Zebrafish Krüppel-Like Factor 4a Represses Intestinal Cell Proliferation and Promotes Differentiation of Intestinal Cell Lineages

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Abstract

Background: Mouse krüppel-like factor 4 (Klf4) is a zinc finger-containing transcription factor required for terminal differentiation of goblet cells in the colon. However, studies using either Klf4−/− mice or mice with conditionally deleted Klf4 in their gastric epithelia showed different results in the role of Klf4 in epithelial cell proliferation. We used zebrafish as a model organism to gain further understanding of the role of Klf4 in the intestinal cell proliferation and differentiation.

Methodology/Principal Findings: We characterized the function of klf4a, a mammalian klf4 homologue by antisense morpholino oligomer knockdown. Zebrafish Klf4a shared high amino acid similarities with human and mouse Klf4. Phylogenetic analysis grouped zebrafish Klf4a together with both human and mouse Klf4 in a branch with high bootstrap value. In zebrafish, we demonstrate that Klf4a represses intestinal cell proliferation based on results of BrdU incorporation, p-Histone 3 immunostaining, and transmission electron microscopy analyses. Decreased PepT1 expression was detected in intestinal bulbs of 80- and 102-hours post fertilization (hpf) klf4a morphants. Significant reduction of alcian blue-stained goblet cell number was identified in intestines of 102- and 120-hpf klf4a morphants. Embryos treated with γ-secretase inhibitor showed increased klf4a expression in the intestine, while decreased klf4a expression and reduction in goblet cell number were observed in embryos injected with Notch intracellular domain (NICD) mRNA. We were able to detect recovery of goblet cell number in 102-hpf embryos that had been co-injected with both klf4a and Notch 1a NICD mRNA.

Conclusions/Significance: This study provides in vivo evidence showing that zebrafish Klf4a is essential for the repression of intestinal cell proliferation. Zebrafish Klf4a is required for the differentiation of goblet cells and the terminal differentiation of enterocytes. Moreover, the regulation of differentiation of goblet cells in zebrafish intestine by Notch signaling at least partially mediated through Klf4a.

Introduction

Mammalian intestinal epithelium undergoes constant proliferation, differentiation, and death. Small intestinal epithelium is composed of crypts of Lieberkuhn and villi [1]. Crypts of Lieberkuhn are pocket-like invaginations into the gut mucosa where proliferative stem cells reside to provide renewal of various differentiated intestinal cells. Differentiated intestinal cells will migrate to villi which are finger-like projections extending into the intestinal lumen. Villi are composed of three types of differentiated intestinal cells: enterocytes can absorb nutrients, goblet cells can secrete mucus as a protective barrier, and enteroendocrine cells can produce different gastrointestinal hormones to regulate growth, repair, and motility of gut epithelium. A fourth type of mature intestinal cells, Paneth cells are located at the crypt base and generate antibacterial peptides [2].

Intestinal stem cell maintenance and cell fate specification have been demonstrated to be regulated by several paracrine signaling pathways and different transcription factors [3]. Krüppel-like factors (KLFs) are evolutionary conserved zinc finger-containing transcriptional factors that regulate a variety of biological processes such as apoptosis, proliferation, differentiation, and development [4]. Among them, Klf4 (also known as GKLF or EZF) mRNA is abundantly expressed in the skin and in epithelial cells of the esophagus, stomach, and colon of newborn mice [5]. Klf4 can function as activator or repressor depending on whether the interacting protein is a co-activator such as CREB-binding protein-related protein (p300/CPB) or co-repressor such as histone deacetylase 3 (HDAC3) [6]. Due to its transactivating capability, Klf4 was shown to function in cell cycle regulation by blocking G1/S progression [7,8]. In response to DNA damage, Klf4 mediated the transactivating effect of p53 on the p21/Cip1/Cip1.
promoter to cause G1/S cell cycle arrest and prevented cell entry into mitosis by repressing cyclin B1 promoter activity [9,10]. Reduced klf4 expression was detected in a number of colorectal cancer cell lines which was attributed to either hemizygous deletion of klf4 gene, hypermethylation of 3' untranslated region or point mutation in the open-reading frame [11]. Decreased klf4 expression was also identified in intestinal adenomas of multiple intestinal neoplasia mice and in colonic adenomas of familial adenomatous polyposis patients [12]. Furthermore, overexpression of klf4 in the human RKO colon cancer cell lines resulted in reduced tumorigenicity, suggesting klf4 is a tumor suppressor gene in colorectal cancer [13]. However, klf4 was also found to be overexpressed in squamous cell carcinoma and was implicated to be potent oncogene [14]. Subsequent studies indicated that Klf4 can switch from a tumor suppressor to an oncogene, depending on the level of p21 in the cell [15,16].

In addition to regulating cell proliferation, Klf4 was shown to modulate the differentiation of various tissues including gastrointestinal tract. Klf4 was shown to regulate expressions of enterocyte differentiation marker gene intestinal alkaline phosphatase and zip4 gene encoding for zinc transporter [17,18]. In klf4+/− mice, significant decrease in the number of goblet cells in the colon was detected on postnatal day1 [19], indicating Klf4 is required for terminal differentiation of goblet cells in the colon. However, other cell types including colonocytes and enteroendocrine cells, which undergo normal maturation and normal cell proliferation was detected in the colon of klf4+/− mice. In contrast, conditional deletion of klf4 gene caused aberrant expression of acidic mucins and TFF2/SP-positive cells marked as premalignant gastric cancer and increased cell proliferation in adult stomach [20].

