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Review

Translating Discovery in Zebrafish Pancreatic Development to Human Pancreatic Cancer: Biomarkers, Targets, Pathogenesis, and Therapeutics

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Abstract

Experimental studies in the zebrafish have greatly facilitated understanding of genetic regulation of the early developmental events in the pancreas. Various approaches using forward and reverse genetics, chemical genetics, and transgenesis in zebrafish have demonstrated generally conserved regulatory roles of mammalian genes and discovered novel genetic pathways in exocrine pancreatic development. Accumulating evidence has supported the use of zebrafish as a model of human malignant diseases, including pancreatic cancer. Studies have shown that the genetic regulators of exocrine pancreatic development in zebrafish can be translated into potential clinical biomarkers and therapeutic targets in human pancreatic adenocarcinoma. Transgenic zebrafish expressing oncogenic *K-ras* and zebrafish tumor xenograft model have emerged as valuable tools for dissecting the pathogenetic mechanisms of pancreatic cancer and for drug discovery and toxicology. Future analysis of the pancreas in zebrafish will continue to advance understanding of the genetic regulation and biological mechanisms during organogenesis. Results of those studies are expected to provide new insights into how aberrant developmental pathways contribute to formation and growth of pancreatic neoplasia, and hopefully generate valid biomarkers and targets as well as effective and safe therapeutics in pancreatic cancer.

Introduction

The goal of this article is to review the biological events and their genetic regulation in exocrine pancreas during development in zebrafish, and discuss the potential of translating discovery in the zebrafish system into preclinical and clinical investigation of human pancreatic cancer. The developmental pathways that control organogenesis play key roles in the multistep carcinogenesis in vertebrate organs, including the pancreas.[1–3] Adenocarcinoma, which resembles the morphological appearance of glands in normal exocrine pancreas, is the predominant type of histopathology of human pancreatic cancer.[4] The mechanisms that control cell division, cytodifferentiation, growth, and migration in exocrine pancreatic epithelia during morphogenesis are expected to be important in development of malignant neoplasia during pancreatic tumorigenesis. However, the genetic regulators of normal and cancerous growth of pancreas remain to be identified and functionally characterized. How the developmental regulators of pancreas contribute to the various steps during initiation of pancreatic neoplasia and progression into invasive adenocarcinoma is still poorly understood. Translation of the developmental findings into clinical biomarkers, molecular targets, and antitumor therapeutics in pancreatic cancer has begun to show promising potentials. Accumulating evidence indicates that developmental studies of the pancreas provide mechanistic insights into pathogenesis of pancreatic tumor, and facilitate development and validation of biomarkers and targets in pancreatic cancer.

Understanding the mechanisms that regulate pancreatic development has been facilitated by studies in model organisms, such as mouse, rat, frog, chick, and fish. Zebrafish (*Danio rerio*) is an established model of vertebrate biology as well as human diseases.[5] With its evolutionarily conserved features and its unique properties like small size, fecundity, external fertilization, and transparency, zebrafish offers advantages for morphologic, genetic, and biochemical studies. Indeed, the zebrafish system is complimentary to the rodent models for genetic analyses of the developmental processes. These include mapping of endodermal specification towards pancreatic cell fate and differentiation[6] as well as studies related to the mechanisms of human diseases.[5,7] The complimentarity of

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the zebrafish system and mouse model is further exemplified by the recent studies that utilize forward genetic screens and genome editing using transcription activator-like effector nucleases (TALENs) in combination with live real-time imaging of development in zebrafish. Not only do the results of these studies highlight the conserved developmental processes between zebrafish and mice, but they also show that these two animal models complement each other as disease models to study organogenesis and diseases.[8–10] The development of pancreas, both endocrine and exocrine, in mammals and zebrafish has previously been reviewed.[1,2,11,12] A focused review of development of the endocrine pancreas in zebrafish has recently been reported.[13] The descriptive and experimental studies indicate that the developmental processes in the pancreas of the zebrafish and the associated genetic regulation are mostly conserved as those in mammals.

The unique properties of the zebrafish system have enabled the discovery of novel genetic elements and their functional roles in development of exocrine pancreas. In this article, we focus on the development of exocrine pancreas in zebrafish and update with findings from recently published literature, followed by discussion of translation of discovery in the zebrafish system into various aspects of human pancreatic cancer. First, we will critically review the developmental biology and genetics of exocrine pancreas, including formation of anlage, cell fate specification, morphogenesis, proliferation, cytodifferentiation, and growth, and compare with those in human where appropriate. Next, the potential of translating the zebrafish studies into clinical biomarkers and therapeutic targets in human pancreatic cancer will be discussed. Lastly, we will assess the zebrafish models of pancreatic cancer and how the models can be exploited for understanding pathogenesis of pancreatic neoplasia and for drug discovery.

Development of Exocrine Pancreas in Zebrafish

In adult zebrafish, the pancreas is diffusely dispersed in the mesentery and within the intestinal loops.[1,14] The anatomy of adult zebrafish exocrine pancreas is similarly shown in transgenic zebrafish expressing green fluorescent protein under control of the *elastase A* promoter.[2,15] For comparison, the adult human pancreas develops as a solitary organ within the duodenal loops. However, the microscopic structure of adult pancreas in zebrafish is very similar to that of adult human (Fig. 1). The exocrine pancreas in adult zebrafish consists of pancreatic ducts and acini with the endocrine islets dispersed within the exocrine pancreatic tissue[1,16] (Fig. 1A). These morphological features are essentially the same as in adult humans (Fig. 1B). Further examination of exocrine pancreas in both larval and adult zebrafish under a transmission electron microscope shows that the pancreatic acini are composed of columnar epithelia. In the acinar cells, the nuclei are located in the basal cytoplasm and zymogen granules in the apical cytoplasm, with the centroacinar cell situated in the acinar lumen.[16] This evidence indicates that the basic structure of exocrine pancreas is mostly conserved in zebrafish and humans even at the ultrastructural level. A detailed comparison of the anatomy of pancreas in adult zebrafish and humans as well as during organogenesis is listed in Table 1. The overall structural similarities of pancreas in zebrafish and humans suggest highly conserved developmental biology and genetics as well as functions of the exocrine and endocrine pancreas in these organisms. Moreover, the morphogenetic processes during pancreatic organogenesis in zebrafish and human embryos are generally conserved at various developmental stages (Table 2). Given the anatomy and developmental processes of exocrine pancreas in zebrafish and human are highly similar, it is reasonable to speculate that the genetic regulators and biological mechanisms are shared between these organisms. These data provide support for zebrafish as a vertebrate model organism to dissect the molecular mechanisms that control pancreatic development and diseases.

In zebrafish, an orchestrated series of signaling events coordinately lead to the formation of a pancreas with exocrine and endocrine components. These signaling events (nodal, sequential, parallel, activating, or repressing) dictate the initial endoderm induction and continue through development of a fully functional pancreas. The early steps in the development of exocrine pancreas in zebrafish include (1) induction and patterning of endoderm and formation of anlage, (2) specification of cell fate, proliferation and cytodifferentiation

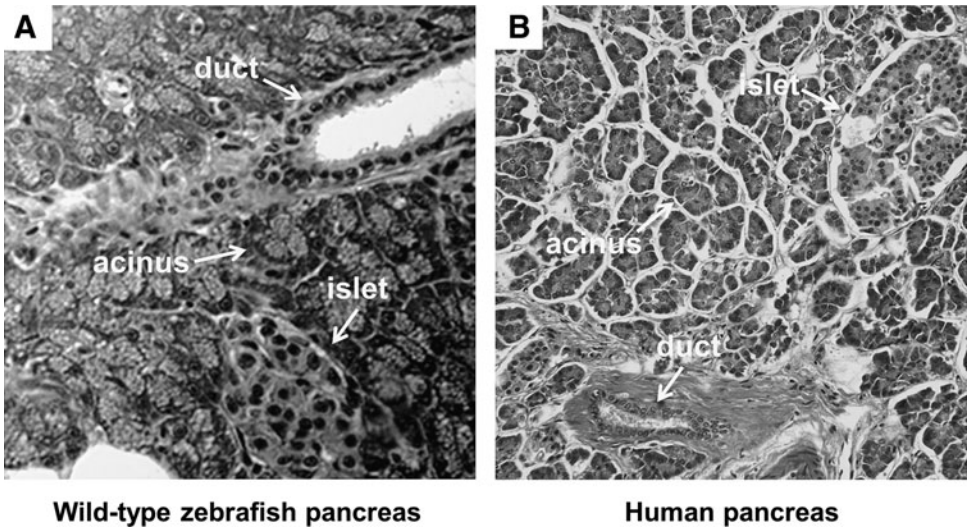

FIG. 1. Comparison of the microscopic anatomy of pancreatic tissues in adult zebrafish and human. Histological sections of **(A)** wild-type zebrafish and **(B)** human were stained with hematoxylin and eosin. The exocrine pancreas consists of ducts and acini, and the islets containing endocrine cells, are as indicated and highly similar between zebrafish and human.

