Exdpf Is a Key Regulator of Exocrine Pancreas Development Controlled by Retinoic Acid and ptf1a in Zebrafish

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Both endocrine and exocrine pancreatic cells arise from pancreatic-duodenal homeobox 1 (pdx1)-positive progenitors. The molecular mechanisms controlling cell fate determination and subsequent proliferation, however, are poorly understood. Unlike endocrine cells, less is known about exocrine cell specification. We report here the identification and characterization of a novel exocrine cell determinant gene, exocrine differentiation and proliferation factor (exdpf), which is highly expressed in the exocrine cell progenitors and differentiated cells of the developing pancreas in zebrafish. Knockdown of exdpf by antisense morpholino caused loss or significant reduction of exocrine cells due to lineage-specific cell cycle arrest but not apoptosis, whereas the endocrine cell mass appeared normal. Real-time PCR results demonstrated that the cell cycle arrest is mediated by up-regulation of cell cycle inhibitor genes p21Cip1, p27Kip1, and cyclin G1 in the exdpf morphants. Conversely, overexpression of exdpf resulted in an overgrowth of the exocrine pancreas and a severe reduction of the endocrine cell mass, suggesting an inhibitory role for exdpf in endocrine cell progenitors. We show that exdpf is a direct target gene of pancreas-specific transcription factor 1a (Ptf1a), a transcription factor critical for exocrine formation. Three consensus Ptf1a binding sites have been identified in the exdpf promoter region. Luciferase assay demonstrated that Ptf1a promotes transcription of the exdpf promoter. Furthermore, exdpf expression in the exocrine pancreas was lost in ptf1a morphants, and overexpression of exdpf successfully rescued exocrine formation in ptf1a-deficient embryos. Genetic evidence places exdpf downstream of retinoic acid (RA), an instructive signal for pancreas development. Knocking down exdpf by morpholino abolished ectopic carboxypeptidase A (cpa) expression induced by RA. On the other hand, exdpf mRNA injection rescued endogenous cpa expression in embryos treated with diethylamino benzaldehyde, an inhibitor of RA signaling. Moreover, exogenous RA treatment induced anterior ectopic expression of exdpf and trypsin in a similar pattern. Our study provides a new understanding of the molecular mechanisms controlling exocrine cell specification and proliferation by a novel gene, exdpf. Highly conserved in mammals, the expression level of exdpf appears elevated in several human tumors, suggesting a possible role in tumor pathogenesis.

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Introduction

The pancreas is a mixed organ with endocrine and exocrine compartments. The endocrine portion contains four distinct hormone-producing cell types organized into islets of Langerhans. Autoimmune-mediated destruction of endocrine β cells causes type 1 diabetes [1,2]. β cell number also gradually declines in type 2 diabetes [2]. The exocrine portion includes acinar cells, which produce digestive enzymes, and duct cells, which form an elaborate duct system that transports these enzymes into the gut. The majority of malignant pancreatic cancers derive from the exocrine portion [3]. Development of all major pancreatic cell types, including endocrine, exocrine, and duct cells, requires the function of the pancreatic-duodenal homeobox 1 (Pdx1), also known as Ipf-1) gene [4,5]. The molecular mechanisms determining early cell fate and the subsequent proliferation of endocrine and exocrine cells are not fully understood. Identification and characterization of novel lineage-specific regulators of exocrine pancreas cell proliferation could shed light on the pathogenesis of pancreatic cancers.

Morphogenesis of the pancreas in zebrafish (Danio rerio) shares some similarities to that in the mouse. In mice, the pancreas develops from one dorsal and one ventral bud that arise from the posterior foregut [6–8] sequentially. The recognizable dorsal pancreatic bud forms from the prepatterned endoderm at around 22–25 somites (embryonic day

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Abbreviations: arRA, all-trans retinoic acid; Cpa, carboxypeptidase A; DEAB, diethylamino benzaldehyde; dph, day(s) postfertilization; E[number], embryonic day [number]; Exdpf, exocrine differentiation and proliferation factor; GFP, green fluorescent protein; hpf, hour(s) postfertilization; Pdx1, pancreatic-duodenal homeobox 1; Ptf1a, pancreatic transcription factor 1, alpha subunit; RA, retinoic acid; RT-PCR, reverse transcriptase PCR; SD, standard deviation

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Pdx1 increased level of pancreas cultures, endocrine pancreatic precursors [29]. In mouse embryonic appears that RA acts directly in the endoderm to induce anterior expansion of endocrine and exocrine cells [25]. It signaling activity [26]. In zebrafish, RA treatment results in exocrine differentiation in the dorsal bud by inhibiting Notch

Xenopus retinoic acid (RA) has been reported in many organisms, [19], Notch [17,20–22], and sonic hedgehog [23] play critical components. Both endocrine and exocrine cells derive from a common pool of progenitors present in the gut endoderm during embryogenesis. The molecular mechanisms regulating cell fate decisions and lineage-specific proliferation are not fully understood. In this work, we report the characterization of a novel gene, exocrine differentiation and proliferation factor (exdpf), as a regulator for exocrine cell fate and differentiation/proliferation. We show that it is a direct target of the transcription factor pancreas-specific transcription factor 1a (Ptf1a), which is expressed in progenitors that give rise to all pancreatic cell types. We find that a deficiency of exdpf results in a severe reduction of exocrine size due to defects in cell proliferation. Consistent with this finding, overexpression of exdpf leads to an increase of exocrine size and a decrease of endocrine size, suggesting a possible change in fate of the endocrine progenitors. The human ortholog of exdpf is highly conserved and its expression level appears elevated in several cancers, including hepatic and pancreatic cancers, implying a possible role in pathogenesis of these malignancies.

9.5 [E9.5]) and the ventral bud arises slightly later at around 30 somites (E10.25 to E10.5). Then the dorsal and ventral buds fuse as a result of gut rotation at E12.5 [9]. Different endocrine cell types are specified at different stages. The α and β cells mature at E9.5 since glucagon and preproinsulin can be detected by immunohistochemistry [10], whereas somatostatin can be detected only at E13.5 [11,12]. Initially, it had been thought that the zebrafish pancreas develops from a single pancreatic anlage that appears at around 15 h postfertilization (hpf) [13–15]. This posterodorsal pancreatic anlage gives rise only to endocrine cells. Using a gut:GFP transgenic line, however, Field et al. observed a second anlage (ventral anlagen) that arose from the foregut at 34 hpf [16] when exocrine cells begin differentiation. In addition to exocrine cells, this anteroventral anlage also contributes to endocrine cells that are scattered outside of the main islet [16].