Notch and Wnt signaling have been implicated in the stem cell regulation and the differentiation of intestinal epithelium [3]. Using γ-secretase inhibitor and promoter assay, it was shown that klf4 expression is inhibited by Notch signaling which controls goblet cell differentiation in mouse gastrointestinal tract [21]. Many colorectal cancers occur under the condition that the presence of mutation in the Wnt pathway and down-regulation of Klf4. A cross talk between Klf4 and Notch and Wnt signaling have been implicated in the stem cell regulation and the differentiation of various tissues including gastrointestinal tract. Klf4 was shown to regulate expressions of enterocyte differentiation marker gene intestinal alkaline phosphatase and zip4 gene encoding for zinc transporter [17,18]. In klf4+/− mice, significant decrease in the number of goblet cells in the colon was detected on postnatal day1 [19], indicating Klf4 is required for terminal differentiation of goblet cells in the colon. However, other cell types including colonocytes and enteroendocrine cells, which undergo normal maturation and normal cell proliferation was detected in the colon of klf4+/− mice. In contrast, conditional deletion of klf4 gene caused aberrant expression of acidic mucins and TFF2/SP-positive cells marked as premalignant gastric cancer and increased cell proliferation in adult stomach [20].

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1-cell zygotes and co-injection of 100 pg lacZ mRNA was performed for comparison.

Polyclonal antibody against Klf4a was generated by immunizing rabbit with His-tagged Klf4a protein without the zinc finger DNA binding domain (Qiagen) and purified by antigen-specific affinity purification using Stul/Link Protein kit (Thermo Scientific).

Total protein isolated from 72-hpf embryos that had been injected with either pCS2-Klf4a-T7 plasmid DNA (100 pg), pCS2-Klf4a-MO1 plasmid DNA (100 pg) and Klf4a-5mmMO2 (4 ng), or pCS2-Klf4a-MO2 plasmid DNA (100 pg) and Klf4a-MO1 and Klf4a-MO2 (1.5 ng each) were separated by 12% SDS polyacrylamide gel electrophoresis. Expression of Klf4a-T7 fusion protein was detected using affinity purified Klf4a antisera (1:100) and peroxidase-conjugated goat anti-rabbit-IgG (1:2000; Jackson) as well as using anti-T7 antiserum (1:5000; Calbiochem) and peroxidase-conjugated goat anti-mouse IgG (1:5000; Jackson). Expression of respective a-tubulin and proliferating cell antigen (PCNA) proteins were detected using either anti-a-tubulin antiserum (1:1000; Sigma) or anti-PCNA antiserum (1:1000; CalTag). Peroxidase-conjugated goat anti-mouse IgG (1:10,000; Sigma) was used to detect expression of both a-tubulin and PCNA proteins. ECL Plus Western Blotting Detection system (Amersham) was used to visualize protein band on Blue XB-1 film (Kodak).

Whole-Mount In Situ Hybridization, Alcian Blue Staining, BrdU Incorporation and p-Histone H3 Immunostaining

Whole-mount in situ hybridization was conducted on embryos treated with 0.003% phenylthiocarbamide and digoxigenin-labeled antisense RNA probes and alkaline phosphatase-conjugated anti-digoxigenin antibodies as described [30]. Various templates were linearized, and antisense RNA probes were produced as follows: her6 (Ban HII/T7); glucagon (Neo 1/SP6); Klf4a (Hind III/T3); PepT1 (NcoI/SP6).

Whole-mount alcian blue staining on klf4a morphants and control embryos were conducted as described [31]. 5-bromo-2-deoxyuridine (BrdU) labeling and p-Histone H3 immunostaining on klf4a morphants and control embryos were conducted as described [31]. In order to evaluate the differentiation of enterodendrocyte and goblet cell at the same time, glucagon-hybridized embryos were embedded for paraffin sectioning. After wax removal and rehydration, these sections were rinsed with acid alcohol and incubated with 0.1% alcian blue 8GX in acid alcohol for 30 min [31]. Finally, they were stained with hematoxylin according to standard procedures.

Histology, Photography, and Electron Microscopy

Paraffin sectioning, hematoxylin and eosin staining were conducted based on standard procedures. Images of embryos were taken using an RT color digital camera (SPOT) on a Zeiss Axioplan 2 microscope equipped with DIC mode. The obtained images were used to count goblet and enterodendrocyte cell numbers and p-Histone H3 and BrdU-labeled cell percentages in the intestine manually. Area of goblet cells was calculated using AxioVision software (Zeiss). BrdU-labeled cell percentages for different parts of intestine were calculated as described [31]. Percentages of p-Histone H3-labeled cells, glucagon-expressing enterodendrocyte cells and alcian blue-stained goblet cells were respectively calculated by counting labeled cell number and the total intestinal cell numbers in those longitudinal sections containing complete intestines of klf4a morphants, klf4a-injected, lacZ-injected, and wild type embryos. Transmission electron microscopy analysis was performed as described previously [31].

Total RNA Extraction and Real-Time Quantitative Reverse-Transcription –PCR (qPCR)

Total RNA was extracted using RNeasy Plant Mini Kit (Qiagen). To quantify expression levels of PepT1, klf4a, and beta-actin, qPCR was conducted as described [31]. klf4a primer pair was F-TCGGAAAG-CGATGCTGCTTTG and R-CCGTCATGCACCATGCTA-TCAAAAC. The primer pair for PepT1 and beta-actin were the same as described [31].