Table 1. Comparison of the Anatomy of Pancreas in Zebrafish and Human

Anatomy	*Zebrafish*	*Human*
Adult		
Gross	• Diffusely distributed in mesentery[16] • Located among intestinal loops	• Solitary • Located between duodenal loops
Microscopic and ultrastructural	• Ducts are composed of cuboidal epithelia. • Acini are made up of columnar epithelia with zymogen granules in apical cytoplasm. • Centroacinar cells are located within acinar lumen. • Endocrine islets are dispersed throughout exocrine tissue[16]	
Embryo		
Gross	• Solitary in embryos and larvae	• Solitary
Microscopic and ultrastructural	• Ducts are composed of cuboidal epithelia • Acini are made up of columnar epithelia with zymogen granules in apical cytoplasm. • Centroacinar cells are located within acinar lumen	
	• During early morphogenesis, endocrine cells starts as a single islet in the head of pancreas. • Islets are composed of insulin-producing cells in periphery, and glucagon- and somatostatin-producing cells in center.[11,20]	• Endocrine islets dispersed throughout exocrine tissue. • Islets are composed of insulin-producing cells in periphery, and glucagon- and somatostatin-producing cells in center

During early embryogenesis in zebrafish (2 d.p.f. to at least 7 d.p.f.), the pancreas develops as a solitary organ like the human pancreas. Unlike human, the zebrafish endocrine pancreas begins morphogenesis as a single islet that proliferates to form multiple islets as observed in human. The pancreas of adult zebrafish and human differs in the gross anatomy; while the human pancreas continues to be solitary as in the embryo; the zebrafish pancreas grows into a diffused structure and is distributed throughout the mesentery. However, microscopically and at the ultrastructural level, the pancreas of zebrafish and human remain highly similar.

h.p.f., hours-post-fertilization; d.p.f., days-post-fertilization.

of progenitors, (3) morphogenesis of ducts and acini, and (4) growth of the organ. Each of these developmental steps is characterized by distinct morphological features and genetic requirements.

The endocrine and exocrine components of the zebrafish pancreas have distinct spatiotemporal origins that start off as first posterodorsal and second anteroventral buds (Fig. 2A). In zebrafish, as in other vertebrate organisms, both the endocrine and exocrine pancreas are derived from the endoderm. For induction of endoderm, cascades of signaling events are set in motion involving transforming growth factor-β, bone morphogenetic protein (Bmp), fibroblast growth factor(s) (Fgfs), and nodal-related 1 (Ndr1), and Ndr2.[6] These regulators induce a common endomesodermal territory via a concentration gradient and act through activin receptor 1b (Acvr1b). As a consequence, the transcription factors, such as Bon and Gata5 are activated and they in-turn regulate expression of Sox32, which mediates patterning of endoderm destined for pancreatic development.[11]

After endodermal specification, the next step in pancreatic development is anteroposterior regionalization of the endoderm that will become the digestive tract (Fig. 2B). The gut patterning events are regulated by the activity of multiple signaling pathways and transcription factors. These include Fgf(s), wingless-type mouse mammary tumor virus integration site family members (Wnt), Bmp, histone deacetylase 1 (Hdac1), pancreatic and duodenal homeobox 1 (Pdx1), sonic hedgehog (Shh), and pancreas-specific transcription factor 1a (Ptf1a). Wnt signaling has been shown to play an important role in cell fate, proliferation, and differentiation; all of which influence tissue patterning.[17] Expression of Wnt inhibits pancreatic growth while promoting hepatogenesis. Similarly, retinoic acid (RA) is important for patterning of anteroposterior endoderm and formation of pancreas. Fgf10 and Fgf24 are required for specification of the ventral pancreas. The TALE homeodomain genes Meis3 and Pbx4[18] act upstream of Shh (another key patterning gene in mammals) to inhibit endocrine development (in particular insulin expression).

The exocrine component of the zebrafish pancreas arises from endodermal progenitor cells that migrate from the primitive intestine to form the pancreatic anlage.[16,19] This process requires the activities of the evolutionarily conserved transcriptional factors, Pdx1 and Ptf1a.[20–23] These factors are also important for pancreatic development in humans and mice, and they are expressed in pancreatic progenitors. Both Ptf1a and Pdx1 are necessary and sufficient for pancreas development.[24] Loss of Ptf1a function in zebrafish (and in humans) results in complete absence of acinar cells[22,23] and significant reduction in insulin expression.[25] Similarly, lack of functional Pdx1 in humans results in pancreatic agenesis.[26] In zebrafish, the level of Ptf1a appears to be critical for determining the cell fate of pancreatic progenitors that will become exocrine and endocrine epithelia.[27] Fgf10 and Notch activate Ptf1a and Pdx1, which regulate differentiation of the exocrine pancreatic progenitors into acinar and ductal cells.[16,20,22,23,27–29] Besides, Hdac1 promotes exocrine pancreas specification and cytodifferentiation as well as endocrine islet morphogenesis.[30]

Proliferation of pancreatic progenitors and epithelia is controlled by various ligands and factors. Notch is required for proliferation of pancreatic progenitors, and expression of Ptf1a is dependent on the activity of Notch signaling.[16,31] Optimal activity of RNA polymerase III is critical for normal epithelial proliferation that is coordinately regulated with acinar and ductal morphogenesis.[2,16,32] The *exocrine*

Table 2. Correlation Between Zebrafish and Humans, Morphogenesis, Developmental Processes and Gene Expression As It Relates to Pancreatic Development at Various Developmental Stages

	Developmental stage			
General morphogenesis	*Zebrafish ZFIN stage*	*Human embryo (CS)*[a,53] *or [W]*	*Developmental process*	*Zebrafish gene expression*
Induction				
Early gastrulation	Shield (50% epiboly)	(CS 6)	Induction of the endoderm	*ndr1/2*, *gata5*, *smad2/4*[b]
Patterning				
Convergence of endoderm	Tail bud (100% epiboly)	(CS 8)	Early endoderm determination and patterning	*sox32*, *fgf24*[b]
Pancreas specific growth (Organogenesis)				
Formation of the bilateral pancreatic primordial	Segmentation (5–7 somite)	(CS 12)	Formation of endocrine precursors (pancreatic anlage)	*sox4b*[b]
Pancreatic primordia convergence	Segmentation (10 somite)	(CS 15); [6][12]	Appearance of ventral pancreatic bud	*pdx1*, *pax6b*, *nkx2.2a*[b]
Continued pancreatic primordia convergence	Segmentation (12 somite)	(CS 17); [8][54]	First appearance of endocrine β cells	*isl1*, *hnf1b*, *ins*[20,b]
Fusion of pancreatic primordia—complete	Segmentation (16 somite)	(CS 17); [8][54]	First appearance of endocrine δ cells	*somatostatin2*[b]
Formation of postero-dorsal endocrine islet	Pharyngula (28 h.p.f.)	(CS 17); [8][54]	First appearance of endocrine α cells	*glucagon a*[20,b]
Formation of antero-ventral pancreatic bud	Pharyngula (40 h.p.f.)	(CS 17)	Initiation of exocrine pancreas formation	*ptf1a*[22,b]
Initiation of bud fusion (engulfment)	Hatching (48 h.p.f.)	"unknown"	Continued exocrine differentiation, formation of epsilon and PP-cell	*ghrelin*, *trypsin*[16], *carboxypeptidase A5*[16,20,b]
Completion of bud fusion	Hatching (72 h.p.f.)	(CS 18)	Formation of duct and secondary endocrine cells	gene involved in further differentiation *ngn3*, *nkx2.2a* (duct formation)[b]
Well defined pancreas, with head and tail	Larval/Juvenile/ Adult (>72 h.p.f.)	(CS 20); [10]	Main and secondary islets	gene involved in further differentiation (expression of hormones and enzymes)[b]

