information

Figure S1 The expression of \textit{fgfrs} and the secretory cell differentiation of \textit{fgfr2b} morphants. (A) \textit{fgfr1a}, \textit{fgfr2}, \textit{fgfr3} and \textit{fgfr4} gene were analyzed in 5 dpf zebrafish gut tissue by RT-PCR. (C) WT embryos and (D) \textit{fgfr2b} morphants were double labeled using 2F11 antibody and WGA. The magnified image shows (C’–D’) enteroendocrine cells and (C”–D”’) goblet cells. DAPI was used for nuclear counter staining (blue). The bar charts show the percentages of 2F11 and WGA positive cells. Error bars indicate SD. Scale bar = 50 \( \mu \)m.

Figure S2 The expression of \textit{fgfr2} and \textit{fgfr4} in adult zebrafish intestine. Section in situ hybridization was used to analyze the expression of \textit{fgfr2} and \textit{fgfr4} genes. (A) \textit{fgfr2} was detected in the lamina propria, and (B) \textit{fgfr4} was expressed mainly in the epithelial layer of the intestine. Scale bar = 50 \( \mu \)m.

Table S1 Primer list for RT-PCR analysis.

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Author Contributions

Conceived and designed the experiments: DWL, WPW. Performed the experiments: DWL, SMT. Analyzed the data: DWL, WPW. Contributed reagents/materials/analysis tools: BFL, YJJ. Wrote the paper: DWL, WPW.

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