DNA Microarray and Gene Ontology (GO) Analyses

Total RNA of 96-hpf wild type and klf4a morphant embryos were extracted with RNeasy Plant Mini Kit (Qiagen) and their qualities were verified by Bioanalyzer 2100 (Agilent). Respective 10 mg total RNA was converted to double-stranded cDNA using SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen). Before precipitation for overnight at –20°C, the synthesized double-stranded cDNA samples were incubated with RNase A at 37°C for 10 min. The pellets were rehydrated with nuclease-free water and their qualities were confirmed by Bioanalyzer 2100. Respective 1 mg cDNA was labeled with Cy5-Random Nonamers using One-Color DNA Labeling Kit (NimbleGen) and then hybridized with Zebrafish Gene Expression 385K Arrays (NimbleGen) that containing 38,489 genes with designed 12 probes of 60 mer oligonucleotides per gene at 42°C for 18 h. The slide was washed in Wash I, II and III (NimbleGen) and then scanned with GenePix 4000B Microarray Scanner (Molecular Devices) after it was dried. A list of up- and down-regulated genes was uploaded to the website of Gene Ontology Enrichment Analysis Software Toolkit (GOEAST, http://omicslab.genetics.ac.cn/GOEAST/tools.php) and analyzed through Batch-Genes program using database of Danio rerio with p-value smaller than 0.1 [32].

Results

Cloning of the Zebrafish klf4a Gene, Phylogenetic, and Syntenic Comparison Analyses

Full-length klf4a cDNA was obtained by screening a zebrafish cDNA library. The deduced amino acid sequence showed that zebrafish klf4a encodes a 396-amino acid long polypeptide. Amino acid sequence comparison among human, mouse Klf4 and zebrafish Klf4a revealed the presence of 3 conserved C-terminal zinc finger DNA binding domains, preceded nuclear localization signal, and the N-terminal activation domain containing acidic residues (Figure S1A). Zebrafish Klf4a shared 66-67% amino acid similarities with human and mouse Klf4. Phylogenetic analysis demonstrated that zebrafish Klf4a is grouped together with human and mouse Klf4 with a high bootstrap value, while a previously identified zebrafish Klf4 was branched together with Klf2 members from mouse and zebrafish (Figure S1B). Syntenic analyses were conducted to compare conservation of genes, including Klf4 homologues, among zebrafish linkage group 21, human chromosome 9, and mouse chromosome 4 (Figure S2). We identified 10 out of 14 genes from zebrafish linkage group 21 sharing equal or greater than 50% amino acid sequence identity with respective homologue in both human chromosome 9 and mouse chromosome 4. However, the orders of loci within the chromosome are quite rearranged. These results indicate that zebrafish Klf4a is the homologue of mammalian Klf4.

Expression Patterns of klf4a and Its Expression was Inhibited by Notch Signaling

Zebrafish klf4a mRNA is a maternal transcript as shown by hybridization signal in 1-cell zygotes (Figure 1A). Ubiquitous klf4a
expression was detected in both shield and bud embryos. At 24-hpf, \textit{klf4a} was expressed in the retina, ventral brain, and epidermis of the whole embryos. \textit{klf4a} mRNA was localized in the olfactory bulbs, retina, pharynx, pectoral fin buds, and epidermis of 48-hpf embryos (Figure 1A and Figure S3). At 72-hpf, \textit{klf4a} was expressed in the olfactory bulbs, retina, left and right habenula.
neurons, cranial neurons, pharynx, pectoral fin, pharyngeal arches, liver, intestinal bulb and exocrine pancreas (Figure 1A and Figure S3). At 96-hpf, klf4a mRNA was distributed in similar tissues as those observed in 72-hpf embryos except that klf4a was expressed in the entire intestine (Figure 1A and Figure S3).

Since Notch signaling has been shown to inhibit mouse klf4 expression [21], we also investigated whether similar regulatory mechanisms exist in zebrafish embryos. Increased klf4a expression in the intestine was detected in 72-hpf DAPT-treated embryos as compared to DMSO-treated embryos (Figure 1B). Decreased expression of her6, a downstream target of Notch, was identified in DAPT-treated embryos by whole-mount in situ hybridization, demonstrating the effectiveness of DAPT treatment. In order to ensure the specificity of whole-mount in situ hybridization, we raised polyclonal antibody by immunizing rabbit with His-tagged KLH4a protein without the zinc finger DNA binding domain. A faint 47 kDa protein band corresponding to expected size of endogenous KLH4a protein can be recognized by KLH antiserum in the lane loaded with total protein isolated from 72-hpf DMSO-treated embryos. In contrast, a prominent 47 kDa protein band was recognized by KLH4a antiserum in the lane loaded with total protein obtained from DAPT-treated embryos (Figure 1B).

In contrast, reduced klf4a expression was detected in intestines of 72-hpf NICD-ectopically expressed embryos as compared to embryos that had been injected with 400 pg of lacZ mRNA (Figure 1C). Increased expression of her6 was identified in NICD-ectopically expressed embryos, demonstrating the effectiveness of NICD overexpression. qPCR further confirmed significant decrease of klf4a mRNA levels. The similarity in the regulation of zebrafish klf4a and mammalian homologue, klf4, by the Notch signaling, further support the notion that they are homologues.

Antisense MO-Mediated Knockdown of klf4a Expression

Two klf4a specific translational antisense MOs (klf4a-MO1 and klf4a-MO2) were designed to study klf4a function by inhibiting its protein synthesis. Respective five mismatches were introduced in these two klf4a-MOs and used as controls in this study. Initial experiments, conducted to determine optimal doses needed, indicated that it required injecting either 4 ng klf4a-MO1 or 16 ng klf4a-MO2 to achieve high (92%; 62%) percentage morphant rate and morphant phenotype (Table S1 and Figure S4). However, co-injection of 1.5 ng each of klf4a-MO1 and klf4a-MO2 can obtain the highest (98%) percentage morphant rate and morphant phenotype. These results indicate that there is a synergistic effect between these two translational klf4a-MOs, and co-injection of 1.5 ng each of klf4a-MO1 and klf4a-MO2 was used to generate klf4a morphants in the following experiments.