References of the cited genes: available in this review and at the ZFIN database at www.zfin.org.
All emerging gene expressions are in zebrafish; where known human expressions are from ZFIN.[54]
Some of the data in this table are partly adapted and modified from Tiso *et al.*[11]
[a]Carnegie Stage (process start stage).
[b]not a complete list; additional and updated details available at www.zfin.org.
[w] = weeks post ovulation.

differentiation and *proliferation factor* (*exdpf*), a target gene of Ptf1a and a regulator downstream of RA, plays an important role in specification and proliferation of exocrine pancreatic epithelia.[33] The transient receptor potential melastatin-subfamily member 7 (Trpm7) ion channel regulates proliferation of exocrine pancreatic progenitors and epithelia through the Mg^{2+}-sensitive pathways that involve Socs3a.[34] Several highly conserved genes involved in ribosomal biogenesis, including the causal gene for Shwachman-Diamond syndrome (*sbds*), *ribosomal protein L3* (*rpl3*), and *pescadillo* (*pes*), have recently been shown to play important roles in pancreatic growth through the expansion of pancreatic progenitor cells.[7] The functional relationship among these genetic regulators of pancreatic progenitors and epithelia remains unexplored and it will be important to determine.

Besides, epigenetic mechanisms that modulate histone acetylation and DNA methylation have been shown to regulate various aspects of pancreatic development in zebrafish. Analysis of zebrafish larvae with loss-of-function in Hdac1 indicates that Hdac1 is critically required for the proliferation of exocrine pancreatic progenitors and epithelia.[35] The germline mutation *hdac1*^{hi1618} causes hyperacetylation of histones H3 and H4, and this is associated with upregulation as well as downregulation of a number of genes, including *p21*^{cdkn1a}.[35] The functional roles of these targets genes of Hdac1 in development of exocrine pancreas are mostly unexplored. Moreover, the zebrafish germline mutations in DNA methyltransferase 1 (Dnmt1) exhibit degeneration of exocrine pancreas and apoptosis of acinar cells without affecting pancreatic ducts and endocrine cells.[36] These findings suggest that DNA methylation mediated by Dnmt1 is required for survival of pancreatic acinar cells during development, and the target genes remain identified. However, the complex relationship among the various ligands, molecular regulators, and the associated signaling pathways has yet to be defined. The mechanisms that control the proliferation and cytodifferentiation of pancreatic progenitors and exocrine epithelia require further investigation.

During morphogenesis of acini and ducts, the exocrine pancreatic progenitors continue to proliferate and

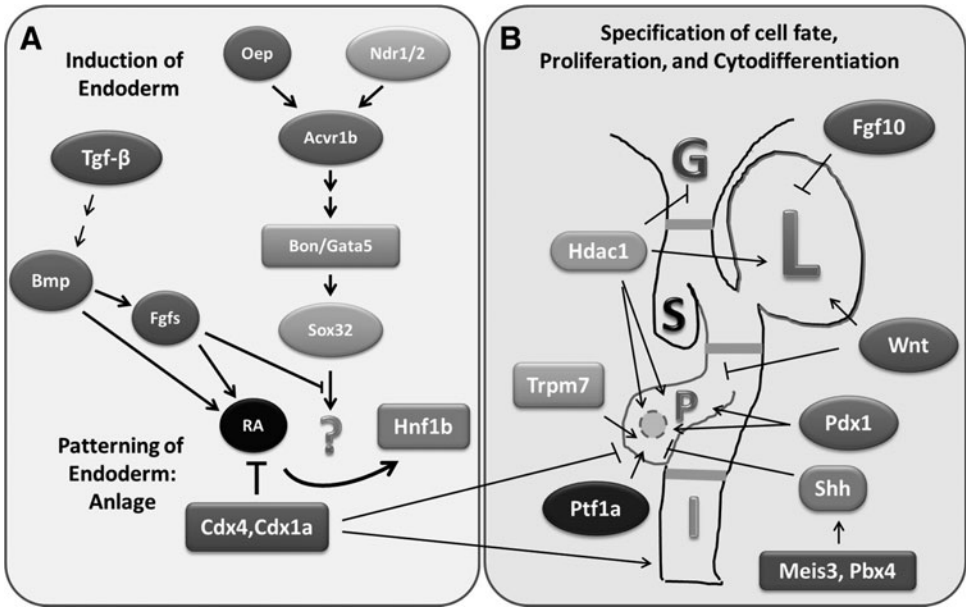

FIG. 2. Genetic and epigenetic regulators of early pancreatic development in zebrafish. Schematic representation showing the signal transducers and transcription factors involved in regulating **(A)** endodermal induction and patterning that lead to formation of the pancreatic endoderm and anlage, and **(B)** specification of cell fate, proliferation, and cytodifferentiation. *Arrows* indicate positive regulation or activation, and *lines* ending in bars denote inhibition. The *gray rectangular* areas represent regionalization (borders) separating the gut tube (G) tube, liver (L), swim bladder (S), pancreas (P), and intestine (I). The *circular structure* in the pancreas represents the endocrine islet. Note: the developmental regulators of pancreas shown in this figure are selected ones and not all those currently known are included for the purpose of clarity.

differentiate (Fig. 3). The acinar epithelia containing zymogen granules form acini (Fig. 3A–E), and the cytokeratin-expressing ductal epithelia develop into the highly branched ductal system (Fig. 3F–J). Experimental evidence indicates that exocrine progenitors migrate and aggregate to form pancreatic ducts, rather than by budding from the exocrine anlage.[16] In agreement with this, "unpolarized" ductal morphogenesis was demonstrated by using the transgenic zebrafish expressing green fluorescent protein under control of the *nkx2.2a* enhancer.[37] Several mutations impair ductal branching and acinar morphogenesis, probably due to primary defects in the proliferation of pancreatic epithelia. For instance, the *polr3b*^{slj} mutation, which reduces the levels of tRNAs and the proliferative capacity of pancreatic epithelia, produces hypomorphic pancreatic ducts, and acini.[2,16,32,38] Analysis of the pancreatic phenotypes of Notch-deficient and Notch-activated larvae establishes a link between ductal branching and acinar morphogenesis.[16] The receptor of Shh, smoothened, is required for proper morphogenesis of exocrine pancreas, as indicated by the phenotype of the *smu* mutation.[15] Besides, the mutations affecting *trpm7* ion channel, including the *trpm7*^{sweetbread} (*trpm7*^{swd}) and *trpm7*^{touchtone} (*trpm7*^{tct}) mutations, diminish pancreatic epithelial cell cycle progression and cell growth, impair ductal branching morphogenesis, and produce small acini.[34] Epigenetic mechanism is also involved, and this is supported by the evidence that the *hdac1*^{hi1618} mutation reduces pancreatic epithelial proliferation, and diminishes acinar and ductal morphogenesis.[35] Furthermore, the zebrafish mutations, including *elys*$^{flotte\ lotte}$ (*elys*^{flo}), *achy*^{ducttrip} (*achy*^{dtp}), *piebald* (*pie*), *mitomess* (*mms*), and *ductjam* (*djm*) disrupt ductal branching and acinar morphogenesis to various extents; and the functional roles of the affected genes remain to be identified.[16] Continued research efforts are indicated to determine the genetic regulation of morphogenesis of exocrine pancreas. Moreover, attempt to define the relationship between acinar morphogenesis and ductal branching is expected to generate data that may shed new light into the cell of origin in human pancreatic adenocarcinoma.

As the pancreas continues to grow, pancreatic ducts further develop and branch, while acini enlarge and mature. The organ size is primarily determined by the amount of exocrine tissues, which is largely dependent on the number and size of acinar cells that express digestive enzymes (Fig. 4). During this stage, the acinar epithelia increase in volume, contain increased number of organelles, such as zymogen granules, rough endoplasmic reticulum, and mitochondria (Fig. 5). Mutations that impair pancreatic epithelial proliferation result in development of relatively small pancreas, as indicated by the mutations that affect *polr3b*,[32] *exdpf*,[33] *trpm7*,[34] and *hdac1*.[35] The mutations, the affected genes, and their functional roles in exocrine pancreatic development are summarized in Table 3. Further characterization of the mutations affecting exocrine pancreas, as well as the associated signaling pathways, is expected to generate new data about the mechanisms that control the growth and thus, the size of the pancreas.

