Hepatocyte Growth Factor Signaling in Intrapancreatic Ductal Cells Drives Pancreatic Morphogenesis

Ryan M. Anderson1a,b, Marion Delous1b, Justin A. Bosch1, Lihua Ye2, Morgan A. Robertson2, Daniel Hesselson1c, Didier Y. R. Stainier1a,b*

Department of Biochemistry and Biophysics, Programs in Developmental and Stem Cell Biology, Genetics and Human Genetics, Liver Center and Diabetes Center, University of California San Francisco, San Francisco, California, United States of America, 1Herman B. Wells Center for Pediatric Research, Department of Pediatrics, Indiana University School of Medicine, Indianapolis, Indiana, United States of America

Introduction

The vertebrate pancreas is an endodermal organ that is part endocrine, releasing hormones that regulate glucose metabolism, and part exocrine, releasing pancreatic juices that aid in digestion. Pancreatic endocrine and exocrine developmental dysmorphogenesis and dysregulation, including diabetes mellitus and pancreatic adenocarcinoma, can result in human diseases with high morbidity and mortality. Thus, a more sophisticated understanding of molecular mechanisms mediating pancreatic development and homeostasis will certainly refine the treatment of these diseases.

In zebrafish as in mammals, all pancreatic endocrine and exocrine tissues derive from the fusion of a dorsal and ventral bud arising at the level of somites 2–9 [1,2,3]. In zebrafish, the dorsal bud generates the principal islet by 24 hours post fertilization (hpf), and fuses with the emerging ventral bud between 40–44 hpf [4,5]. Around 52 hpf, acinar and ductal cells start to expand caudally to form the tail of the pancreas [5,6,7]. The pancreatic mesenchyme is essential for the induction, growth, branching, and cytodifferentiation of the pancreatic epithelium [8]. While several mesenchymal signals mediating pancreatic induction have been identified (reviewed in [9]), our knowledge of how the mesenchymal/epithelial signaling pathways regulate pancreatic growth and branching is more limited [8].

Hepatocyte Growth Factor (HGF) is a stromally-produced ligand which binds Met, a receptor tyrosine kinase that is predominantly expressed in epithelia. Upon receptor dimerization and autophosphorylation, Met activates a bevy of cellular processes including motogenesis, tubulogenesis, mitosis, chemotaxis, and cell survival [10]. During organogenesis, HGF/Met signaling has been shown to be involved in liver and placenta formation, as well as in the migration of hypaxial muscle precursors into limbs [11,12,13,14]. However, the role of HGF/Met signaling in vertebrate pancreas development remains unclear. Both HGF and Met are expressed in the developing rodent pancreas [15,16], but pancreatic phenotypes have not been characterized in global knockout mice. Studies have been mostly focused on the role of HGF/Met signaling in pancreatic tumorigenesis and beta-cell survival. Indeed, pancreas-specific Met knockout mice are euglycemic and morphologically unaltered.

Abstract

In a forward genetic screen for regulators of pancreas development in zebrafish, we identified donut<sup>s908</sup>, a mutant which exhibits failed outgrowth of the exocrine pancreas. The s908 mutation leads to a leucine to arginine substitution in the ectodomain of the hepatocyte growth factor (HGF) tyrosine kinase receptor, Met. This missense mutation impedes the proteolytic maturation of the receptor, its trafficking to the plasma membrane, and diminishes the phospho-activation of its kinase domain. Interestingly, during pancreatogenesis, met and its hgf ligands are expressed in pancreatic epithelia and mesenchyme, respectively. Although Met signaling elicits mitogenic and migratory responses in varied contexts, normal proliferation rates in donut mutant pancreata together with dysmorphic, mislocalized ductal cells suggest that met primarily functions morphogenically in pancreatic tail formation. Treatment with PI3K and STAT3 inhibitors, but not with MAPK inhibitors, phenocopies the donut pancreatic defect, further indicating that Met signals through migratory pathways during pancreas development. Chimera analyses showed that Met-deficient cells were excluded from the duct, but not acinar, compartment in the pancreatic tail. Conversely, wild-type intrapancreatic duct and “tip cells" at the leading edge of the growing pancreas rescued the donut phenotype. Altogether, these results reveal a novel and essential role for HGF signaling in the intrapancreatic ducts during exocrine morphogenesis.