To test the effectiveness of MOs inhibiting the expression of klf4a, we carried out western blot analysis. Since endogenous KLH4a protein level is low, we overexpressed klf4a by injecting a pCS2+-KLH4a-T7 plasmid DNA into 1–2 cell zygotes and detected a 52 kDa protein band corresponding to the expected size of KLH4a-T7 fusion protein from 72-hpf injected embryos (Figure 2A). Synthesis of the 52 kDa KLH4a-T7 fusion protein was inhibited in embryos that had been co-injected with pCS2+-KLH4a-T7 plasmid DNA and klf4a-MO1 and klf4a-MO2 while the 52 kDa KLH4a-T7 fusion protein still can be detected in embryos that had been co-injected with pCS2+-KLH4a-T7 plasmid DNA and klf4a-5mmMO2. These results demonstrated the specificity of two translational klf4a MOs used in this study.

Notable morphological changes such as smaller eyes and otic vesicles, reduced dorsal-ventral axis, and the enlargement of midbrain ventricle were detected in 24-hpf klf4a morphants as compared to wild type and embryos that had been injected with respective 4 ng klf4a-5mmMO1 or klf4a-5mmMO2 (Figure 2B and Figure S4). In 96-hpf klf4a morphants, no intestinal folding and no inflation of swim bladder could be detected (Figure 2C and Figure S4). Histological analyses further demonstrated hypoplastic development of liver, intestines and exocrine pancreas in 96-hpf klf4a morphants (Figure 2D and data not shown).

Decreased PepT1 Expression Detected in Enterocytes of Intestines of klf4a Morphants

Zebrafish intestines contain three types of differentiated epithelial cells including enterocytes, enteroendocrine and goblet cells. The oligopeptide transporter, PepT1 is considered as a terminal differentiation marker of enterocytes [33]. The expression intensity of PepT1 in the intestinal bulb of 80-hpf klf4a morphants was decreased substantially when compared to control embryos (Figure 3). While PepT1 expression levels in 102- and 120-hpf klf4a morphants were not substantially decreased as compared to respective control embryos, however qPCR showed significant decreased PepT1 mRNA levels in both 80- and 102-hpf but not in 120-hpf klf4a morphants. These data indicates that KLF4a regulates enterocyte terminal differentiation.

Significant Decreased Goblet Cell Number Detected in Intestines of klf4a Morphants

To investigate role of klf4a in the differentiation of goblet cells, we used alcian blue staining to detect sulfated and carboxylated sialomucins in intestinal goblet cells. Since the differentiation of goblet cells involved the initial formation of pre-goblet cells which contains numerous mucous granules, we defined those alcian blue-stained goblet cells with area equal or larger than 24.0 ± 0.5 μm² (n = 86) as mature goblet cells and those with area less than this value as immature goblet cells. We observed 90% (n102 = 18; n200 = 29) reduction of mature goblet cell number and respective 80% to 46% decrease of immature goblet cell number in 102- and 120-hpf klf4a morphants as compared to control embryos (Figure 4A and 4B).

In order to demonstrate the differentiation defect of goblet cell is due to the absence of KLF4a protein, we conducted rescue experiment. Co-injection klf4a mRNA with both klf4a-MO1 and klf4a-MO2 can rescue 13.7% (n = 57) of mature goblet cell number in 102-hpf injected embryos which is comparable to 14.3% (n = 54) mature goblet cell number detected in wild type embryos. In contrast, co-injection lacZ mRNA (n = 55) did not have any effect (Figure 4C). Furthermore, we conducted klf4a overexpression experiment and discovered that a 2.8 fold (n = 60) increase of mature goblet cell number was detected in 102-hpf embryos that had been injected with klf4a mRNA but not with lacZ mRNA (n = 53) (Figure 4D). These results demonstrate that decreased goblet cell number observed in klf4a morphants is specifically due to deficiency of KLF4a protein and klf4a expression is important for the differentiation of goblet cells in intestines of zebrafish embryos.

A previous study was shown that Delta-Notch signaling controls the intestinal secretory cell fate commitment in zebrafish, and intestinal cells almost adopted secretory cell fate when Delta-Notch signaling was blocked [34]. We detected reduction in both immature and mature (6.7 fold decrease; n = 39) goblet cell numbers in NICD-ectopically expressed 102-hpf embryos as compared to wild type embryos (Figure 4E). However, adequate recovery of both immature and mature (11.9%, n = 46) goblet cell numbers was identified in 102-hpf embryos that had been co-injected with both klf4a and Notch 1a.NICD mRNA as compared to
wild type embryos \( (n = 40) \) that containing 20.9% mature goblet cells. While, no rescue effect can be observed in embryos that had been co-injected with both lacZ and Notch 1a NICD mRNA \( (n = 35) \). These results indicate that the regulation of differentiation of goblet cells in zebrafish intestine by Notch signaling at least partially mediated through Klf4a.

### Decreased glucagon-Expressing Enteroendocrine Cell Number Detected in Intestines of klf4a Morphants

Intestinal enteroendocrine cells are another type of secretory intestinal cells, they produce different hormones, including glucagon, which is also synthesized in pancreatic \( \alpha \) cells. A small
but significant decreased numbers of glucagon-expressing enteroendocrine cells were detected in the posterior intestinal bulb and mid-intestine of 96-hpf klf4a morphants as compared to control embryos (Figure 5). These results suggest that klf4a expression is also somewhat important for the differentiation of enteroendocrine cells in intestines of zebrafish embryos.