A brief homology and ontology, along with known functions of these developmental regulators in zebrafish, are presented in Table 4. These data indicate that the developmental regulators are structurally conserved between zebrafish and human on the basis of amino acid sequences and protein domains/motifs. Essentially, they suggest that the functions of these developmental regulators characterized in

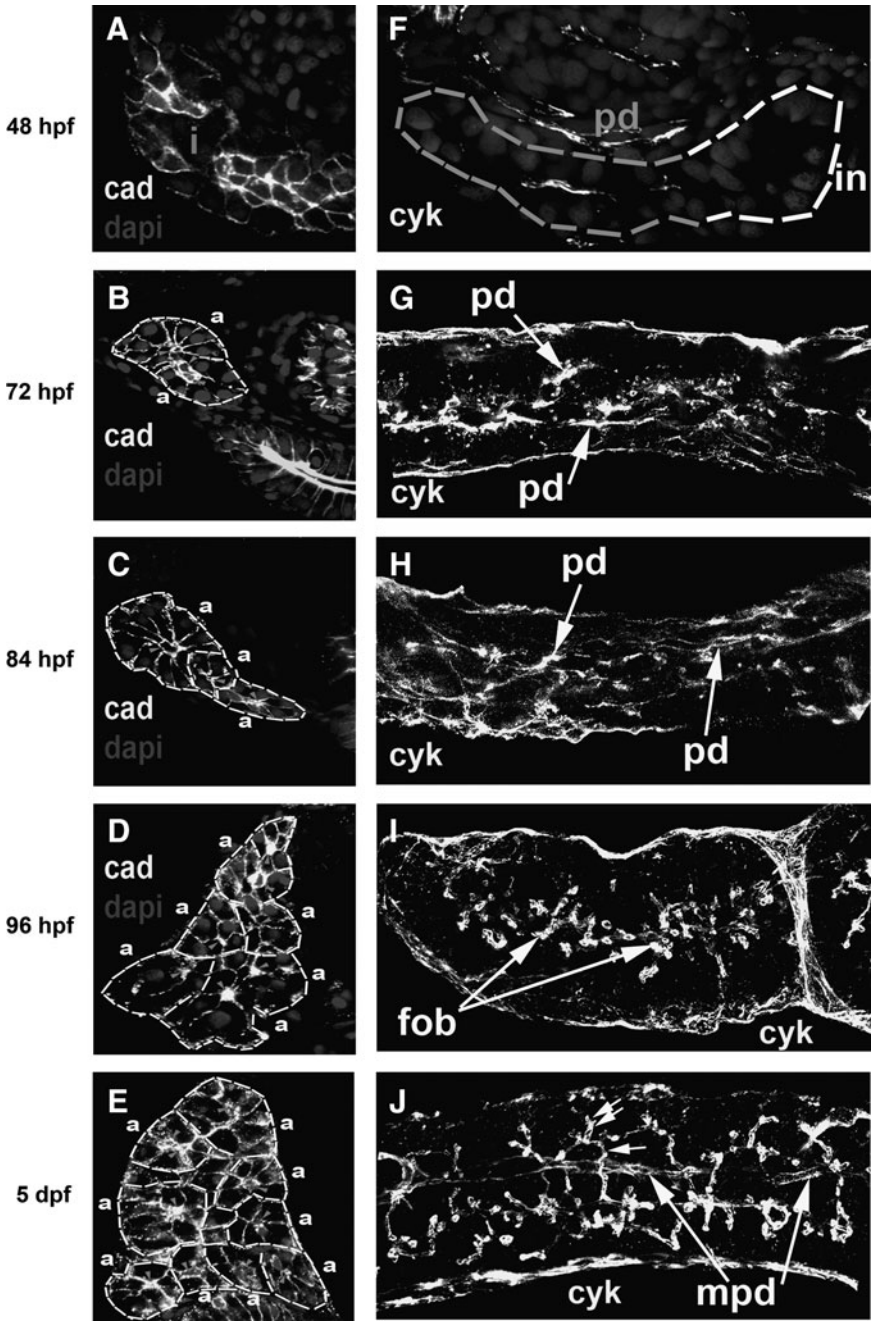

FIG. 3. Morphogenesis of pancreatic acinar glands and ducts in wild-type zebrafish. **(A–E)** Histological transverse-sections of the pancreas [**(A)**, through islet; **(B–E)**, caudal to islet] of larvae processed for immunohistochemistry using anticadherin (cad, *white*) antibodies as a marker of intercellular adhesion. At 48 h.p.f., there is no identifiable acinus in the exocrine tissues surrounding the islet (i). Starting at 72 h.p.f., the number of acini (a, as demarcated by *white dashed lines*) increases as development progresses through 5 d.p.f. The centroacinar cells are indicated by *gray arrowheads*. **(F–J)** Histological transverse-sections **(F)** or confocally analyzed optical sections **(G–J)** of the pancreas of larvae processed for immunohistochemistry using anti-cytokeratin (cyk) antibodies as a marker of pancreatic ducts. At 48 h.p.f., the main pancreatic duct (pd, *gray outline*) is contiguous with the intestine (in, *white outline*). Starting at 72 h.p.f., small pancreatic ducts (pd) become evident, and there are more ducts seen at 84 h.p.f. Later at 96 h.p.f, the first order branches (fob) of pancreatic ducts can be identified. By 5 d.p.f., the first order branches (*single arrow*) and the second order branches (*double arrows*) of pancreatic ducts are evident. Note: dapi, *gray* staining, showing the cell nuclei **(A–F)**. The images for 48 h.p.f. are not to scale with the other images captured at various time points. These images are adapted from Yee *et al.*[16] with reprint permission from the publisher. h.p.f., hours-post-fertilization; d.p.f., days-post-fertilization.

the zebrafish model are potentially translated to human development and diseases. We anticipate that continued research focusing on the regulatory mechanism of pancreatic development and growth in zebrafish may help understand how the aberrant control mechanism in human pancreatic epithelia and the microenvironment leads to cancerous growth and metastasis.

Developmental Regulators As Biomarkers and Targets of Pancreatic Cancer

It has been well established that the common genetic pathways are shared between embryonic development and cancer that arises in various organs—in particular, the pancreas. The developmental studies help facilitate the understanding of the pathogenetic mechanism underlying the multistep carcinogenesis. Besides, the genetic regulators of organogenesis may be exploited to develop biomarkers and targets in various human malignancies, including pancreatic cancer.

The potential of targeting the epigenetic and genetic regulators of exocrine pancreas development as a therapeutic approach in pancreatic cancer has been demonstrated in preclinical and clinical studies (Table 5). Chemical inhibitors of the epigenetic regulators of exocrine pancreas, particularly those controlling histone deacetylation and DNA methylation, have been investigated as therapeutic agents in pancreatic cancer. For instance, Hdac1 regulates gene transcription

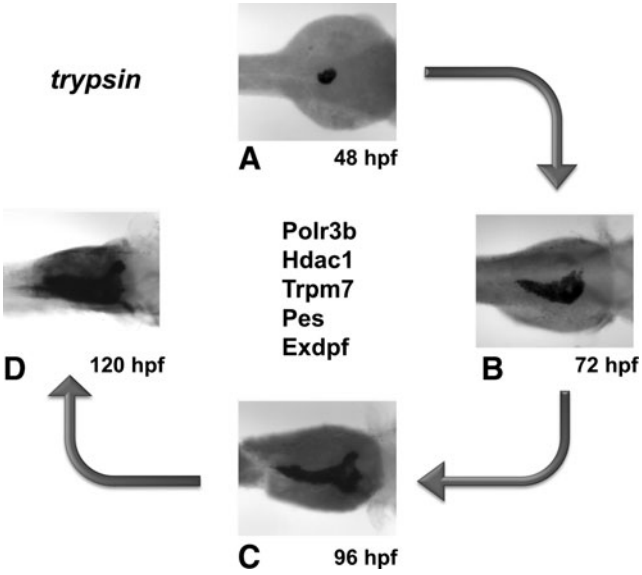

FIG. 4. Growth of exocrine pancreas during morphogenesis in zebrafish **(A–D)**. Dorsal views of wild-type larvae processed for whole mount *in situ* hybrdization using an antisense *trypsin* riboprobe as a marker of exocrine pancreas. The developmental regulators as listed have been shown to play important roles in the growth of exocrine pancreas during morphogenesis. These images are adapted from Yee *et al.*[16] with reprint permission from the publisher.