The dynamic process of pancreatic development is controlled by extrinsic signals from the adjacent tissues and intrinsic transcription factors. Multiple signals including fibroblast growth factor [17,18], bone morphogenetic protein [19], Notch [17,20–22], and sonic hedgehog [23] play critical roles for proper pancreas formation. A conserved role of retinoic acid (RA) has been reported in many organisms, including zebrafish [24,25], Xenopus [26], and mouse [27,28]. There are conflicting data, however, on the relative effects of RA on endocrine and exocrine pancreas differentiation. In Xenopus, RA treatment promotes endocrine at the expense of exocrine differentiation in the dorsal bud by inhibiting Notch signaling activity [26]. In zebrafish, RA treatment results in anterior expansion of endocrine and exocrine cells [25]. It appears that RA acts directly in the endoderm to induce endocrine pancreatic precursors [29]. In mouse embryonic pancreas cultures, all-trans retinoic acid (aTRA) inhibits branching morphogenesis and exocrine cell differentiation but accelerates endocrine differentiation, possibly due to increased level of Pdx1 in the endocrine clusters [30]. The differential effects may be explained by the distribution of the RAR and RXR receptors in the developing mouse pancreas [31].

A network of intrinsic transcription factors that act in a cascade fashion to initiate and maintain cell-specific gene expression patterns determines the ultimate lineage-specific cell fate. One of the earliest transcription factors functioning in the developing pancreatic epithelium is PDX1, which plays an essential role during the early phase of pancreas development. Mice with a targeted mutation in the Pdx1 gene exhibited no development of pancreatic tissue [4]. The agenesis of the pancreas is caused by an early arrest right after initial bud formation [4,5]. Furthermore, multiple roles of Pdx1 in cell lineage determination during pancreas formation has been revealed by lineage tracing using a modified version of Cre/lox technology [32]. Cells labeled between E9.5 and E11.5 give rise to all three pancreatic cell lineages including islet, exocrine acini, and ducts. Conversely, cells labeled at E8.5 and E12.5 or thereafter give rise only to endocrine and acinar cells [32]. These results suggest that temporal regulation of Pdx1 expression is critical for cell fate determination. In addition, several transcription factors have been identified as endocrine specific determinants. Neurogenin 3 (Ngn3) is one of the most important endocrine specific transcription factors [20,33]. In contrast to the endocrine lineage, little is known about the mechanisms that control the differentiation of exocrine and ducal lineages. Initially, pancreatic transcription factor 1, alpha subunit (Ptf1a) had been considered an exocrine specific transcription factor since its expression becomes restricted to exocrine cells by E13.5 in mice [34]. However, cell lineage tracing experiments revealed that ptf1a-expressing cells give rise to all pancreatic cell types [35].

Here, we provide several lines of evidence indicating that exocrine differentiation and proliferation factor (exdpf), a novel gene identified from zebrafish but highly conserved in mouse and human, is an exocrine cell determinant and required regulator of cell proliferation. The protein encoded by exdpf is a putative signaling molecule and is expressed highly in the exocrine cells during pancreas formation in zebrafish. Knocking down exdpf by antisense morpholino caused significant reduction or loss of expression of exocrine markers. In contrast, overexpression of exdpf resulted in the overgrowth in size of the exocrine pancreas and remarkable decrease of endocrine cells. This result suggests that misexpression of exdpf in the endocrine precursors is able to transform their fate. We further show that the reduction of exocrine cells in exdpf morphants is due to lineage-specific cell cycle arrest. Real-time PCR revealed that the expressions of cell cycle inhibitor genes p21Cip and p27Kip are dramatically increased in the exdpf morphants. To test the effect of RA on exocrine cell differentiation, we performed an epistatic study of exdpf and the RA pathway. The results show that exdpf acts genetically downstream of RA. Exogenous RA treatment induced anterior ectopic exocrine cells via exdpf induction. Moreover, injection of exdpf mRNA partially restored exocrine cell differentiation at the endogenous area in embryos treated with exogenous RA, whereas the expansion of endocrine cells were reduced. Our data establish a critical role of exdpf in exocrine cell fate determination and proliferation. Being a vertebrate-specific gene, the exdpf orthologs are highly conserved from fish to human. A search of National Center for Biotechnology Information (NCBI) database revealed that the human exdpf ortholog expression is up-regulated in several human cancers including hepatic,
pancreatic, and renal cancers, suggesting that overexpression or mutation in the exdpf gene might be involved in the pathogenesis of cancers.

**Results**

**Vertebrate Orthologs of the exdpf Gene Are Highly Conserved**

In our effort to identify pancreas specific genes, we isolated the exdpf gene (GeneID: 338304 [http://www.ncbi.nlm.nih.gov/sites/entrez]) from a RNA whole-mount in situ hybridization screen in zebrafish (our unpublished data). A BLAST search of the zebrafish genome revealed a homolog of exdpf named endocrine differentiation and proliferation factor (exdpf, not described here). The deduced peptide encoded by the exdpf gene contains 117 amino acids. Multiple sequence alignments using ClustalW showed that the Exdpf protein is specific to vertebrates and highly conserved across the vertebrates including zebrafish, mouse, and human (Figure S1A). The human and mouse orthologs are known as uncharacterized novel open reading frames (c20orf149, Gene ID: 79144 and AK154758). Overall, the deduced protein is about 42% identical across different species. The N terminus is highly conserved whereas the C terminus is more diversified, which suggests that subtle functional differences might lie in the C terminus. In addition, synteny analysis showed that a cluster of homologous genes is also conserved between zebrafish and human at the exdpf loci (Figure S1B).

**The exdpf Gene is Highly Expressed in the Exocrine Cells during Pancreas Formation in Zebrafish**

We studied the temporal and spatial expression of exdpf by reverse transcriptase PCR (RT-PCR) and whole-mount in situ hybridization. RT-PCR results (Figure S2) indicated that the exdpf transcript is maternally deposited since it was detected at the one-cell stage. The amount of exdpf transcript reduced following the one-cell stage, and the lowest level was detected at the shield stage. Then exdpf expression gradually increased from shield stage and a strong level was detected between 1 d postfertilization (dpf) and 2 dpf when the exocrine pancreas starts to develop; the highest level of expression was detected between 2 dpf and 5 dpf, the longest time point of this study. This result suggests that zygotic expression of the exdpf gene starts at around the shield stage.