Analyses of Secretory Cell Differentiation along Anterior-Posterior Axis of Intestines in klf4a Morphants

To further investigate the effect of KLF4a on the differentiation of two secretory cells along anterior-posterior axis of intestines, 102-hpf wild type and klf4a morphant embryos were first hybridized with glucagon RNA probes to label glucagon-expressing enteroendocrine cells by whole-mount in situ hybridization. Respective glucagon-hybridized embryos were then processed for paraffin embedding and sectioning. Paraffin sections were used for alcian blue staining to label goblet cells and subsequent hematoxylin staining were used to identify all intestinal cells including enterocytes. Cell numbers of alcian blue-stained goblet cells, glucagon-expressing enteroendocrine cells and the rest of enterocytes were quantified from a number of longitudinal sections containing complete intestines along anterior to posterior axis. As shown in Figure 6, glucagon-expressing enteroendocrine cells were scattered from distal intestinal bulb/foregut to posterior intestine/hindgut while goblet cells were mainly distributed in the mid-intestine/midgut. The distribution of glucagon-expressing enteroendocrine cells remained to be similar while the distribution of goblet cells was altered in 102-hpf klf4a morphants (n = 10) as compared with wild type embryos (n = 7) (Figure 6A). Significant decreases in the number of goblet cells in klf4a morphants within intestinal bulb (1.6 fold), mid-intestine (1.9 fold), and posterior intestine (1.6 fold) were identified as compared to wild type embryos (Figure 6B). These data showed that the differentiation of goblet cells along the anterior-posterior axis of intestines was altered by klf4a knockdown.

BrdU-Labeled S Phase and p-Histone H3-Labeled M Phase Cell Population Detected in klf4a Knockdown and Overexpressed Embryos

To investigate the role of KLF4a in zebrafish intestinal cell proliferation, BrdU labeling and p-Histone H3 immunostaining were performed. A significant 1.6 fold (n = 10) increase of p-Histone H3-stained M phase cell percentages was observed in intestines of 72-hpf klf4a morphants as compared to wild type (n = 7) embryos (Figure 7A). A significant 1.8 fold increase of BrdU-labeled S phase cell percentages was detected in intestines of 96-hpf klf4a morphants (n = 6) with respective increases in the intestinal bulb/foregut (1.5 fold), mid-intestine/midgut (2.1 fold), and posterior intestine/hindgut (2.2 fold) regions as compared to

Figure 3. Decreased PepT1 expression is detected in klf4a morphants. PepT1 expression comparison among 80- (A), 102- (B), and 120-hpf (C) klf4a-MO1 and klf4a-MO2-injected morphants, klf4a-5mmMO1-, klf4a-5mmMO2-injected, and wild type embryos. (D) PepT1 expression level analyzed by qPCR. Error bars indicate the standard error. Student’s t-test was conducted to compare klf4a morphants with either wild type, klf4a-5mmMO1- or klf4a-5mmMO2-injected embryos. doi:10.1371/journal.pone.0020974.g003
control embryos ($n = 5$) (Figure 7A and 7B). In contrast, a significant 1.9 fold ($n = 21$) decrease of p-Histone H3-stained M phase cell percentages was observed in intestines of 72-hpf $klf4a$ overexpressed embryos as compared to lacZ-ectopically expressed ($n = 11$) embryos (Figure 8A). And a significant 1.2 fold ($n = 8$) decrease of BrdU-labeled S phase cell percentages was detected in intestines of 72-hpf $klf4a$-ectopically expressed embryos with respective decreases in the intestinal bulb (1.1 fold), mid-intestine (1.1 fold), and posterior intestine (1.2 fold) regions as compared to lacZ-ectopically expressed embryos ($n = 6$) (Figure 8A and 8B). These results indicate that Klf4a negatively regulates intestinal cell proliferation in zebrafish embryos.

In view of the fact that the presence of abnormal appearance of intestinal epithelium by histological analyses and decreased goblet cell number in $klf4a$ morphants, we used transmission electron microscopy to better define the ultrastructures of enterocytes and goblet cells. A single layer of columnar shaped intestinal epithelium with basal nuclei was detected in intestinal bulbs of 72- and 102-hpf wild type embryos (Figure 9). In contrast, three layers of stratified cuboidal shaped epithelium were detected in the intestinal bulbs of 72- and 102-hpf $klf4a$ morphants. In the mid-intestines of 102-hpf wild type embryos, matured goblet cells with many mucous granules which grouped into swollen thecae can be detected, while the majority of goblet cells were pre-goblet cells...
with a small number of mucous granules in the mid-intestines of 102-hpf klf4a morphants. These results further demonstrate that zebrafish Klf4a inhibits intestinal cell proliferation and regulates the differentiation of goblet cells.

Gene Expression Profile Comparison between klf4a Morphants and Wild Type Embryos via DNA Microarray and Gene Ontology (GO) Analyses

To investigate the difference of gene expression profile between wild type and klf4a morphants, DNA microarray analyses were conducted by hybridization with Cy3-labeled double-stranded cDNAs synthesized from 96-hpf wild type and klf4a morphant embryos. We obtained 219 genes that are up- or down-regulated for over two-fold changes between wild type and klf4a morphants from DNA microarray analyses. However only 156 out of 219 genes contained gene symbols that were uploaded to the website of Gene Ontology Enrichment Analysis Software Toolkit and analyzed through Batch-Genes program using the database of Danio rerio with p-value smaller than 0.1. Among them, 67 genes were mapped to different GO terms (Table 1). Expression of Stk36, a serine/threonine kinase that stabilizing full-length Gli, was down-regulated in klf4a morphants [35]. This data suggests that Klf4a regulates Hedgehog signaling. Decreased expressions of caprin2 and tcf3b, two mediators of canonical Wnt signaling, were identified in klf4a morphants. This result implies that Klf4a modulates Wnt signaling [36,37]. In klf4a morphants, down-regulated expression of cldnj which is important for the formation of the otoliths and ear function suggests the involvement of Klf4a in the development of otic vesicle [38]. Decreased expression of dnah9, which is a component of outer dynein arm complex of cilia was also identified in klf4a morphants [39]. These results demonstrated that Klf4a may be involved in the regulation of hedgehog and Wnt signaling pathways and the development of brain, otic vesicle, and ciliogenesis.