by modulating the acetylation status of nucleosomal histones and other proteins, and it is implicated in normal physiology and disease states.[39] The germ-line mutation in *hdac1* or antisense oligos-mediated disruption of *hdac1* expression causes reduced growth of exocrine pancreas by impairing epithelial proliferation.[30,35] Treatment of zebrafish larvae with chemical inhibitors of HDACs, such as trichostatin A (TSA), impairs epithelial proliferation in the exocrine pancreas.[40] Preclinical studies indicate that the chemical inhibitor of HDACs exerts antiproliferative effects on human pancreatic adenocarcinoma, both in culture and in mouse xenograft model.[41] Clinical trials have been conducted to investigate the therapeutic efficacy of the combination of the HDAC inhibitor (suberoylanilide hydroxamic acid, SAHA) and the standard chemotherapeutic drug gemcitabine with pending result.[41]

Combination of HDAC inhibitor with chemical modulators of other developmental regulators of exocrine pancreas has been shown to produce enhanced cytotoxicity in human pancreatic adenocarcinoma cells. We and others have demonstrated that HDACs and POLR3 play critical roles in the signaling mechanisms that control embryonic and neoplastic development.[32,35,39,42] It was unknown if POLR3 and HDACs regulate growth of exocrine pancreas in a coordinated fashion. Whether targeting POLR3 can enhance the antitumor activities of HDAC inhibitors has not been reported. We have recently provided evidence that the combination of HDAC inhibitors (TSA or SAHA) and the POLR3 inhibitor (ML-

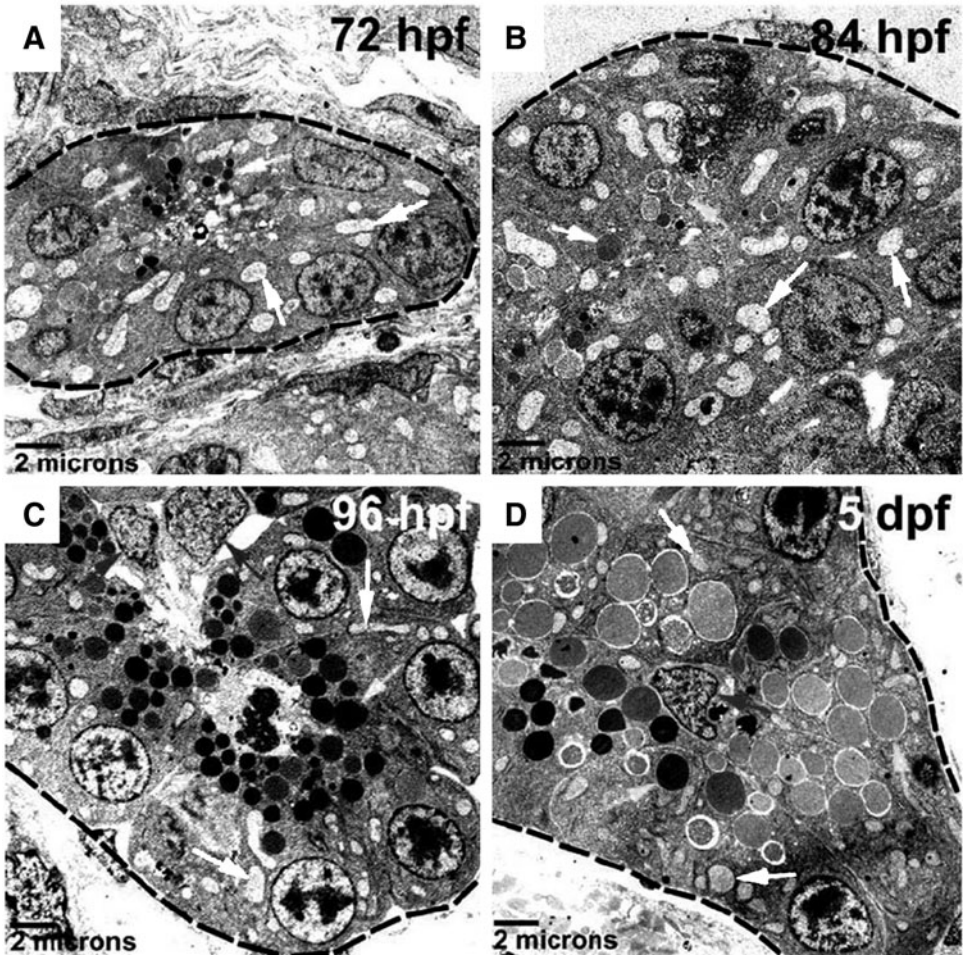

FIG. 5. Ultrastructure of acinar epithelia in exocrine pancreas during morphogenesis **(A–D)**. Transmission electron micrographs of pancreatic acini (as outlines by *black dashed lines*) in wild-type zebrafish larvae. The acinar cells progressively mature from 72 h.p.f. through 5 d.p.f. as indicated by cell polarity, number, and size of zymogen granules (*dark* or *gray* fairly *round circles* shown with *light gray arrows*), mitochondria (*white arrows*), and rough endoplasmic reticulum. The centroacinar cells at 96 h.p.f. and on 5 d.p.f. are denoted by *dark gray arrows*. These images are adapted from Yee *et al.*[16] with reprint permission from the publisher.

Table 3. Zebrafish Germ-Line Mutations Affecting Exocrine Pancreas

Mutation	*Exocrine pancreas phenotype*	*Genes*	*References*
akreas	Arrested growth	*ptf1a*	27
apc	Small	*adenomatous polyposis coli* (*apc*)	17
ductjam (*djm*)	Small acini, dysmorphic ducts	unidentified	16
ducttrip (*dtp*)	Diminished acini, dysmorphic ducts	*S-adenosylhomocysteine hydrolase* (*ahcy*)	2,16,55
def	small, arrested growth	*digestive-organ expansion factor* (*def*)	56
dandelion (*ddn*)	Degeneration, apoptosis of acinar cells	*DNA methyltransferase I*	36
exdpf	Small, arrested growth	*exocrine differentiation and proliferation factor* (*exdpf*)	33
flotte lotte (*flo*)	Small, degenerated	*embryonic lethal yolk sac* (*elys*)	2,16,57
hdac1^{s436}	Small	*histone deacetylase 1* (*hdac1*)	30
hdac1^{hi1618}	Small, arrested growth	*histone deacetylase 1* (*hdac1*)	35
K-ras^{G12V}	Arrested growth	*K-ras*	49
mind bomb (*mdb*)	Hypomorphic ducts, enlarged acini	*ubiquitin ligase*	16
mitomess (*mms*)	Decreased zymogen, hypomorphic ducts	unidentified	16
nil per os (*npo*)	Small	*RNA binding protein 19* (*rbp 19*)	58
pescadillo (*pes*)	Small, defect in progenitors	*pescadillo* (*pes*)	7
rpl3	Small, defect in progenitors	*ribosomal protein L3* (*rpl3*)	7
slimjim (*slj*)	Small, degenerated	*RNA polymerase III subunit 2* (*polr3b*)	2,16,32,38
smu	Duplicated, no posterior extension	*smoothened* (*smo*)	15
sweetbread (*swd*) *trpm7*^{j124e1}, *trpm7*^{b508}	Reduced growth, impaired proliferation Small acini, hypomorphic ducts	*trpm7*	2, 16, 34

60218) produces synergistic suppression of epithelial proliferation in the exocrine pancreas of zebrafish (Fig. 6A). This finding in zebrafish could be translated to human pancreatic adenocarcinoma, in which the combination of SAHA and ML-60218 produced enhanced antiproliferative and proapoptotic effects (Fig. 6B). Our data provide a proof of principle for enhancement of the antitumor activity of HDAC inhibitors by counteracting their "pro-oncogenic" side effects, as SAHA-induced upregulation of tRNAs was repressed by ML-60218.[40]