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* E-mail: ryanande@iu.edu (RMA); didier.stainier@mpi-bn.mpg.de (DYRS)

a Current address: Herman B Wells Center for Pediatric Research, Department of Pediatrics, Indiana University School of Medicine, Indianapolis, Indiana, United States of America.

b Current address: Department of Developmental Genetics, Max Planck Institute for Heart and Lung Research, Bad Nauheim, Germany.

c Current address: Garvan Institute of Medical Research, Metabolic Diseases Division, Sydney, New South Wales, Australia.
Author Summary

The pancreas functions as an endocrine and exocrine gland that secretes hormones regulating blood glucose homeostasis, and pancreatic juice that aids the digestion and absorption of nutrients, respectively. Contrary to endocrine tissue development, that of the exocrine pancreas has received less attention. We conducted a forward genetic screen in zebrafish and identified HGF/Met signaling as a key regulator of exocrine development. We called the mutant donut because the body of the pancreas fails to elongate and thus remains rounded. The mutation leading to this phenotype affects the extracellular domain of Met, the hepatocyte growth factor (HGF) receptor, impairing its maturation, plasma membrane localization and phospho-activation. Although HGF/Met signaling may elicit many context-dependant cellular responses, our data indicate that HGF/Met signaling triggers the migration, but not the proliferation, of the pancreatic ductal cells to drive the extension of the pancreatic tail.

Results/Discussion

Identification and genetic mapping of donut<sup>908</sup> mutants

To find novel regulators of endodermal organ morphogenesis and differentiation, we conducted a forward genetic screen utilizing doubly transgenic (2CLIP; 2-color liver, insulin, exocrine pancreas) zebrafish with EGFP-expressing acinar cells and DsRed-expressing pancreatic beta-cells and hepatocytes [20]. Using this approach, we identified donut<sup>908</sup> mutants, which show shortened (type 1) or absent (type 2) exocrine pancreatic (xp) tails. Tg(ptf1a:GFP)<sup>908</sup> [21] was crossed into the donut<sup>908</sup> background to reveal the short exocrine pancreas phenotype at 3 days post fertilization (dpf; Fig. 1A–E), prior to the terminal differentiation of acinar cells. Differentiation of the principal islet (beta-cells), beta, acinar and ductal cells, and liver (l) appeared unaffected in donut mutants (Fig. 1B,C; and see below). Nearly 25% of progeny from heterozygous intercrosses showed the donut phenotype, evenly split between types 1 and 2 (Fig. 1F). The variable expressivity suggested either a hypomorphic mutation in donut, and/or a genetic interaction with other loci.

To isolate the molecular lesion responsible for the donut phenotype, we used bulk segregant analysis and localized donut on linkage group 25; we then used z-markers and customized CA-repeat markers to define a critical interval containing 14 candidate genes (Fig. 1G). In sequencing these candidates, we identified a T2324G variant in the coding sequence of the met gene, resulting in an L775R missense substitution (Fig. 1H). The Met<sup>L775R</sup> substitution in donut<sup>908</sup> mutants represents a significant shift in amino acid charge and polarity at a residue that is conserved in vertebrates as a leucine or an isoleucine (Fig. 1I). Importantly, L775 is localized to the IPT3 (immunoglobulin-like regions in plexins and transcription factors 3) domain in the Met ectodomain, which together with IPT4 comprises a high affinity HGF binding site (Fig. 1L). [22,23]. Moreover, protein domain modeling based on human MET IPT3 indicates that the analogous residue, I777, resides in a solvent inaccessible region of IPT3, and that its substitution with arginine may be sterically unfavorable (Fig. 1J,K), suggesting that Met folding may be compromised in zebrafish donut mutants.