We then performed RNA whole-mount in situ hybridization using an exdpf probe to obtain detailed expression analysis of exdpf in the developing pancreas. Double in situ hybridization was performed using either a preproinsulin probe to locate the endocrine β cells or a trypsin probe to mark the exocrine cells in combination with the exdpf probe. Exdpf transcripts were first detected in the developing somites at 8.5 hpf (Figure 1A). From the three-somite to 21-somite stage, exdpf was expressed in somites, adaxial cell, slow muscle fiber, and epiphysis (Figure 1B–1E). Interestingly, exdpf started to express in the pancreatic area at 33 hpf (Figure 1F), just before exocrine specification begins. Later in development, the strongest domain of exdpf expression appeared in the pancreas. Cells expressing exdpf (blue staining in Figure 1F) were located about one somite anterior to the cluster of preproinsulin-positive cells (red staining in Figure 1F). By 36 hpf, exdpf-expressing cells started to contact the preproinsulin-positive cells (Figure 1G) as a result of gut rotation. By 2 dpf, exdpf-expressing cells embraced the cluster of preproinsulin-positive cells (unpublished data). These exdpf-expressing cells continue to grow posteriorly to form a typical pancreas-like shape at 3 dpf (Figure 1H–1J). From 33 hpf to 3 dpf, there was no overlap between exdpf-expressing cells and preproinsulin-positive cells, indicating that exdpf expression is excluded from endocrine cells. Conversely, exdpf transcripts completely overlap with trypsin expression at 3 dpf (Figure 1J). To confirm the exocrine-specific expression of exdpf at 4 dpf, we performed a double in situ hybridization using an exdpf probe and a probe against carboxypeptidase A (cpa), another exocrine marker. As expected, exdpf expression completely overlaps with cpa expression at 4 dpf (unpublished data). Together, these data suggest that exdpf is expressed exclusively in the exocrine cells during pancreas development.

**Reduced exdpf Impairs Exocrine Cell Differentiation and Growth**

Based on the expression pattern of exdpf, we postulated that it is required for exocrine pancreas development. To test this hypothesis, we knocked down exdpf by injection of antisense morpholino oligonucleotides (MO1exdpf and MO2exdpf, designed to interfere with its translation. We tested both morpholinos to assure that the phenotypes observed are due to the specific knockdown of exdpf. A morpholino standard control oligonucleotide from Gene Tools was used to inject the control embryos. No specific phenotypes were observed in these control embryos. Double in situ hybridization using a trypsin probe and a preproinsulin probe was performed to assess the effect on exocrine and endocrine development simultaneously. In the control embryos, β cells formed a cluster (islet) that was surrounded by exocrine cells at the anterior area of the pancreas (head) at 3 dpf (Figure 2A). Both the β cell mass and exocrine mass increased at 5 dpf in the control embryos (Figure 2B). In addition, exocrine cells expanded posteriorly to form a typical pancreas like shape (Figure 2A and 2B). The majority of embryos injected with 2 ng of exdpf morpholin (MO1exdpf) exhibited no trypsin expression at 3 dpf (86%, n = 50) or 5 dpf. However, there were a few embryos (14%, n = 50) with reduced exdpf function that showed severe reduction of trypsin expression at 3 dpf and the remaining exocrine cells were restricted to the anterior pancreatic area engulfing the endocrine cells (Figure 2C). Furthermore, the exocrine cells failed to grow and expand posteriorly by 5 dpf (Figure 2D). In contrast, the endocrine cells looked largely normal in exdpf morphants (Figure 2C and 2D). Only a small fraction of exdpf morphants (10%, n = 50) showed scattered preproinsulin cells at 5 dpf (unpublished data). These results indicate that exdpf is required specifically for exocrine cell differentiation and growth. Over 90% of MO2exdpf morphants exhibited similar phenotypes (Figure S3). To investigate the function of exdpf in the differentiated exocrine cells, we used MO1exdpf for the rest of this study because it gave milder phenotypes.

We then studied early exocrine differentiation using a ptf1a probe. The protein encoded by ptf1a is a basic helix-loop-helix (bHLH) transcription factor that plays a critical role in exocrine pancreas development. A null mutation of Ptf1a in mouse leads to complete agenesis of exocrine pancreas and spatially disorganized endocrine pancreas [34,35]. In zebrafish, ptf1a loss of function by antisense morpholino injection suppresses exocrine markers without affecting endocrine...
markers and the organization of the main islet [36,37]. Since ptf1a can serve as an early marker of exocrine development [36], we analyzed ptf1a expression in exdpf morphants at 2 dpf and 3 dpf by whole-mount in situ hybridization. In the control embryos, ptf1a-expressing cells formed a loose cluster at 2 dpf (Figure 2E) and the expression expanded toward the posterior with exclusion from the endocrine cells at 3 dpf (Figure 2F). Expression of ptf1a was missing in the vast majority of exdpf morphant embryos (85%, n = 50). In a small fraction of exdpf morphants (15%, n = 50), initial expression of ptf1a appeared normal at 2 dpf (Figure 2G). But the expression of ptf1a failed to expand towards the posterior by 3 dpf (Figure 2H) and remained in the same area as in 2 dpf. This result confirms that exdpf is critical for exocrine cell specification and expansion.

The exdpf gene is expressed in the developing somites as well as the exocrine pancreas (Figure 1). To clarify whether the exocrine pancreas defect is due to pleiotropic abnormalities, we used a recently obtained transgenic fish MP760GFP (our unpublished data) that expresses green fluorescent protein (GFP) in the developing liver, gut, and pancreas. In this transgenic line, pancreatic expression of GFP is restricted in the exocrine portion. A minimal amount of exdpf morpholino was used to achieve the least amount of defects in overall body morphology. At 24 hpf, strong expression of GFP was observed in the presumed pancreatic area in both control embryos and exdpf mRNA injected embryos (Figure S5A and S5B, arrows). By 2 dpf, pancreatic GFP expression became more obvious in the control and exdpf mRNA-injected embryos (Figure S5A and S5B, arrowheads). In contrast, only residual or no pancreatic GFP expression was observed in exdpf morphants (Figure S5C). However, gut GFP expression in exdpf morphants (Figure S5C) remained comparable to that in the control embryos (Figure S5A).