Moreover, we have demonstrated that a combination of chemical inhibitors of HDACs and the receptor of SHH

Table 4. Conserved Genetic Regulators of Exocrine Pancreatic Development

Protein ID	*Identical position*	*% identity*	*Similar position*	*Synteny*	*Paralog*	*Ortholog*	*Functional roles in exocrine pancreatic development in zebrafish*	*Role in human pancreatic disease*	*References*
Trpm7	1304	69.32	277	[a]	9	57	Epithelial proliferation, acinar growth, ductal branching, organ growth, Mg^{2+} homeostasis	Over-expressed in pancreatic adenocarcinoma; required for proliferation, migration, and prevention of replicative senescence in pancreatic cancer cells	34,47,59
Ptf1a	173	52.26	43	[b]	14	49	Cytodifferentiation, morphogenesis	Diabetes mellitus, pancreatic agenesis	22,23,25
Pdx1	135	45.76	42	[a]	28	51	Patterning, morphogenesis	Susceptibility to diabetes mellitus, Mature Onset Diabetes of the Young (MODY), pancreatic agenesis	60–62
Shh	285	61.55	66	[a]	4	61	Positional information, cellular differentiation, signal transduction	Over-expressed in pancreatic adenocarcinoma; required for cellular proliferation	63–65
Fgfs	586	70.77	144	[b]	8	61	Patterning, morphogenesis	Pancreatic cancer, chronic pancreatitis	66–68
Pes	406	68.58	98	[a]	[b]	57	Growth	Unknown	69–71

Identical position = primary structure of protein and identity of amino acids conserved among human, zebrafish, mouse, and rat.
Similar position = conserved amino acid property for example, may alter between L and I amino acid.
[a]Shared synteny describes preserved co-localization of genes on chromosomes of human, zebrafish, and mouse Ortholog and paralog information obtained from Ensemble at useast.ensembl.org.
[b]Unknown; protein IDs are listed as in zebrafish nomenclature.
Ontologies source Homologene www.ncbi.nlm.nih.gov/homologene/and zfin.org

Table 5. Genetic Regulators of Zebrafish Exocrine Pancreas with Potential Roles As Clinical Biomarkers and Therapeutic Targets in Human Pancreatic Adenocarcinoma

Zebrafish genes and mutations	*Human orthologue*	*Expression in human pancreatic adenocarcinoma*	*Preclinical studies as therapeutic targets in human pancreatic adenocarcinoma*	*References (preclinical studies)*	*Clinical studies of therapeutic targeting in human pancreatic adenocarcinoma*	*References (clinical studies)*
trpm7^{swd}, *trpm7*^{j124e1} *trpm7*^{b508}	*TRPM7*	Over-expressed in tissues and cell lines	siRNA impairs proliferation by arresting cell cycle arrest and inducing replicative senescence.	34,46–48	None	Not applicable
polr3b^{slj}	*POLR3b*	Over-expressed in cell lines	Chemical inhibitor of POLR3 in combination with HDAC inhibitor produces enhanced cytotoxicity.	40	None	Not applicable
hdac1^{hi1618}	*HDAC1*	Over-expressed in cell lines	siRNA-mediated silencing of *HDAC1* or chemical inhibitors of HDACs reduce proliferation.	35,41,43	Vorinostat, a small molecule inhibitor of HDACs, tested in locally advanced and non-metastatic tumor; result pending.	41
smoothened^{smu}	*SMO*	Over-expressed in cell lines	Chemical antagonist of SMO inhibits proliferation, produces enhanced cytotoxicity with HDAC inhibitor, and inhibits stem cells characteristics.	43, 72–75	Vismodegib and saridegib, small molecules inhibitors of SMO, tested in metastatic cancer; recruiting.	41
ubiquitin ligase^{mdb}	*NOTCH*	Over-expressed in cell lines	Inhibition of Notch signaling by blocking the activity of γ-secretase, subsequent signaling, and cellular proliferation.	76,77	RO4929097 and MK-0752, Small molecules inhibitors of γ-secretase, tested in tumors of various stages; recruiting.	41
apc	*WNT*	Up-regulated in pancreatic circulating tumor cells	Inhibition of acyltransferase called Porcupine that adds fatty acid to WNT, prevents secretion of WNT and subsequent signaling.	78	LGK974, a small molecule Inhibitor of WNT secretion, Tested in advanced WNT-dependent solid tumors, including pancreatic adenocarcinoma; recruiting.	ClinicalTrials.gov Identifier: NCT01351103
K-ras	*K-RAS*	*K-RAS*^{G12D} or *K-RAS*^{G12V} mutations	Inhibiton of farnesyl transferase prevents attachment of K-RAS to plasma membrane, inducing proliferative arrest and apoptosis.	41, 79	Tipifarnib, a small molecule inhibitor of farnesyltransferase, prevents activation of RAS, ineffective for treatment of advanced cancer.	41