Discussion

Mammalian Klf4 has been shown to play important roles in the regulation of proliferation, differentiation and tumorigenesis of gastrointestinal tract epithelium [40]. In this study, we characterized function of zebrafish klf4a, a zebrafish homologue of mammalian klf4, to gain further understanding of the role of Klf4 in the intestinal cell proliferation and differentiation. Our loss-of-function, overexpression, rescue, histological and transmission electron microscopy results demonstrated that zebrafish Klf4a is essential for the repression of intestinal cell proliferation as well as for the promotion of goblet cell differentiation and the terminal differentiation of enterocytes. In addition, zebrafish klf4a expression is negatively regulated by Notch signaling.

Zebrafish Klf4a is Essential for the Differentiation of Different Intestinal Cell Lineages

Mouse klf4 is expressed abundantly in the colon. A previous study using klf4−/− mice and a recent report using klf4−/− mice

Figure 5. Reduction in glucagon-expressing enteroendocrine cell number is detected in klf4a morphants. glucagon RNA probe is used in whole-mount in situ hybridization to detect enteroendocrine cells in 96-hpf (A) wild type, (B) klf4a-5mmMO1-, (C) klf4a-5mmMO2-injected, and (D) klf4a-MO1 and klf4a-MO2-injected embryos. (E) Quantification of glucagon-expressing enteroendocrine cell number in 96-hpf embryos with different treatments. Error bars indicate the standard error. Student’s t-test was conducted to compare klf4a morphants with either wild type, klf4a-5mmMO1-, or klf4a-5mmMO2-injected embryos.
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with intestine-specific Klf4 deletion all showed failure of differentiation of goblet cells in the colon [19,41]. Significant decreased mature and immature goblet cell numbers were detected in intestines of 102- and 120-hpf klf4a morphants (Figure 4). In addition, goblet cells were mainly distributed in the mid-intestine and their cell number was significantly affected in 102-hpf klf4a morphants (Figure 6). Although a small but significant decreased glucagon-expressing enteroendocrine cell number was also detected in intestines of 96-hpf klf4a morphants, nevertheless, such decline is relatively small compared to those observed for goblet cell (Figure 5). Therefore, our results demonstrate an evolutionary conserved role of Klf4 in the differentiation of goblet cell in intestinal epithelium.

Since a change in cell fate from Paneth/goblet cells to enteroendocrine cells was observed in Gfi1−/− mice [42], we hypothesize that a portion of decreased goblet cells may change their fate in development and become enteroendocrine cells. This process results a small but significant decrease in the number of glucagon-expressing enteroendocrine cells observed in klf4a morphants. Meanwhile, the other portion of decreased goblet cells may enter apoptosis in klf4a morphants, which remains to be determined.

Notch signaling has been shown to be important in cell fate decision between secretory versus absorptive intestinal cells [34]. Our results show that Notch signaling inhibits klf4a expression in the intestine of zebrafish embryos (Figure 1), this finding is consistent with previous reports that klf4 expression is prevented by the Notch signaling in mouse intestinal epithelium [21,43]. Since the absence of Notch activation favors the differentiation of secretory cells in zebrafish intestine [34], we detected decreased goblet cell number in NICD-ectopically expressed 102-hpf embryos (Figure 4E). However, we were able to detect recovery of goblet cell number in 102-hpf embryos that had been co-injected with both klf4a and Notch 1a NICD mRNA (Figure 4E). These results further demonstrate that the regulation of differentiation of goblet cells in zebrafish intestine by Notch signaling at least partially mediated through Klf4a.

Math1 is required for the commitment of different secretory cell lineages in mouse intestine and its expression is inhibited by Hes1 mediator of Notch signaling [44]. Although orthologue of the Math1 has not been identified in zebrafish, we consider that zebrafish Klf4a may situate downstream of Math1 orthologue to regulate the differentiation of goblet cells in zebrafish embryos.

**Figure 6. Analysis of secretory cell differentiation along anterior-posterior axis of intestines in klf4a morphants.** 102-hpf wild type and klf4a morphant embryos were hybridized with glucagon RNA probe and processed for paraffin embedding and sectioning. Paraffin sections were then used for alcian blue and hematoxylin staining. (A) A section shows complete intestine including intestinal bulb (IB)/foregut, mid-intestine/midgut and posterior intestine/hindgut of a wild type embryo. Corresponding enlarged images of foregut, midgut, and hindgut of both wild type and klf4a-MO1 and klf4a-MO2-injected embryos are shown. Arrows indicate glucagon-expressing enteroendocrine cells and arrowheads indicate alcian blue-stained goblet cells. (B) Comparison of the percentages of goblet cell and glucagon-expressing enteroendocrine cell numbers within different segments of intestines in 102-hpf wild type and klf4a-MO1 and klf4a-MO2-injected embryos. Error bars indicate the standard error. Student’s t-test was conducted to compare klf4a morphants with wild type embryos. A, anus; IB, intestinal bulb.