Exdpf is also expressed in the developing liver (Figure 1) during embryogenesis. To assess whether it is required for liver development, in situ hybridization was performed using a ceruloplasmin (cp) probe. At 3 dpf, clear expression of cp was detected in the livers of control embryos injected with standard morpholino control (Figure S5D). No detectable change of cp expression was observed in exdpf morphants (Figure S5E–S5G). This might be due to the redundant function of exdpf homolog endpf since it is also expressed in the developing liver.
The exdpf Gene Is a Direct Target of ptf1a

We further studied the genetic interaction between ptf1a and exdpf. In mild exdpf morphants (injected with 2 ng of MO1 exdpf), ptf1a expression was initiated but restricted to the anterior area in 72% of embryos (Figure 2G, n = 90), indicating that exocrine cell differentiation can start but expansion fails. However, only 15% of embryos (n = 50) still exhibited ptf1a expression when injected with 4 ng of MO1 exdpf, suggesting a role for exdpf in exocrine cell differentiation. Knocking down ptf1a by morpholino injection (Figure 3) resulted in agenesis of the exocrine pancreas (Figure 3C); only about 5% of ptf1a morphants exhibited weak cpa expression (n = 189, Table 1). In the control embryos, injection of exdpf mRNA led to a great expansion of the exocrine pancreas (Figure 3B). Interestingly, injection of exdpf mRNA into ptf1a morphants successfully restored expression of exocrine marker cpa to about 70% of embryos (Figure 3D–3F, n = 168). About 35% of embryos exhibited nearly full restoration of cpa expression in the ptf1a morphants when exdpf mRNA was injected, whereas another 35% exhibited partial restoration (Table 1). In a reciprocal experiment, injection of ptf1a mRNA into exdpf morphants failed to rescue expression of exocrine markers (unpublished data). Together, these results place exdpf genetically downstream of ptf1a in exocrine development.

To test whether ptf1a functions through exdpf in exocrine specification, we performed in situ hybridization analysis of exdpf in ptf1a morphants (Figure 4). Indeed, exdpf expression in the exocrine pancreas was abolished in such embryos, as expected (Figure 4B). However, the epiphysis expression of exdpf remained unchanged in the ptf1a morphants (Figure 4B, inset), indicating that ptf1a specifically controls pancreatic expression of exdpf. Ptf1 is an unusual heterotrimeric bHLH transcription factor composed of Ptf1a/P48, a common class A bHLH protein (such as HEB, E2–2, E12, or E47, also called...
E-proteins), and a third protein that can be either the mammalian Suppressor of Hairless RBP-J or its paralog, RBP-L [38,39]. Ptf1 binding sites are bipartite with an E-box (CANN TG) and a TC-box (TTTCCC) spaced one or two helical turns apart, center to center [39–41]. The heterodimeric subcomplex of Ptf1a and the E-protein binds to the E-box and RBP-J or RBP-L binds to the TC-box [39,41]. Binding of the Ptf1 complex to DNA requires both boxes, and the spacing between these elements is critical for Ptf1 binding [42]. Functional binding sites for the Ptf1 complex are present in the 5′ promoter regions of all of the acinar digestive enzyme genes examined [40,41]. To determine whether Ptf1a might indeed control the transcription of exdpf, we searched the 5-kb 5′ flanking region and intronic sequences of this gene for potential PTF1-binding sites comprising an E-box and a TC-box spaced one or two helical DNA turns apart. Three potential binding sites were detected. Binding site 1 is about 3 kb upstream of the transcriptional start site (Figure 4C) and binding site 2 is around 1 kb upstream of the transcriptional start site (Figure 4C); binding site 3 is about 500 bp downstream of the transcriptional start site in the first intron (Figure 4C).

A 3.6-kb exdpf promoter region containing the transcriptional start and three potential PTF1-binding sites increased the activity of the luciferase reporter plasmid 41-fold compared to the promoterless pGL3-Basic in HEK 293 cell lines tested by transfection (Figure 4D, seq1). While deletion of binding site 1 reduced the activity to 29-fold, a 1.5 kb promoter region that retained binding site 2 and binding site 3 still maintained an activity of 26-fold (Figure 4D, seq 2 and seq 3). However, deletion of binding site 1 and binding site 3 reduced the activity to 8-fold (Figure 4D, seq4). Not surprisingly, deletion of all three binding sites further reduced the transcription of the reporter gene almost to a basal level (Figure 4D, seq 5). These results provide strong evidence that Ptf1a can promote the transcription of exdpf, and Ptf1-binding site 1 and binding site 3 are especially critical for the activation of the promoter. Taken together, these data demonstrate that exdpf is a direct target gene of Ptf1a.

Excess exdpf Causes Increases in Exocrine Pancreas Mass

To assess whether exdpf is sufficient for exocrine specification, we carried out overexpression experiments by injecting synthetic exdpf mRNA into embryos at the one-cell stage (Figure 5). Elastase A:GFP transgenic fish were used to facilitate the identification of exocrine cells. In this fish, GFP expression is controlled by the elastase A (elaA) regulatory sequence which allows exocrine specific GFP expression in larvae and adult [43]. At 3 dpf, the average exocrine cell number in the control embryo is 197.8 ± 8.2 (Figure 5C; Table 1. Quantitative Data of cpa Expression in Ptf1a Morphants

| Group                  | Full or Near Full cpa Expression | Partial cpa Expression | No cpa Expression | Total Embryo Number |
|------------------------|----------------------------------|------------------------|-------------------|---------------------|
| Ptf1a MO               | 0 (0%)                           | 10 (5.29%)             | 179 (94.7%)       | 189                 |
| Ptf1a MO + exdpf mRNA  | 58 (34.5%)                       | 60 (35.7%)             | 50 (29.8%)        | 168                 |
| exdpf mRNA             | 114 (93.4%)                      | 3 (2.5%)               | 5 (4.3%)          | 122                 |

Figure 4. Exdpf Expression Is Regulated by Ptf1a
(A and B) In situ hybridization using an exdpf probe in 3 dpf embryos. (A) An embryo injected with standard morpholino control. Inset: epiphysis expression. (B) An embryo inject with Ptf1a morpholino. Inset: epiphysis expression. Note that no exocrine expression of exdpf was detected. All embryos are shown in lateral view, anterior to the left. Scale bar: 50 μm. (C) Locations and sequences of the three potential PTF1 binding sites in zebrafish exdpf gene and different exdpf promoter segments used in luciferase assay. The E-box and TC-box are underlined and capital letters indicate conserved nucleotides. The nucleotides that are not confirmed to the conserved nucleotides are in red. Dots indicate the spaces between the E-box and TC-box. (D) Comparison of the activities of the segments containing different Ptf1 binding sites when transfected into 293 cells. These segments were inserted just before the ORF of luciferase gene. Luciferase reporter activity was adjusted for transfection efficiency and expressed relative to the promoterless pGL3-Basic vector. Error bars represent standard deviations.