We hypothesized that the donut<sup>908</sup> mutation would result in a Met loss of function. To test this hypothesis, we injected zygotes with translation-blocking met morpholinos (metMO). We found that even low doses of metMO (2 ng) could reproduce the shortened pancreatic tail phenotype observed in donut mutants with high penetrance (Fig. 1M,N). Importantly, when donut<sup>908/908</sup> embryos were injected with a near-threshold (1 ng) dose of MO, 53.4% displayed a short pancreas phenotype, as compared to 29.1% of injected wild-type embryos (Fig. 1S1). Since HGF is the only demonstrated ligand for Met, we reasoned that knockdown of both zebrafish hgf paralogs, hfga and hgfb, should also mimic donut mutant phenotypes. Similar to donut mutants, we found that embryos injected with a mixture of MOs directed against hfga and hfgb (hgfaMO) also developed shortened pancreata (Fig. 1O,P). We observed a positive correlation between the injected quantity of each morpholino and the penetrance and expressivity of the pancreatic tail phenotypes (Fig. 1Q), with expressivity variation resolving towards the more severe type 2 phenotype at higher doses. Finally, donut mutants frequently lack muscle tone in the pectoral fins, suggesting a failed morphogenesis of hypaxial muscle (Fig. 1D,E, arrowheads; Fig. S2), which corroborates previous studies of Met function in myogenic precursor cells in mouse and zebrafish [11,12]. In sum, these data are consistent with the interpretation that the phenotypic spectrum observed in donut<sup>908</sup> mutants is due to a hypomorphic effect of the L775R substitution.

To clarify how HGF signaling is implemented in pancreatogenesis, we examined the expression of met, hfga and hgfb during several stages of pancreas formation. We also analyzed the expression of both furin genes, furina and furinb, which encode the proteases that cleave Met into a mature form. At 34 hpf, which is prior to pancreatic bud fusion, met is expressed throughout the endodermal organ forming region, including pancreas, liver, and intestine anlagen (Fig. 2A). Additionally, met expression is detected in pancreatic endoderm before (44 hpf), during (52 hpf), and after its caudal extension (80 hpf; Fig. 2A). We found that hfga and hgfb were expressed adjacent to the dorsal pancreatic bud at 34 hpf, and that their expression remains in register with the leading edge of the growing pancreatic tail (red arrowhead) between 52 and 80 hpf (Fig. 2B,C). Importantly, both furina and furinb are expressed in endodermal tissue throughout pancreatogenesis, with furina present in liver and pancreas at 75 hpf (Fig. S3), indicating that the process of Met maturation is active in these organs. To determine which exocrine tissues express met during pancreatic tail morphogenesis, we performed fluorescent in situ hybridization in the Tg(nkx2.2a:3.5 kb:GFP) line (hereafter duct:GFP) which highlights the intrapancreatic ducts (IPDs) [6]. We found that within the pancreatic tail, met was expressed in both IPD and acinar cells at 60 hpf, a time of active pancreatic tail extension (Fig. 2D-F). A role for Met signaling in pancreatic exocrine morphogenesis has not been reported in mammalian model systems. Hgf and Met knockout mice die during organogenesis precluding analysis [11,13,14], and a pancreas specific knockout (PancMet KO) showed no clear morphological defect of the adult pancreas [18]. However, as pancreas development was not described in PancMet KO, it is possible that a transient phenotype or developmental delay could have been overlooked. Furthermore, it is likely that early mosaic recombine activity inherent to the Pdx1-Cre line that was utilized in these experiments leaves a population of wild-type cells in these mice. These cells may be competent to effect normal morphogenesis, and would mirror what we observed in chimera studies (see below). Alternatively,
residual Met protein, which is expressed in endoderm prior to the onset of Pdx1 expression, or a parallel/redundant mechanism could drive exocrine outgrowth in mammals. Additional targeted studies are needed in mice to determine whether the role of Met in exocrine pancreas growth is conserved.