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It seems that the prevention of mouse Klf4 expression by Notch signaling favors the formation of intestinal enterocytes, however, studies have shown that Klf4 regulates expression of several terminal differentiation marker genes of enterocytes [17,18]. In addition, a recent report showed that a reduction in alkaline phosphatase activity in enterocytes of small intestine and a reduction of carbonic anhydrase-1 expression in colonocytes of Klf4 mouse [41]. Likewise, decreased PepT1 expression observed in klf4a morphants indicates that Klf4a is required for the terminal differentiation of enterocytes in zebrafish embryos (Figure 3).

Zebrafish Klf4a Represses Intestinal Cell Proliferation

Although low Klf4 expression levels were identified in different human colorectal cancer cell lines, there are controversial results regarding in vivo Klf4 functions as a tumor suppressor from different mouse studies [11,19,20,41]. In klf4a morphants, increased intestinal cell proliferation and the presence of stratified enterocytes were detected while decreased intestinal cell proliferation was identified in klf4a-ectopically expressed embryos (Figure 7, 8 and 9). We consider that substantial decreased PepT1 expression level was not detected in 120-hpf klf4a morphants is likely attributed to increased layers of enterocytes with low level of PepT1 expression. Mammalian Klf4 was shown to mediate the cell cycle progression by repress expressions of cyclin D1 and cyclin B1 either directly or indirectly through the activation of p21 [45]. In addition, Klf4 was found to interact with β-catenin and repress β-catenin-mediated gene expression such as cyclin D1 [22]. Recent studies in zebrafish embryos showed that expression of p21 and cyclin G1 inhibited cell proliferation in the liver, intestine and exocrine pancreas [46,47]. Furthermore, Wnt-β-catenin signaling was shown to maintain cell proliferation in intestines of zebrafish embryos [48]. Therefore, we consider that Klf4a may regulate expressions of p21, cyclin G1 and/or β-catenin-mediated genes to affect intestinal cell proliferation in zebrafish embryos.

Zebrafish Klf4a Regulates the Development of Brain, Otic Vesicle and Ciliagenesis

In 24-hpf klf4a morphants, notable morphological changes including smaller eyes and otic vesicles, reduced dorsal-ventral axis, and the enlargement of midbrain ventricle were detected (Figure 2). Results of DNA microarray and GO analyses revealed decreased expressions of caprin2 and tcf3b in klf4a morphants (Table 1) [37]. Furthermore, elevated expression level of vim was observed in the hindbrain of 24-hpf klf4a morphants (unpublished observation). All these results imply that Klf4a is involved in the brain development.

Decreased expression level of cldnj that was shown to be essential for normal ear function in zebrafish, and small otic vesicles with the presence of small otoliths were identified in klf4a morphants (Table 1) [38]. These data suggest that Klf4a is required for the proper development of otic vesicles. DNA microarray and GO analyses showed reduced expression levels of stk36 and dnah9 in klf4a morphants (Table 1). Since zebrafish Stk36 was demonstrated to be required for cilia biogenesis [49],

Figure 7. Increased cell proliferation is detected in intestines of klf4a morphants. (A) Images of p-Histone H3-stained cells in intestines of 72-hpf wild type and klf4a-MO1 and klf4a-MO2-injected embryos are shown. Comparison of p-Histone H3-stained cell percentages in intestines of wild type and klf4a morphants is shown. Arrows indicate p-Histone H3-stained cells. Error bars indicate the standard error. Student’s t-test was conducted to compare klf4a morphants with wild type embryos. (B) Images of BrdU-labeled cells in intestines and in respective foregut, midgut and hindgut of 96-hpf klf4a-5mmMO2-injected and klf4a-MO1 and klf4a-MO2-injected and klf4a morphants are shown in panel A. Arrows indicate BrdU-labeled cells. Error bars indicate the standard error. Student’s t-test was conducted to compare klf4a morphants with klf4a-5mmMO2-injected embryos. doi:10.1371/journal.pone.0020974.g007
Figure 8. Decreased cell proliferation is detected in intestines of klf4a-overexpressed embryos. (A) Images of p-Histone H3-stained cells in intestines of 72-hpf lacZ- and klf4a-overexpressed embryos are shown. Comparison of p-Histone H3-stained cell percentages in intestines of lacZ- and klf4a-overexpressed embryos is shown. Arrows indicate p-Histone H3-stained cells. Error bars indicate the standard error. Student’s t-test was conducted to compare klf4a- with lacZ-overexpressed embryos. (B) Images of BrdU-labeled cells in intestines of 72-hpf lacZ- and klf4a-overexpressed embryos are shown. Comparison of BrdU-labeled cell percentages in intestines and in respective foregut, midgut, and hindgut of 72-hpf lacZ- and klf4a-overexpressed embryos are shown. Arrows indicate BrdU-labeled cells. Error bars indicate the standard error. Student’s t-test was conducted to compare klf4a- with lacZ-overexpressed embryos.

Figure 9. Transmission electron microscopy shows stratification of intestinal cells and abnormal goblet cell structures in klf4a morphants. Foregut images of a 72-hpf wild type (A) and a klf4a-MO1 and klf4a-MO2-injected (D) embryos are shown. Images of foregut (B) and midgut (C) of 102-hpf wild type and foregut (E) and midgut (F) of klf4a-MO1 and klf4a-MO2-injected embryos are shown. Arrows indicate mature goblet cells and arrowhead indicates an immature goblet cell.
we hypothesize that Klf4a is also involved in the regulation of ciliogenesis.