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Figure 5. Reduced exdpf Causes Defects of Exocrine Cell Proliferation

(A) BrdU labeling result in 33 hpf embryos. Green: MP760:GFP, Red: BrdU staining. Blue: DAPI staining. Dorsal view, anterior to the left. Enlargement represents higher magnification of boxed area in each row. Arrows indicate non-proliferating cells; arrowheads indicate proliferating cells as evidenced by overlapping of red and green colors.

(B) BrdU labeling result in 3 dpf embryos. Green: elastase A:GFP, Red: BrdU staining. Lateral view, anterior to the left. Scale bar: 50 μm. Arrows indicate non-proliferating cells; arrowheads indicate proliferating cells as evidenced by overlapping of red and green colors.
Table 2. Quantitative Data of BrdU Assay

| Group          | 33 hpf                  | 3 dpf                  |
|----------------|-------------------------|------------------------|
|                | BrdU Positive | Total Cell Number | Percentage of BrdU Cells | BrdU Positive | Total Cell Number | Percentage of BrdU Cells |
| Control        | 26 ± 3.05       | 32 ± 3.28            | 79.61%                  | 169.6 ± 8.2    | 197.8 ± 8.2       | 84.93%                  |
| edbp mRNA      | 34 ± 3.28       | 38.67 ± 5.04         | 87.92%                  | 249.4 ± 3.53   | 253 ± 4.2         | 98.58%                  |
| edbp MO        | 3 ± 0.58        | 9.67 ± 0.88          | 31.02%                  | 3.6 ± 3.43     | 21.6 ± 3.4        | 16.67%                  |

Average cell number ± SD.

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(C) Quantitative graphs for BrdU incorporation rate in 33 hpf or 3 dpf embryos. The average number of GFP positive cells with BrdU incorporation was obtained by counting BrdU-labeled GFP positive cells from five embryos. Y axis: Mean ± SD.

(D) Semi-quantitative RT-PCR examination of expression of cyclin D1 and cell cycle inhibitors p21, p27 and cyclin G1 in control (control), edbp morphants (MO), and edbp mRNA injected embryos (RNA) at 33 hpf, 2 dpf, 3 dpf, and 5 dpf. In each group, 30 embryos were used to extract total RNA for RT-PCR. dx.doi.org/10.1371/journal.pbio.0060293.g005
not as much as p21Cip expression (Figure 5D, second row). In addition, the expression of cyclin G1 also slowly increased in the exdpf morphants from 2 dpf to 5 dpf. Taken together, our results suggest that knocking down exdpf leads to cell cycle arrest through up-regulation of p21Cip, p27Kip, and cyclin G1.

We have shown in our previous result that overexpression of exdpf increased exocrine cell number due to overproliferation of these cells (Figure 5B, [center]). We then confirmed this result by checking the expression level of cyclin D1 through semiquantitative RT-PCR. At 33 hpf (just before exocrine cell differentiation), cyclin D1 expression was slightly increased in the exdpf mRNA injected embryos (Figure 5D). From 2 dpf to 5 dpf, the increase of cyclin D1 expression in the exdpf mRNA injected embryos became stronger. Interestingly, we also detected elevated expression of cyclin D1 in the exdpf morphants from 2 dpf to 5 dpf. We postulate that this type of increase may be caused by exdpf independent mechanisms that control organ size. Due to the strong increase of cell cycle inhibitors in exdpf morphants, an increased level of cyclin D1 is not enough to drive the cell into the proliferating phase. The semi-quantitative RT-PCR results are further confirmed by real-time PCR (Table 3). Similar up-regulation of cell cycle inhibitors including p21, p27, and cyclin G1 were detected in the exdpf morphants (Table 3).

To examine where the increase of p21 expression comes from, we performed in situ hybridization using a p21 probe. At 33 hpf, p21 expression was detected in the developing brain (Figure S7A and enlargement). No p21 expression was observed in the developing pancreatic area. A similar p21 expression pattern was observed in exdpf mRNA injected embryos (Figure S7B and enlargement). Conversely, noticeable p21 expression could be seen in the developing pancreatic area in exdpf morphants (Figure S7C and enlargement). Thus, cell proliferation deficits observed in the developing exocrine pancreas caused by knocking down exdpf are likely mediated by increased level of p21 expression in the cells.

The exdpf Gene Acts Genetically Downstream of RA in Regulating Exocrine Pancreas Development

We have shown that exdpf is essential for the exocrine pancreas differentiation and expansion; overexpression of exdpf gene leads to increased exocrine size. This effect is similar to that of RA treatment (Figure 6). Embryos treated with RA exhibited expanded endocrine pancreas [25]. Exogenous RA treatment also caused ectopic formation of exocrine at positions anterior to the presumptive pancreatic area (Figure 6B). But the exocrine pancreas failed to expand toward the posterior at 5 dpf (unpublished data; only a small number of embryos survived) as it did in untreated control embryos. Interestingly, RA treatment of exdpf morphants failed to induce exocrine formation (Figure 6D). This result indicates that exocrine pancreas formation in RA-treated embryos requires exdpf function.

In reciprocal experiments, treatment with 10−6 M diethylaminoobenzaldehyde (DEAB) to block the RA pathway often resulted in no exocrine pancreas formation at 3 dpf (Figure 6F, 85%, n = 181 embryos). Only about 15% (n = 181) of DEAB treated embryos exhibited weak expression of an exocrine marker at 3 dpf. This result suggests that RA is required for early exocrine formation during normal pancreas development. To test whether overexpression of exdpf can rescue exocrine pancreas formation in the DEAB treated embryos, we injected synthetic exdpf mRNA into embryos at the 1-cell stage followed by treatment with 10−6 M of DEAB from 9 hpf up to the time of analysis. At 3 dpf, exdpf mRNA injection increased the percentage of embryos exhibiting expression of the exocrine marker cpa to 35% (Figure 6G and 6L, n = 166). This result suggests that exdpf acts downstream of the RA pathway during normal pancreas development. RA treatment often resulted in bilateral ectopic exocrine formation at the anterior area (Figure 6N, 85%, n = 191 embryos). To test whether exogenous RA treatment induces exdpf expression, in situ hybridization using an exdpf probe was performed. Interestingly, similar bilateral ectopic expression of exdpf was observed in RA treated embryos (Figure 6K). On the other hand, blocking RA synthesis by DEAB completely abolished exdpf expression in the pancreatic area (Figure 6L), whereas the epiphysis expression appeared normal (Figure 6L, arrowhead). These data suggest RA signaling influences exdpf expression in the developing pancreas.