MetL775R shows impaired plasma membrane targeting and activation

To model how the donut mutation affects Met function, we generated an I776R point mutation in murine Met, an analogous lesion to the donuts908 mutation, and then transfected either MetWT or MetI776R into TOV112D cells. This cell line lacks endogenous Met activity, but expresses the intracellular components of the Met signaling pathway [22,24]. Met is translated as a single polypeptide chain which is cleaved by furin into alpha and beta chains [25]. In TOV112D cells transfected with MetWT, most of the nascent polypeptide was cleaved to the mature form (150 kDa + 30 kDa), while a small proportion of the receptor remained unprocessed (190 kDa). Since the I776R lesion lies far from the furin cleavage site (residues 302–307) [26], we were surprised to find that the MetI776R precursor polypeptide failed to be cleaved (Fig. 3A, bottom panels). Since cleavage-incompetent Met mutants (i.e.
MetL775R cannot signal normally [27], we next assessed the signaling efficacy of MetWT and MetL775R using specific anti-phosphorylated Met antibodies directed against several key tyrosine residues. Upon HGF binding, Met dimerizes and its cytoplasmic catalytic region is activated by trans-phosphorylation of tyrosines Y1234 and Y1235. Subsequently, residues Y1349 and Y1356 are phosphorylated in the Met multifunctional docking site, which connects Met to multiple downstream branches, including Ras/MAPK and PI3K, through several adapter proteins. In addition, phosphorylation of tyrosine Y1003 negatively regulates Met signaling by promoting receptor turnover. Compared to MetWT, phosphorylation of Y1234-5, Y1349, and Y1003 were significantly diminished in MetL775R (Fig. 3A,B). Finally, since cleavage by Furin occurs in the Golgi apparatus [28], we hypothesized that intracellular trafficking of MetL775R from the endoplasmic reticulum to the plasma membrane was impaired. To investigate this hypothesis, we next examined the presentation of Met at the plasma membrane using an antibody that binds specifically to the Met ectodomain (anti-Met ab1). Antibody staining was evident at the membrane of unfixed, unpermeabilized HEK293T cells expressing MetWT or cleavage incompetent MetK306A, but not MetL775R (Fig. 3C–E). However, all of these Met variants were detected in fixed and detergent-permeabilized cells using an antibody that recognizes an intracellular epitope (anti-Met ab2), thereby indicating similar translocation efficiencies of the three constructs (Fig. 3F–H). Thus, these data suggest that MetL775R is retained in an intracellular compartment, and that the lack of MetL775R cleavage per se is not causing this retention. To further test this hypothesis, RNA encoding zebrafish MetWT or MetL775R fused to mCherry were injected in zebrafish embryos and the localization of the proteins analyzed at blastula stages (Fig. 3I–J). As observed in cell culture, MetL775R was not detected at the plasma membrane in vivo. This defect is not simply a delay of membrane targeting, as similar results were observed at later time points (data not shown). Together, these data support the hypothesis that deficient signaling through MetL775R causes the hypomorphic effect observed in dontus908 mutants. Likely, reduced Met signaling is due to (1) localization of MetL775R away from the plasma membrane, (2) impaired binding of HGF to the high-affinity binding site in IPT3-4, and/or (3) impaired assembly of Met co-receptor moieties, that would then lead to defective phosho-activation of Met.