Using zebrafish embryos, our results provide in vivo evidence that zebrafish Klf4a functions to inhibit cell proliferation, and confirm its role as a tumor suppressor. Together with observation in human and mouse, Klf4a and its homologues are proved essential for the differentiation of goblet cells and the terminal differentiation of enterocytes in zebrafish embryos. Furthermore, klf4a expression is negatively regulated by Notch signaling and Klf4a acts as a mediator in the regulation of differentiation of goblet cells in zebrafish intestine by Notch signaling.

Supporting Information

**Figure S1** Amino acid sequence comparison and phylogenetic analyses. (A) Amino acid sequence comparison among human and mouse Klf4 and zebrafish Klf4a. Identical amino acid sequences are shown in black boxes. Conserved acidic residues important for activation are indicated by star. Three zinc finger DNA binding motifs are underlined. (B) Phylogenetic tree analyses. The bar represents the number of estimated differences for a unit branch length. Bootstrap values are shown.

**Figure S2** Maps showing gene order with conserved syntenic groups between zebrafish linkage group 21 and respective human chromosome 9 and mouse chromosome 4. Genes and their start positions (million base pair) on respective zebrafish linkage group 21 (LG 21), human chromosome 9 (Has 9) and mouse chromosome 4 (Mmu 4) are shown. The entire chromosome (grey box) is shown, but the scale is different in different chromosomes.

**Figure S3** Developmental expression patterns of klf4a. Lateral view of 48- (A), 72- (B), and 96-hpf (C), and dorsal view of 96-hpf embryos (D), and ventral view of 72- (E) and 96-hpf (F) deyolked embryos are shown. CN, cranial neuron; E, epidermis; EP, exocrine pancreas; HN, habenular neuron; I, intestine; L, liver; OB, olfactory bulbs; P, pharynx; PA, pharyngeal arches; PF, pectoral fin; R, retina.

**Table S1** Morphant phenotype characterization based on injection of different doses of klf4a MO1 and klf4a MO2.

| Gene Ontology Term | Down-regulated gene name | Up-regulated gene name | p-value |
|--------------------|--------------------------|------------------------|---------|
| Biological process | smoothened signaling pathway | stk36 | 0.0441 |
| ciliary or flagellar motility | dhah9 | 0.0441 |
| biomineral tissue development | cldnj | 0.0345 |
| positive regulation of Wnt receptor signaling pathway | caprin2 | 0.0345 |
| spinal cord dorsal/ventral patterning | TCF-3-B | 0.0225 |
| pseudouridine synthesis | pus7l | 0.05 |
| unsaturated fatty acid metabolic process | alox5 | 0.05 |
| lipid localization | | | |
| DNA-dependent DNA replication initiation | mcm7 | 0.0441 |
| circadian rhythm | nfil3 | 0.0668 |
| response to xenobiotic stimulus | amt | 0.0904 |
| Molecular function | peptidase activity | ctsbb, ctskl, cpa2 | 0.00025 |
| serine-type endopeptidase activity | homolog of CLIPB11 (Anopheles gambiae) | granzyme E, homolog of CELA1 (Homo sapiens) | 0.000162 |
| hyaluronic acid binding | hapln2 | 0.0441 |
| FMN binding | hao2 | 0.0904 |
| single-stranded DNA binding | ercc5 | 0.0441 |
| Cellular component | large ribosomal subunit | rpl13a | 0.0441 |

**Figure S4** Phenotype comparison among 4 ng klf4a-MO1-injected and 16 ng klf4a-MO2-injected morphants and 4 ng klf4a-5mmMO1-injected embryos. (A) Phenotype of 24-hpf embryos with different treatments. * Indicates enlargement of the midbrain ventricles and arrows indicate reduced otic vesicles. (B) Intestinal phenotype of 96-hpf klf4a-MO1-injected and klf4a MO2-injected morphants. Arrows indicate the absence of intestinal folding and arrowheads indicate no inflation of swim bladder.

**Table S2** Genes up- and down-regulated in klf4a morphants.

| Gene Ontology Term | Down-regulated gene name | Up-regulated gene name | p-value |
|--------------------|--------------------------|------------------------|---------|
| Biological process | smoothened signaling pathway | stk36 | 0.0441 |
| ciliary or flagellar motility | dhah9 | 0.0441 |
| biomineral tissue development | cldnj | 0.0345 |
| positive regulation of Wnt receptor signaling pathway | caprin2 | 0.0345 |
| spinal cord dorsal/ventral patterning | TCF-3-B | 0.0225 |
| pseudouridine synthesis | pus7l | 0.05 |
| unsaturated fatty acid metabolic process | alox5 | 0.05 |
| lipid localization | | | |
| DNA-dependent DNA replication initiation | mcm7 | 0.0441 |
| circadian rhythm | nfil3 | 0.0668 |
| response to xenobiotic stimulus | amt | 0.0904 |
| Molecular function | peptidase activity | ctsbb, ctskl, cpa2 | 0.00025 |
| serine-type endopeptidase activity | homolog of CLIPB11 (Anopheles gambiae) | granzyme E, homolog of CELA1 (Homo sapiens) | 0.000162 |
| hyaluronic acid binding | hapln2 | 0.0441 |
| FMN binding | hao2 | 0.0904 |
| single-stranded DNA binding | ercc5 | 0.0441 |
| Cellular component | large ribosomal subunit | rpl13a | 0.0441 |

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**Author Contributions**

Conceived and designed the experiments: IL SH. Performed the experiments: IL CC YL YW YC GL. Analyzed the data: IL YC CL. Contributed reagents/materials/analysis tools: IL CC YL YW YC GL CL. Wrote the paper: IL SH.
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