Taken together, these results place exdpf genetically downstream of RA in exocrine pancreas development.

Overexpression of exdpf Inhibits Endocrine β Cell Development

The expression of the exdpf gene is excluded from the endocrine islet during pancreatic development (Figure 1I). To evaluate the effect of exdpf overexpression on endocrine cell differentiation, we performed in situ hybridization against preproinsulin in 3 dpf embryos (Figure 7). In the exdpf mRNA injected embryos, preproinsulin expression was dramatically reduced (Figure 7B). Preproinsulin::GFP transgenic fish were injected with exdpf mRNA to quantify the GFP-positive cells. In the control embryos, the average number of

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**Table 3. Real-Time PCR Analysis Of Cell Cycle Control Genes**

| Gene   | 33 hpf | 2 dpf | 3 dpf | 5 dpf |
|--------|--------|-------|-------|-------|
|        | exdpf MO | exdpf RNA | exdpf MO | exdpf RNA | exdpf MO | exdpf RNA | exdpf MO | exdpf RNA |
| p21    | 13.06 (0.0047) | 1.45 (0.04) | 23.65 (<0.001) | 1.19 (0.028) | 35.03 (0.001) | 1.64 (0.03) | 4.73 (<0.001) | 1.06 (0.0117) |
| p27    | 0.74 (0.019) | 1.23 (0.045) | 0.51 (0.001) | 0.95 (0.039) | 2.69 (<0.001) | 0.81 (0.001) | 28.68 (<0.001) | 1.22 (<0.001) |
| cyclin G1 | 1.14 (0.01) | 1.24 (0.042) | 3.39 (0.042) | 1.10 (0.006) | 14.84 (<0.001) | 1.45 (0.08) | 15.29 (0.04) | 3.71 (0.045) |
| Cyclin D1 | 1.01 (<0.001) | 1.97 (<0.001) | 30.79 (<0.001) | 38.79 (<0.001) | 4.63 (<0.001) | 9.84 (<0.001) | 8.25 (0.006) | 7.68 (0.049) |

Ratio (p-value).
doi:10.1371/journal.pbio.0060293.t003
GFP positive cells was 45.15 ± 7.04 (Figure 7F, mean ± SD, n = 20). As expected, overexpression of\textit{exdpf} resulted in a significant reduction of GFP positive cell number by about 40%; the average number of GFP-expressing cell was 27.55 ± 6.02 (Figure 7F, n = 20). The stage of 3 dpf is relatively late for β cell development. To examine whether the reduction of β cell number at 3 dpf is due to defects in cell proliferation or specification, we quantified\textit{preproinsulin:GFP} positive cells at 24 hpf (see Figure S8 for panel of embryos used for quantification) when the majority of β cells come from newly specified cells. Overexpression of\textit{exdpf} reduced β cell number by about 43% (Figure 7E, from 21.4 ± 4.16 to 9.2 ± 4.04 cells per embryo). However, no significant change in β cell number was observed in the\textit{exdpf} morphants (Figure 7E, 19.5 ± 3.39 cells per embryo). These data indicate that overexpression of\textit{exdpf} inhibits β cell specification and suggests a possible transformation of cell fate in the endocrine pancreatic precursors.

Exogenous RA treatment of WT embryos dramatically increased\textit{preproinsulin}-expressing cell number (Figure 7C and 7F, 212.15 ± 14.88 cells per embryo, n = 20) compared with that in the DMSO treated control embryos (Figure 7A and 7F, 45.15 ± 7.04, n = 20). Interestingly, overexpression of\textit{exdpf} in the RA treated embryos inhibited the anterior expansion of the endocrine pancreas (Figure 7D and 7F, 105.2 ± 10.88 cells per embryo, n = 20). This result suggests that overexpression...
The expression of exdpf in the anterior ectopic pancreas induced by RA treatment balanced RA signaling and turned more cells into the exocrine fate.

**Discussion**

Our results provide strong evidence that a novel gene exdpf is specifically employed by the zebrafish exocrine progenitors to promote cell differentiation and proliferation. Moreover, epistasis experiments support that exdpf functions downstream of ptf1a in exocrine cell specification. RA also interacts genetically with Exdpf during exocrine formation.

How does Exdpf regulate exocrine cell specification? First, exdpf starts to express in exocrine progenitors at 33 hpf, just before exocrine cell differentiation. Second, exdpf is required for exocrine cell differentiation. Knocking down exdpf by antisense morpholino resulted in the absence of exocrine markers in a majority of injected embryos. The remaining smaller fraction of exdpf morphants exhibited significantly reduced expression of exocrine markers. Third, exdpf is both necessary and sufficient for exocrine cell proliferation. We observed exdpf overexpression increased the proliferation of exocrine cells (98% versus 85% in the control embryos), whereas knocking down exdpf severely impaired proliferating...
ability due to the increased level of cell cycle inhibitors p21C\textsuperscript{CIP}, p27K\textsuperscript{IP}, and cyclin G1. Although exdpf is necessary for exocrine cell differentiation, it is not sufficient to induce ectopic formation of the exocrine pancreas. A possible explanation is that exdpf only functions in the committed exocrine progenitors. Our results show that Exdpf promotes exocrine cell proliferation by regulating cyclin D expression.