Cell migration but not proliferation is impaired by the donuts908 mutation

Growth and elongation of the pancreatic tail involves both proliferation and directed migration of exocrine cells [6,7]. To analyze which mechanism is impaired in donuts mutants, we first assayed cell proliferation using 30 min incorporation of the thymidine analog Edu at two time points during pancreatic tail growth: 56 and 75 hpf (Fig. 4A–D). Quantification of labeled cells showed no significant difference in exocrine cell proliferation between WT and donuts mutant animals (Fig. 4E). To determine whether acinar or ductal cell migration was affected in donuts mutants, we next examined the distribution of acinar and ductal cells using ptf1a:GFP and duct:GFP, respectively, in 84 hpf wild-type (WT) and metMO-injected (Fig. 4F–I) larvae. Although ptf1a:GFP+ acinar cells were always confined to the pancreas, duct:GFP+ cells were clearly localized in the hepatic region in both type 1 and type 2 mutants. Furthermore, while IPD cells were elongated and spindle-shaped in WT, they showed a more rounded morphology in metMO-injected larvae (Fig. 4H,I, insets). We marked hepatocytes and biliary ducts with Prox1 and Alcam antibodies, respectively, and found that duct:GFP+ pancreatic cells had extensively invaded the liver, tracking along the biliary duct network (Fig. 4J,K). Taken together, our data show that HGF/Met signaling promotes invasive cell behavior during pancreatic tail morphogenesis, rather than the proliferation of exocrine cells.

The invasive cell behaviors activated by HGF/Met signaling, such as motility and proliferation, work through multiple parallel downstream branches of Met signaling. Three key branches involve the PI3K, ERK, and STAT3 pathways, which may be activated by direct interaction with Met, or through adapter proteins: the PI3K/Akt pathway elicits cell migration via Rac1, as well as cell survival; the ERK branch promotes proliferation and transformation; and STAT3 signaling mediates branching morphogenesis and proliferation [reviewed in (10)]. To dissect which specific branches might be critical for Met-mediated exocrine...
Figure 3. Orthologous murine donut mutation, I776R, impairs HGF signaling and receptor trafficking. (A) Immunoblots of Met protein prepared from lysates of TOV112D cells transfected with murine MetWT or MetI776R. Upper panels blotted with anti-pY show diminished phosphorylation at tyrosines critical for signaling and lower panels blotted with anti-Met show loading controls. Precursor (white arrows) and mature (black arrows) forms of MetWT are both detected, but MetI776R is only present in the precursor form. (B) Quantification by densitometry of phosphorylated Met versus total Met in panel A (t test, * p<0.05; **p<0.01). (C–H) Immunofluorescence staining of HEK293 cells transfected with MetWT (C,F), MetI776R (D,G), or furin cleavage-incompetent MetR306A (E,H). Unfixed, unpermeabilized cells were stained with anti-Met ab1, which binds an extracellular epitope (C–E), and fixed, permeabilized cells were stained with ab2, which binds an intracellular epitope (F–H). MetWT and MetR306A

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are localized to the plasma membrane (C, E, insets in F, H), but MetI776R is not (D), and is mostly retained in the cytoplasm (G, inset). Intracellular staining of Met demonstrates similar transfection efficiency. DAPI staining (blue) marks nuclei. (I–J) Live imaging of zebrafish blastulae injected with zebrafish MetWT-mCherry (I) and MetL775R-mCherry (J) at 5 hpf. Zebrafish MetL775R-mCherry largely fails to localize to the plasma membrane as compared to MetWT. Scale bars, 20 μm.

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morphogenesis, we treated larvae during the formation of the pancreatic tail with specific inhibitors (Fig. 4L). Inhibition of ERK signaling with the MEK inhibitor UO126 or the p38 MAPK inhibitor SB203580 resulted in a mild phenocopy of donut type 1 and type 2 mutants, ranging from 25–40%. However, treatment with the potent PI3K inhibitor LY294002 or the STAT3/Src family kinase inhibitor SU6656 generated a nearly perfect phenocopy of type 1 and 2 donut mutants. These data confirm our previous observations that cell proliferation via MAPK/MEK is not the primary mechanism driving pancreatic tail outgrowth. The furin protease inhibitor CMK caused only a minor effect on pancreatic tail formation confirming that failure of proteolytic cleavage of Met may not cause the reduced signal transduction capacity of MetL775R.