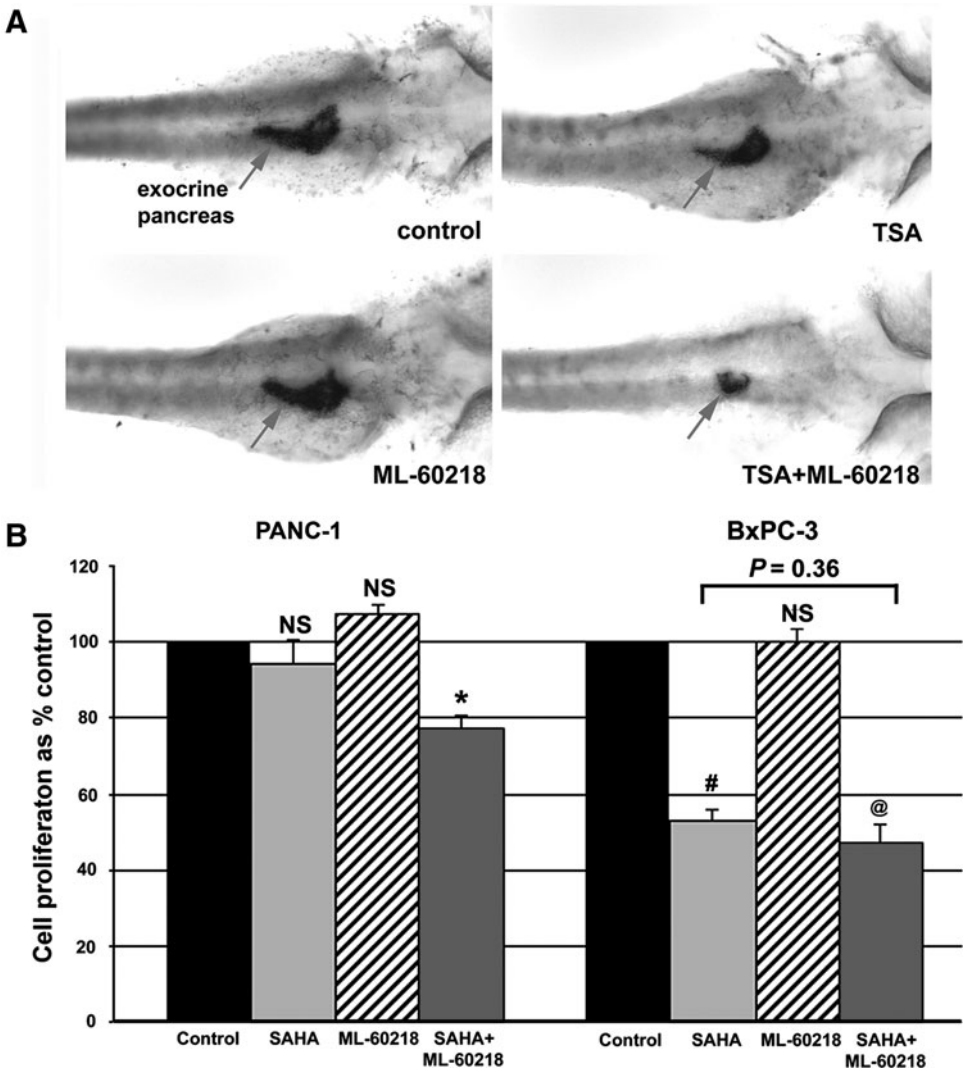

FIG. 6. Combined targeting Polr3 and Hdacs produces synergistic suppression of growth suppression of exocrine pancreas in zebrafish and enhanced cytotoxicity in human pancreatic adenocarcinoma. **(A)** Combination of TSA and ML-60218 synergistically inhibits expansion of exocrine pancreas in zebrafish. Dorsal view of larvae with *gray arrows* pointing at the exocrine pancreas. Starting at 48 h.p.f., wild type larvae were incubated for 24 h in the presence of TSA, ML-60218, TSA + ML-60218, or control (dimethyl sulfoxide or untreated). The exocrine pancreas was analyzed by whole mount *in situ* hybridization using antisense *trypsin* riboprobes. **(B)** SAHA and ML-60218 inhibit anchorage-independent colony formation and induce cellular morphology consistent with cellular senescence and cell death. Soft agar colony assay. PANC-1 and BxPC-3 were treated with 5 μM SAHA, 100 μM ML-60218, 5 μM SAHA + 100 μM ML-60218, or untreated (control), and grown in soft agar for 14 days. Each column represents the mean number of colonies in each treatment group from three independent experiments, with each treatment group in triplicate; bars represent standard error of mean. Statistical analysis was performed using Student's *t*-test to compare between each treatment and control. $^{*}p < 0.05$; $^{\#}p < 0.005$; $^{@}p < 0.0001$. Reprinted from Yee *et al.*[40] with the publisher's permission. TSA, trichostatin A; SAHA, suberoylanilide hydroxamic acid.

(Smoothened) produced enhanced cytotoxicity in human pancreatic adenocarcinoma. In this study, the combination of SAHA with the small molecule antagonist of Smoothened (SANT-1) produced supra-additive suppression of proliferation and induction of apoptotic death in human pancreatic cancer cells in a soft agar assay and in culture.[41,43] The enhanced cell cycle arrest was associated with upregulated expression of the cyclin-dependent kinase inhibitors $p21^{CDKN1A}$ and $p27^{CDKN1B}$, and downregulation of *cyclin D1*.[43] The proapoptotic effect was associated with nuclear localization of survivin, increased expression of BAX, and activation of caspases 3 and 7.[43] The potentiated cytotoxicity by the combination of SAHA and SANT-1 in pancreatic cancer cells may involve cooperative suppression of the hedgehog pathway, as shown by SAHA-induced upregulation of *HHIP* and repression of *PTC-1* mRNAs,[43] and possibly acetylation of GLI proteins.[44]

Furthermore, translation of the recent discovery of the Trpm7 ion channel in the growth of exocrine pancreas in zebrafish has led to the identification of the human ortholog TRPM7, and also its subfamily member TRPM8 as potential tissue biomarkers and molecular targets in human pancreatic adenocarcinoma (Table 5). The Trpm7 ion channel is required for normal growth of exocrine pancreas through

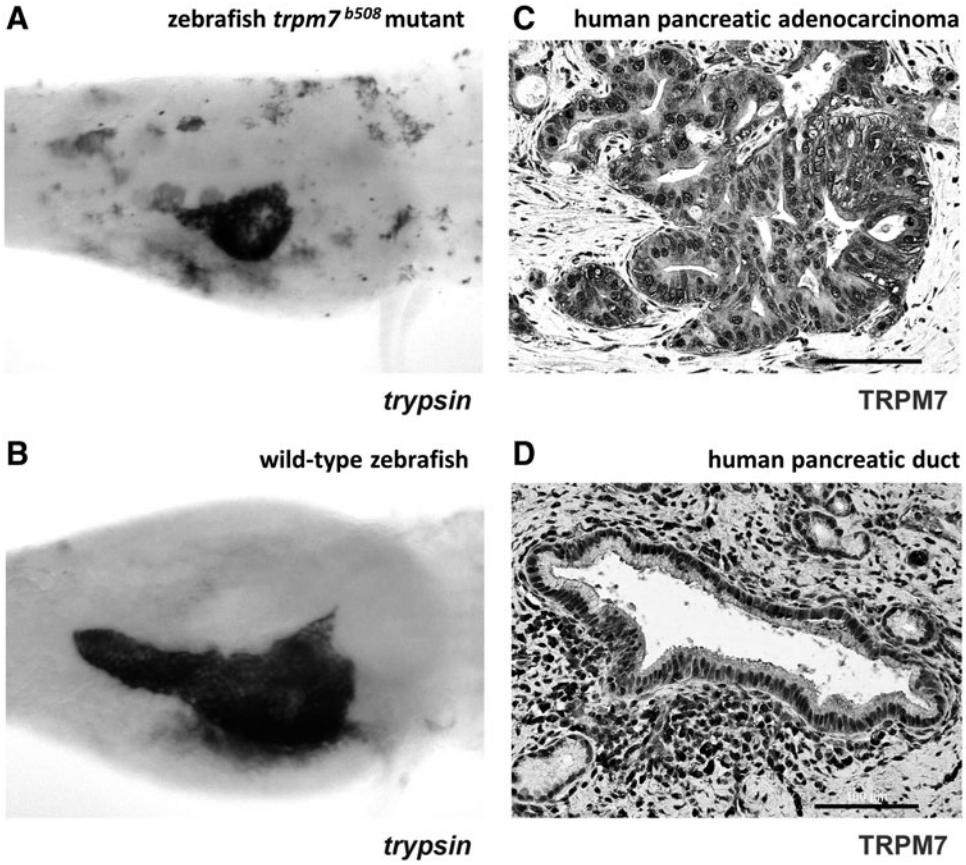

FIG. 7. Trpm7 is critically required for growth of exocrine pancreas in zebrafish, and TRPM7 is over-expressed in human pancreatic adenocarcinoma. **(A, B)** Zebrafish larvae on 5 d.p.f. processed for whole-mount *in situ* hybridization using an antisense trypsin riboprobe as a marker of exocrine pancreas. The *trpm7*^{b508} mutation is a hypomorphic allele; it produces a premature stop codon that leads to deletion of the kinase domain in Trpm7. As compared to wild-type **(B)**, the exocrine pancreas of the *trpm7*^{b508} mutant **(A)** is diminished in size. In this experiment, the wild-type larvae were incubated in E3 embryo medium containing PTU that inhibits skin pigmentation and allows visualization of the pancreas. The *trpm7*^{b508} mutants have defect in skin pigmentation, such that addition of PTU is unnecessary during incubation of the larvae in E3 medium. **(C, D)** Histological sections of human pancreatic adenocarcinoma and normal human pancreatic tissue were processed for immunohistochemistry using anti-TRPM7 antibodies. Immunoreactivity against TRPM7 is relatively strong in pancreatic adenocarcinoma **(C)**, as compared to that in normal pancreatic ducts **(D)**. Scale bar, 100 μm. These images are adapted from Yee *et al.*[34] with permission from the publisher. PTU, 1-phenyl-2-thiourea.

regulation of cell cycle progression and epithelial growth[34] (Fig. 7A, B). Based on these study results, we discovered that TRPM7 and its subfamily member TRPM8 are aberrantly over-expressed in human pancreatic adenocarcinoma[34,45–47] (Fig. 7C, D). Small interfering RNA-mediated silencing of *TRPM7* or *TRPM8* reduces cellular proliferation, impairs cell cycle progression, and induces replicative senescence in the pancreatic cancer cells.[34,45–48] These data further demonstrate the potential of human orthologs to developmental regulators of exocrine pancreas in zebrafish as clinical biomarkers and therapeutic targets in human pancreatic cancer.[47]

Zebrafish Models for Studying Pathogenesis and Drug Discovery in Human Pancreatic Cancer

The histopathology of pancreatic adenocarcinoma in zebrafish resembles that in human (Fig. 8), suggesting similar pathogenetic mechanisms for the development of pancreatic tumor in both organisms. Zebrafish models for exocrine pancreatic tumors have been generated using germ-line mutation of oncogenic *K-ras*[49] and by microinjection of human pancreatic adenocarcinoma cells.[2] These zebrafish models of pancreatic cancer will be useful tools for dissecting the signaling pathways that mediate the multistep pancreatic carcinogenesis. They can be exploited for discovery and development of antitumor therapeutics in human pancreatic cancer.