Exdpf acts genetically downstream of ptf1a in exocrine specification. It is likely that ptf1a controls the expression of exdpf in exocrine progenitors. The expression pattern of exdpf in exocrine pancreas is similar to that of ptf1a [36]. ptf1a is expressed in the exocrine progenitors at 32 hpf. Similarly, ptf1a-expressing cells also surround \( \beta \) cells as a result of gut rotation [36]. In addition, three Ptf1a binding sites have been identified in the promoter region of the exdpf gene (Figure 4C). Using luciferase assay, we were able to show that these Ptf1a binding sites are functional in culture cells. Thus, exdpf is a direct target gene of Ptf1a. Other transcription factors such as Pdx1 might also control exocrine progenitor specific expression of exdpf. During embryogenesis, ptf1a is expressed in the ventral aspect of pdx1-expressing domain from 32 hpf to 36 hpf [36]. Moreover, two Pdx1 binding sites have also been identified within exdpf promoter region (data not shown). Knocking down of ptf1a leads to the agenesis of the exocrine pancreas, which is consistent with previous results [36,37]. Injection of exdpf mRNA into the ptf1a morphants successfully restored the expression of the exocrine marker. This is likely due to the expansion and differentiation of residual progenitor cells promoted by exdpf mRNA in the Ptf1a morphants. In our rescue experiments, the injected concentration of ptf1a morpholino only created a hypomorph situation and should still contain a residual pool of progenitors. Co-injection of exdpf mRNA promotes proliferation and differentiation of these cells, resulting rescue of the defect. It is also likely that Ptf1a activity recovers following cessation of the morpholino effect, and that this allows for full exocrine differentiation to occur in progenitor cells that have been rescued by exogenous exdpf. Nonetheless, our results provide evidence that Exdpf acts downstream of ptf1a in exocrine formation. In addition, overexpression of exdpf by mRNA injection inhibited endocrine cell fates (Figure 7B, 7E, and 7F). This endocrine repression result is in agreement with a recent report by Dong et al. Using partial loss of function analysis for ptf1a, Dong et al. found that high levels of ptf1a promote exocrine fate whereas low levels promote endocrine fate [44]. It is likely that Ptf1a exerts its function through exdpf in this aspect.

The exdpf gene encodes a putative signaling molecule containing two SH2 and two SH3 domains as well as other conserved domains (Figure S4), suggesting that Exdpf may function in response to signals from adjacent mesoderm tissues. Multiple intercellular signals including transforming growth factor beta, Hedgehog, and Notch are critical for the proper specification of endocrine and exocrine cell fates during pancreas development. It is not clear which signal or signals exdpf responds to in order to make the exocrine cell fate decision. Our results support the idea that exdpf promotes exocrine cell proliferation. As a putative signaling molecule, exdpf might be involved in transducing signals from growth factors that are required for exocrine cell proliferation. However, the identities of such growth factors remain elusive.

Genetic evidence places exdpf downstream of RA. Exogenous treatment with RA caused anterior expansion of endocrine cells as well as ectopic anterior exocrine cells, which is consistent with previous results [25]. Contradictory results from RA treatment have been reported using different organisms regarding exocrine development. In mouse embryonic culture, RA treatment suppresses exocrine differentiation and branching morphogenesis [30,45]. Others reported that allRA treatment leads to endocrine and duct differentiation from the pancreatic bud, but inhibits exocrine differentiation [45]. In Xenopus, exogenous RA treatment causes endocrine expansion in the dorsal bud at the expense of exocrine tissue but stimulates exocrine differentiation in the ventral bud [26]. These contradictory results might derive from different organisms and different concentrations of either RA or 9cis RA. In our experiment, we find that excessive 9cis RA caused ectopic anterior exocrine formation. Exogenous treatment with RA induced ectopic pancreatic cells including endocrine and exocrine cells. Anterior ectopic formation of the exocrine pancreas by RA treatment requires exdpf function since exdpf morpholino injection blocks ectopic exocrine formation and RA also induces anterior ectopic expression of exdpf. Overexpression of exdpf in wild-type embryos significantly suppresses endocrine cell differentiation, suggesting exdpf transforms the cell fate of pancreatic progenitors (Figure 7G). The balance of RA and overexpressed exdpf in the progenitors results in reduced endocrine cells compared with RA treated wild-type embryos.

Pancreatic cancer is one of the leading causes of cancer deaths because it is often highly aggressive and resistant to treatments available at the time of diagnosis [46]. Genetic studies have identified structural mutations in pancreatic cancers; the alterations include the activation of K-Ras proto-oncogene as well as inactivation of tumor suppressor genes such as TP53 or INK4a locus [47–49]. By carefully searching the NCBI database, we found that the human exdpf ortholog is expressed in relatively high levels in multiple tissues including the pancreas, colon, and mammary glands. Interestingly, the EST expression profile also indicates that higher level of exdpf ortholog has been detected in several cancers including pancreatic cancer, breast cancer and kidney cancer (Figure S9). In addition to structural mutations, many growth factor receptors and their ligands are overexpressed in pancreatic cancers. Since exdpf can promote acinar cell proliferation, it is worth studying the role of this gene in pancreatic cancer. Zebrafish has proven to be a useful model system to study pancreatic cancers. In 2004, Yang et al. reported that human MYCN caused pancreatic neuroendocrine tumors in transgenic zebrafish that expressed MYCN in \( \beta \) cells, muscle cells and neurons [50]. Recently, transgenic fish that express oncogenic KRASG12V under the ptf1a promoter was generated. In these fish, KRASG12V blocked the differentiation of pancreatic progenitor cells and this undifferentiated progenitor pool lead to invasive pancreatic cancer [51]. These examples demonstrate that the zebrafish model is useful in advancing our understanding of pancreatic cancers.

Together, our results reveal a specific requirement for Exdpf in exocrine pancreas formation in zebrafish. The gene exdpf is expressed exclusively in the exocrine progenitors and differentiated exocrine cells. We demonstrate that exdpf is necessary for exocrine cell differentiation. Furthermore, exdpf is both sufficient and necessary for the proliferation of
differeniated exocrine cells. We speculate that the study of the function of exdpf in pancreatic cancers could shed light on the pathogenesis of this malignancy.

Materials and Methods

Zebrafish husbandry. Zebrafish were raised and kept under standard laboratory conditions at about 28 °C. Embryos were staged according to Kimmel et al. [52]. The elasate AGFP fish was a gift from Gong’s laboratory in Singapore [43]. The wild-type line used was AB.

In situ hybridization. In situ hybridization was performed essentially as previously described [53]. For double in situ hybridization, Fast Red (Roche) and NBT/BCIP (50 mg/ml; Promega) were used as alkaline phosphatase substrates. The following probes were synthesized, Fast Red (Roche) and NBT/BCIP (50 mg/ml; Promega) were used as alkaline phosphatase substrates. The following probes were synthesized: exdpf-RNA probe (5’-CACCTTACCTCAGTACAATTTATA-3), and MO2. Note that the transcripts are maternally deposited into the eggs. Zygotic expression of exdpf starts at around shield stage and increases during subsequent embryogenesis. A strong level of exdpf expression can be detected at 2 dpf and lasts until 5 dpf, the longest time point of this analysis.