Figure 4. Cell migration, but not proliferation, is dysregulated in donut908 mutants. (A–D) EdU incorporation assay in WT (A,C) and donut908 mutant (B,D) animals at 56 (A,B) and 75 (C,D) hpf. (E) Quantification of proliferation assay data shows no significant change in acinar cell proliferation at early or late stages of pancreatic tail formation. (F–I) Morphology of exocrine tissues at 84 hpf in WT (F,H) and metMO-injected (G,I) larvae revealed by Tg(ptf1a:GFP) (acinar; F,G) or Tg(nkx2.2a(-3.5 kb):GFP) (duct; H,I) expression. In metMO-injected embryos, acinar and ductal cells fail to migrate caudally, and remain near the principal islet (β). In metMO larvae, ductal cells exhibit a more rounded morphology in the exocrine pancreas (insets) and are also observed in the liver region (asterisk). (J,K) 84 hpf WT (J) and metMO-injected (K) Tg(duct:GFP) larvae stained with Prox1 (blue) and Alcam (red). Pancreatic ductal cells are ectopically localized in the liver (li) along tracts of biliary ducts. (L) Quantification of pancreatic tail defects in small molecule treated larvae; inhibitors of the MAPK pathway (UO126, SB203580) and furin function (CMK) had little effect on pancreatic tail outgrowth, while inhibitors of PI3K (LY294002) and STAT3 (SU6656) function mimicked the type 1 and type 2 donut phenotypes. epd, extrapancreatic duct.

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Met signaling is autonomously required in IPD cells and “tip cells” for pancreatic tail elongation

The differentiating exocrine pancreas is highly organized at the onset of pancreatic tail elongation [6,7]. Acinar and ductal cells are the only two cell types found in the growing pancreatic tail, and they are always closely associated. Even though met is expressed in both cell types, we hypothesized that it was required primarily in ductal cells, since these cells were mislocalized and dysmorphic in metMO-injected larvae. To test this hypothesis, we performed cell transplantation experiments to generate chimeras and determine the autonomy of Met function in the pancreas (Fig. 5A). First, blastomeres isolated from control or metMO-injected Tg(hsc:mCherry) (see Methods) embryos were directed toward an endodermal fate by cas mRNA coinjection [29,30], and were transplanted into WT hosts. Incorporated donor cells ubiquitously express mCherry RFP upon heat shock induction, and were quantified based on their contribution to pfl1a::GFP—ductal cell compartments in the distal or proximal portion of the exocrine pancreas (Fig. 5B). In contrast to WT cells that contributed evenly to both acinar and ductal compartments, metMO-injected cells were strongly biased toward the acinar cell and proximal ductal compartments, and were largely excluded from the distal ductal compartment (Fig. 5C–E; Tables 1,2). We observed similar results when we transplanted endodermal cells from donut mutant embryos into Tg(hsc:mCherry) hosts (data not shown). Next, we assessed the rescue of exocrine pancreatic tail outgrowth by transplanting varying numbers of WT Tg(hsc:mCherry) cells into the endoderm of metMO-injected hosts (Tables 3,4). metMO-injected larvae lacking WT contribution showed a type 1 donut phenotype (Fig. 5F), whereas a high contribution of WT endoderm cells led to a complete rescue of the pancreatic tail outgrowth (Fig. 5G). Notably, the tip of the rescued pancreatic tail appeared to be composed exclusively of WT donor cells (Fig. 5G, inset). In larvae with fewer integrated WT cells, the pancreatic tail was also rescued, with the WT cells being preferentially found in the IPD (Fig. 5H, inset). Finally, we confirmed that WT endoderm could not similarly rescue the short exocrine pancreas phenotype observed in hgfMO-injected embryos, consistent with a mesenchyme-specific role for hgfα and hgfβ (Fig. S4). These data indicate that endodermal Met signaling, preferably preferentially in distal IPD cells, directs the caudal extension of the pancreatic tail.