A transgenic zebrafish model (ptf1a:eGFP-Kras^{G12V}) was established by expressing oncogenic *K-ras* under control of *ptf1a* promoter.[49] In the oncogenic K-Ras expressing zebrafish, there is abnormal persistence of pancreatic progenitor cells which fail to differentiate in larvae, and they subsequently develop invasive carcinoma with acinar and/or ductal histological features. The pancreatic tumors in the transgenic adult zebrafish exhibit abnormally activated hedgehog signaling, which has become a therapeutic target of human pancreatic adenocarcinoma[43] (Table 5). The oncogenic *K-ras*

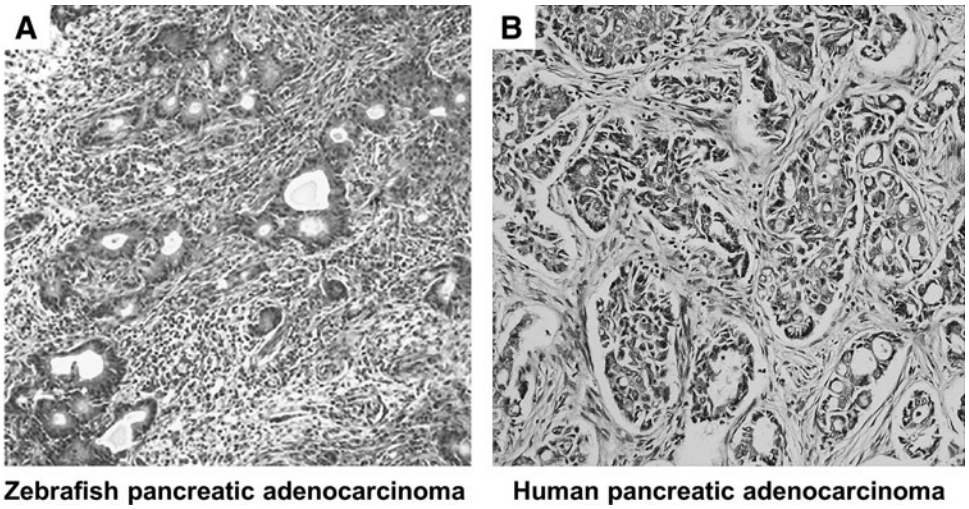

FIG. 8. Histopathology of pancreatic adenocarcinoma in zebrafish resembles that in human. **(A)** Pancreatic adenocarcinoma in zebrafish carrying the transgene *K-ras*^{G12V} mutation. **(B)** Human pancreatic adenocarcinoma. The image **(A)** is adapted from Park *et al.*[49] with the publisher's permission.

expressing transgenic zebrafish model provides support for conserved histopathology and genetic pathways in pancreatic cancer between zebrafish and human. With generation of gene expression data from the human pancreatic cancer genome projects,[50,51] the zebrafish model can be further utilized to advance understanding of the pathogenetic mechanisms of pancreatic adenocarcinoma. This can be accomplished by modulating the candidate genes in the transgenic zebrafish, and determining how they influence initiation, development, and progression of pancreatic tumor.

Complementary to the transgenic zebrafish model, tumor xenografts have been developed by microscopic injection of human pancreatic cancer cells into zebrafish embryos.[2] We have microinjected fluorescently labeled human pancreatic adenocarcinoma cells into the yolk sac of wild-type zebrafish larvae on 2 days-post-fertilization (d.p.f.), and tumor growth was monitored every 12 h. Between 2 and 5 d.p.f., the injected cancer cells remained as a solitary mass (Fig. 9). On 6 d.p.f., two smaller cell masses were observed; and by 7 d.p.f., at least three discrete cell masses are evident, indicating segregation of the tumor cells that resembles tumor invasion and metastasis (Fig. 9). Generation of such zebrafish xenograft model of human pancreatic adenocarcinoma should be feasible with pancreatic cancer stem cells or cell lines derived from freshly resected surgical specimens from human patients.

While mouse models of pancreatic cancer have accelerated our understanding of its pathogenetic mechanism and allowed preclinical testing of drug efficacy,[52] transgenesis and tumor xenografts in zebrafish offer a unique opportunity to model pancreatic cancer in a complementary fashion. In particular, the intrinsic features of zebrafish (small size,

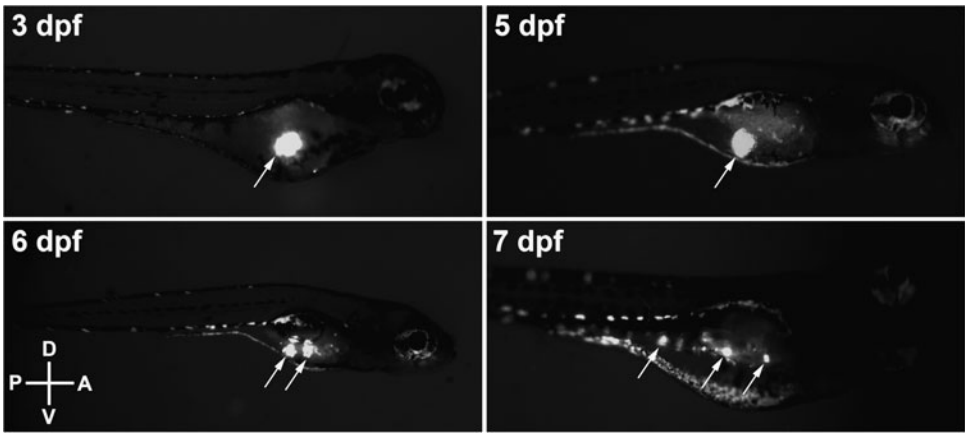

FIG. 9. Zebrafish xenograft of pancreatic cancer generated with human pancreatic adenocarcinoma cells PANC-1. A suspension of 50 dil-CM labeled PANC-1 cells in 50 nL culture medium was injected under a stereo-dissecting microscope (MZ16F; Leica) into the yolk sac of zebrafish larvae on 2 d.p.f. The larvae were incubated in embryo medium at 35°C. The individual larva was kept in each well of a 24-well tissue culture plate. The tumor xenograft (*arrows*) in each zebrafish larvae was examined every 12 h under a stereo-dissecting microscope with fluorescent illumination at 568 nm (MZ16F; Leica). Images of the xenograft in the same larva were captured at the indicated time intervals. D, dorsal; V, ventral; A, anterior; P, posterior. Reprinted with permission from Yee[2].

fecundity, optical transparency) enable chemical-genetic interrogation and real-time visual monitoring of pancreatic tumor growth and metastasis *in vivo*.[2] Moreover, these zebrafish models provide new platforms for investigation of the complex interaction between the external factors (such as environmental pollutants and chemical carcinogens) and genetic constituency in transformation of pancreatic epithelia into premalignant neoplasia and eventually invasive carcinoma. By application of chemical genetics and small molecules screening to the zebrafish models, we can improve our capability of developing effective and safe antitumor therapeutics toward the goal of prevention and treatment of human pancreatic cancer.

Conclusion and Future Perspectives

In summary, descriptive and experimental studies in zebrafish have generated new knowledge about genetic and epigenetic regulation of exocrine pancreas during development. Emerging evidence has demonstrated the potential of translating the developmental regulators of exocrine pancreas in zebrafish into clinical biomarkers and therapeutic targets in human pancreatic adenocarcinoma. Future studies using the zebrafish models of pancreatic cancer are expected to advance our understanding of the molecular mechanisms underlying tumorigenesis in a developmental context, as well as identifying and validating candidate therapeutic agents. Application of technologies, such as zinc finger nucleases, TALENs, and micro-computed tomography, in combination with inducible pancreas-specific gene expression, will further enhance the power of the zebrafish model for dissecting the mechanisms that mediate pancreatic development and cancer. These concerted efforts are expected to facilitate maximal utility of the zebrafish model, along with the mammalian models, toward the goal of personalized therapy in pancreatic cancer by targeting the molecular phenotype of tumor and its associated stroma in the individual patient.[41]

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Disclosure Statement

No competing financial interests exist.

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