Supporting Information

Figure S1. Vertebrate Orthologs of Exdpf are Highly Conserved (A) Peptide sequences of zebrafish Exdpf and Exdpf (Endpfr) aligned with mouse and human c20orf149 proteins using the ClustalW WWW Service at the European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw; (Thompson et al., 1994). ‘:’ indicate positions that have a single, fully conserved residue. ‘*’ and ‘.’ indicate positions that have strong (·) and weak (·) similarities. Dashes indicate gaps.

Figure S2. RT-PCR Result of exdpf During Embryogenesis Note exdpf can be detected at one-cell stage, indicating that the transcripts are maternally deposited into the eggs. Zygotic expression of exdpf starts at around shield stage and increases during subsequent embryogenesis. A strong level of exdpf expression can be detected at 2 dpf and lasts until 5 dpf, the longest time point of this analysis.

Figure S3. Exdpf Antisense Morpholino Oligonucleotide MO2 Inhibits Exocrine Pancreas Formation (A and B) In situ hybridization using a carboxypeptidase A (cpa) probe. (A) A wild-type (WT) zebrafish injected with 2 ng of standard morpholino oligonucleotide control. White arrow head: cpa expression in pancreas. (B) An embryo injected with 2 ng of exdpf MO2. Note severely reduced expression of cpa (white arrow). (C) An embryo injected with 4 ng of exdpf MO2. (D) An embryo injected with 6 ng of exdpf MO2. Found at doi:10.1371/journal.pbio.0060293.s002 (165 KB TIF).

Figure S4. Predicted Domains of Exdpf Protein PROSITE (http://www.expasy.org/prosite) was used to predict functional domains in Exdpf protein. The predicted domains and phosphorylation site as well as ATP binding site are listed.

Found at doi:10.1371/journal.pbio.0060293.s004 (949 KB TIF).
Figure S5. **Exdpf** is Specifically Required for Exocrine Pancreas Formation but not Gut or Liver

(A–C) Expression of GFP MP760GFP transgenic fish. (A) A control embryo injected with standard control morpholino. Note strong GFP expression in the pancreatic area at 24 hpf (arrow) and 2 dpf (arrowhead). (B) An example of embryo injected with **exdpf** mRNA (100 pg). Note strong GFP expression in the pancreatic area at 24 hpf (arrow) and 2 dpf (arrowhead). **exdpf** expression in the gut is still comparable to that in control embryo. (C) An example of embryo injected with **exdpf** morpholino (2ng of MO1**°**). Note no strong GFP expression in the presumptive pancreatic area at 24 hpf (arrow). A weak GFP positive pancreatic bud can be observed at 2 dpf (arrowhead). **exdpf** expression in the gut is still comparable to that in the control and **exdpf** RNA injected embryos.

(D–G) In situ hybridization using a *ceruloplasmin* probe with 3 dpf embryos. (D) A control embryo injected with standard morpholino control. (TUNEL assay result in 5 dpf embryos. (D–G) In situ hybridization using a *ceruloplasmin* probe with 3 dpf embryos. (D) A control embryo injected with standard morpholino control. (E–G) Examples of embryos injected with **exdpf** morpholino. Note comparable expression of *ceruloplasmin* in all embryos. Found at doi:10.1371/journal.pbio.0060293.sg005 (6.2 MB TIF).

Figure S6. Reduced **exdpf** Does not Affect Apoptosis in the Developing Pancreas

(TUNEL assay result in 5 dpf embryos. (A–C) A control embryo injected with standard morpholino control. Enlargement: of boxed area in (C). Note a few cell debris representing cells undergoing apoptosis (arrows in C enlargement).

(D–F) An embryo injected with 100 pg of **exdpf** mRNA. Enlargement: of boxed area in (F). Note comparable number of cells undergoing apoptosis (arrows in F).

(G–I) An embryo injected with **exdpf** morpholino. Enlargement: of boxed area in (I). Note both exocrine cells (arrows) and non-exocrine cells (arrowhead) undergoing apoptosis. Lateral view, anterior to the left. Scale bar: 50 μm.

(J) A quantitative graph of TUNEL assay. Found at doi:10.1371/journal.pbio.0060293.sg006 (2 MB TIF).

Figure S7. Reduced **exdpf** Specifically Increases β2i Expression in the Pancreatic Area

In situ hybridization using a p21 probe.

(A) A control embryo injected with standard morpholino control. Note p21 expression in the head area. No p21 expression is detected in the pancreatic area.

(B) An embryo injected with **exdpf** mRNA (100 pg). Note p21 expression in the head area. No p21 expression is detected in the pancreatic area.

(C) An embryo injected with **exdpf** morpholino. Note p21 expression in the pancreatic area (arrow). Found at doi:10.1371/journal.pbio.0060293.sg007 (3.5 MB TIF).

Figure S8. Examples of *preproinsulin*GFP Embryos Used for Cell Counting

Green: *preproinsulin*GFP. Blue: DAPI staining. All embryos are at 24 hpf, de-yolked and flat mounted.

Found at doi:10.1371/journal.pbio.0060293.sg008 (6 MB TIF).

Figure S9. Expression of Human **exdpf** in Normal Tissues and in Tumors

Human **exdpf** ortholog is expressed in different organs including colon, kidney, liver, and pancreas. Relatively higher level of **exdpf** has been detected in several tumors including colorectal cancer, kidney tumor, liver tumor, and pancreatic tumor.

Found at doi:10.1371/journal.pbio.0060293.sg009 (741 KB TIF).

Table S1. Primers Used for RT-PCR and Real-Time PCR

The sequences of forward and reverse primers and product length for each gene tested are included.

Found at doi:10.1371/journal.pbio.0060293.st001 (27 KB DOC).

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**Author contributions.** ZJ, JS, and SL conceived and designed the experiments. ZJ, JS, FQ, AX, and XA performed the experiments. ZJ, JS, BL, and SL analyzed the data. N-AI and ZZ contributed reagents/materials/analysis tools. JS, BL, and SL wrote the paper.

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**Competing interests.** The authors have declared that no competing interests exist.

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