We thus propose a model for pancreatic tail outgrowth in which ductal cells would respond to HGF secreted from the mesenchyme adjacent to the pancreatic primordium and initiate migration caudally. The HGF signal may constitute a chemotactic signal for the ductal cells, or simply a motogenic signal, with directionality imparted by distinct signaling pathways. Even though Met signaling appears to be required in IPD cells for pancreatic outgrowth, Met signaling may also be active in a small population of “tip” cells that are mature or differentiating acinar cells. From the analysis of a specific subset of chimeric embryos, ductal cells may thus be directing the migration of the pancreatic tail from a

Figure 5. HGF/Met signaling is required in intrapancreatic ducts for pancreatic tail morphogenesis. (A) Schematic of cell transplantation experiments. WT or metMO-injected donor cells were transplanted into WT hosts (top), and WT donor cells were transplanted to metMO-injected hosts (bottom); cas mRNA injection biased donor cells toward endodermal differentiation. (B) Scheme used to identify and quantify contribution of transplanted cells in chimeras. Donor cells are marked by Tg(hs:mCherry) (red), acinar cells are marked by Tg(ptf:GFP)h1 (blue), and Alcam immunostaining (green) delineates the ducts. (C–E) Single plane confocal images of WT (C) and metMO–injected larvae: (D) high contribution of WT donor cells (inset), the pancreatic tail morphology can be rescued with only WT donor cells (inset of single confocal slice); with moderate contribution of WT donor cells, the pancreatic tail growth is rescued (G), and the tip of the tail is almost entirely comprised of WT donor cells (inset of single confocal slice, with moderate contribution of WT donor cells, the pancreatic tail morphology can be rescued with only WT ductal cells (H), and no WT acinar cells are observed in the tip (inset of single confocal slice).

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Table 1. Endodermal contributions in WT->WT chimeric embryos.

| donor contribution | prox. duct (n, %) | dist. duct (n, %) | prox. acinar (n, %) | dist. acinar (n, %) |
|--------------------|------------------|------------------|-------------------|-------------------|
| high               | 4/4, 100%        | 4/4, 100%        | 4/4, 100%         | 4/4, 100%         |
| low                | 1/1, 100%        | 2/3, 67%         | 0/1, 0%           | 2/2, 100%         |
| total*             | 5/5, 100%        | 6/7, 86%         | 4/3, 80%          | 6/6, 100%         |

Abbreviations: prox., proximal; dist., distal.

*Not every chimeric pancreas could be clearly assessed in all regions due to variations in mounting technique and limitations of confocal microscopy. Ambiguous samples were excluded from analysis.

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trailing position, possibly exhibiting cellular extensions, such as cytonemes or filipodia, that were not resolved in our studies. Hence, our data expand the understanding of the role of HGF/Met signaling in mitogenic, motogenic and morphogenetic events required for the development, homeostasis and regeneration of different tissues. In addition, they provide a developmental framework to dissect the role of Met in pancreatic cancer stem cells [31,32].

Materials and Methods

Zebrafish strains

Fish were raised and maintained under standard conditions [33]. Pigmentation was inhibited with 0.02 mM phenylthiourea (Sigma). We used the following published strains: Tg(fabp10:2RFp, ela3l:EGFP)g22 [34], Tg(ins:CFP-NTR)s892 [35], Tg(els:Cerulean) [36], Tg(nlx2.2a(-3.5 kb):GFP)mm3 (a.k.a. “duct:GFP”) [6], and Tg(ptf1a:xGFP)mm1 [21]. To generate the Tg(hsp70l:loxP-mCherry-loxP-H2B-GFP-)cryaa:Cerulean line (a.k.a. Tg(hs:mCherry), H2B-GFP was replaced in the plasmid hsp70l:loxP-mCherry-loxP-H2B-GFP-cryaa:Cerulean [37] by an in-frame PCR fusion product of NICD [38], P2A [39], and Emerald GFP (Invitrogen, Carlsbad, CA). Transgenesis was achieved as described [40].

Genetic screen and mapping

An ENU mutagenesis screen was executed as described [20]. Bulk segregant analysis was performed using pooled DNA extracted from 20 WT or donut mutant embryos. donut mutants were subsequently genotyped using a dCAPS strategy: a PCR product amplified by the primer set: 5’-CGTTT GTGTG GGTTG TATAG-3’ and 5’-TCCAG CCCAA-3’. For transgenesis, ES cells (HEK293T) were plated on polylysine-coated coverslips and transfected with lipofectamine with either murine pBabe-puro-MetWT (Addgene) or site directed mutagenesis-generated pBabe-puro-MetI776R. Cells were transfected 48 h before immunostaining; unfixed cells, as well as 4% paraformaldehyde-fixed and 0.1% Triton-permeabilized cells were incubated before immunostaining: unfixed cells, as well as 4% paraformaldehyde-fixed and 0.1% Triton-permeabilized cells were incubated with indicated antibodies for 2 hours prior to incubation with appropriate fluorescent secondary antibodies.

Immunoprecipitation and Western blot analysis

TOV112D human ovarian carcinoma cells were obtained from ATCC (Manassas, VA) and cultured using a 1:1 mixture of MCDB 105 medium and medium 199 supplemented with 15% FBS. MetWT and MetI776R were transfected into TOV112D cells with lipofectamine. Coimmunoprecipitations and protein blot analyses were performed as previously described [43]. The antibodies used for immunoprecipitation, protein blot and immunofluorescence are: anti-Met (25H2) and anti-phosphoMet (Cell Signaling), goat anti-Met (extracellular epitope, R&D Systems), rabbit anti-Met (SP260 – intracellular epitope, Santa Cruz).

Embryo treatment with pathway specific inhibitors

Tg(nlx2.2a(-3.5 kb):GFP)mm3; Tg(els:Cerulean) embryos, distributed in 12-well plates (20 embryos per well), were incubated with different chemicals from 60 to 76 hpf, during the growth of the pancreatic tail. Chemicals were specific inhibitors of MEK

| Table 2. Endodermal contributions in metMO>WT chimeric embryos. |
|---|
| donor contribution | prox. duct (n, %) | dist. duct (n, %) | prox. acinar (n, %) | dist. acinar (n, %) |
| high | 4/10, 40% | 2/10, 20% | 9/10, 90% | 8/10, 80% |
| low | 7/14, 50% | 2/14, 14% | 12/14, 86% | 12/14, 86% |
| total* | 11/24, 46% | 4/24, 17% | 21/24, 88% | 20/24, 83% |

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| Table 3. Endodermal contributions in WT>metMO-injected chimeric embryos. |
|---|
| donor contribution | prox. duct (n, %) | dist. duct (n, %) | prox. acinar (n, %) | dist. acinar (n, %) |
| high | 15/16, 94% | 16/18, 89% | 16/16, 100% | 18/18, 100% |
| low | 4/7, 57% | 7/9, 78% | 1/7, 14% | 1/9, 11% |
| total* | 19/23, 83% | 23/27, 85% | 17/23, 74% | 19/27, 70% |

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HGF Signaling in Exocrine Pancreas Formation
Embryo microinjection and cell transplantation

The morpholinos (Gene Tools) used for gene knockdown were described in [12,44]. For experiments at the blastula stage, mRNAs encoding zebrafish wild-type or L775R Met fused to desmin were injected with 200 pg of mRNA was synthesized in vitro, and donor and furin (CMK, at 65 µM). Cell transplantations were performed as described [42]. When cas-injected mutant donor cell were transplant- ed, donors were genotyped after transplantation.

Supporting Information

Figure S1 donut mutants variably lack muscle tone in pectoral fins. (A-C) 9 dpf Tg(fgf11a:GFP) larvae imaged from dorsal aspect to show fin morphology (arrows). In WT larvae, fins are folded closely against the body (A), while in type 1 (B) and type 2 (C) donut mutant larvae, the pectoral fins lack tone, and display an open wing configuration. Bar = 0.5 mm. (TIF)

Table 4. Breakdown of rescued embryos by WT tissue contribution.

|     | total (n, %) | duct only (n, %) | acinar only (n, %) | duct & acinar (n, %) |
|-----|-------------|-----------------|-------------------|---------------------|
| 19/27 | 70%        | 5/19, 26%       | 1/19, 5%          | 13/19, 68%          |

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