Transient receptor potential ion channel Trpm7 regulates exocrine pancreatic epithelial proliferation by Mg$^{2+}$-sensitive Socs3a signaling in development and cancer

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SUMMARY

Genetic analysis of pancreatic development has provided new insights into the mechanisms underlying the formation of exocrine pancreatic neoplasia. Zebrafish sweetbread (swd) mutants develop hypoplastic acini and dysmorphic ducts in the exocrine pancreas, with impeded progression of cell division cycle and of epithelial growth. Positional cloning and allelic complementation have revealed that the swd mutations affect the transient receptor potential melastatin-subfamily member 7 (trpm7) gene, which encodes a divalent cation-permeable channel with kinase activity. Supplementary Mg$^{2+}$ partially rescued the exocrine pancreatic defects of the trpm7 mutants by improving cell-cycle progression and growth and repressing the suppressor of cytokine signaling 3a (socs3a) gene. The role of Socs3a in Trpm7-mediated signaling is supported by the findings that socs3a mRNA level is elevated in the trpm7 mutants, and antisense inhibition of socs3a expression improved their exocrine pancreatic growth. TRPM7 is generally overexpressed in human pancreatic adenocarcinoma. TRPM7-deficient cells are impaired in proliferation and arrested in the G0-G1 phases of the cell division cycle. Supplementary Mg$^{2+}$ rescued the proliferative defect of the TRPM7-deficient cells. Results of this study indicate that Trpm7 regulates exocrine pancreatic development via the Mg$^{2+}$-sensitive Socs3a pathway, and suggest that aberrant TRPM7-mediated signaling contributes to pancreatic carcinogenesis.

INTRODUCTION

Genetic analyses of the developmental processes in model organisms have elucidated the mechanisms of human diseases. The vertebrate pancreas is a model organ for dissecting the signaling pathways that are common to development and cancer. The genetic control of cell division and growth in exocrine pancreatic epithelia is crucial for ductal and acinar morphogenesis, and is directly relevant to the initiation and progression of malignant neoplasia during pancreatic tumorigenesis. However, the regulatory mechanisms involved in the control of the growth and thus the size of the exocrine pancreas are still poorly understood.

Understanding the mechanisms that regulate pancreatic development has been facilitated by studies in model organisms, including zebrafish (Danio rerio). The exocrine component of the zebrafish pancreas arises from endodermal progenitor cells that migrate from the gut tube, requiring the transcriptional activities of pancreatic and duodenal homeobox (Pdx1) and pancreas-specific transcription factor (Ptf1a) (Yee et al., 2001; Biemar et al., 2001; Lin et al., 2004; Zecchin et al., 2004; Yee et al., 2005; Ward et al., 2007). Signaling pathways, including wingless-type mouse mammary tumor virus integration site family members (Wnt), fibroblast growth factor (Fgf) and sonic hedgehog (Shh), are implicated in the induction and patterning of exocrine pancreatic tissues (Goessling et al., 2008; Manfroid et al., 2007; Dong et al., 2007; Chung and Stainier, 2008). Differentiation of the exocrine pancreatic progenitors into acinar and ductal cells involves the complex interactions of Ptf1a, Pdx1, Fgf10 and Notch (Yee et al., 2001; Field et al., 2003; Lin et al., 2004; Zecchin et al., 2004; Yee et al., 2005; Dong et al., 2007; Dong et al., 2008) (for a review, see Yee and Pack, 2005; Tiso et al., 2009; Yee, 2010). During morphogenesis, the exocrine pancreatic epithelial cells proliferate and form the zymogen-granule-secreting acini and the highly branched ductal system (Yee et al., 2005). Whereas Notch is required for proliferation of pancreatic progenitors (Esni et al., 2004; Yee et al., 2005), optimal activity of RNA polymerase III is crucial for normal epithelial proliferation that is coordinately regulated with acinar and ductal morphogenesis (Yee et al., 2005; Yee et al., 2007; Yee, 2010). Although the molecular mechanisms underlying maintenance and regeneration of β-cells in pancreatic islets have been intensively studied, genetic control of the exocrine pancreatic growth that is directly relevant to cancer is still largely unexplored.

Ion channels control diverse cellular processes and physiological functions during embryogenesis and in adult life, but little is known about their roles in pancreatic development and cancer. The
transient receptor potential (TRP) family is a superfamily of cation channels (Venkatachalam and Montell, 2007), and TRPM7 is member 7 of the melastatin-like subfamily. TRPM7 is a widely expressed divalent cation channel with protein serine/threonine kinase activity, and it regulates cellular Mg\(^{2+}\) and Ca\(^{2+}\) homeostasis (Nadler et al., 2001; Runnels et al., 2001; Schmitz et al., 2003). The kinase of TRPM7 can autophosphorylate, and its activity is regulated by Mg\(^{2+}\)-ATP (Takezawa et al., 2004; Ryazanova et al., 2004; Matsushita et al., 2005; Demeuse et al., 2006). At the cellular level, TRPM7 regulates survival of lymphocytes, neurons and mast cells (Nadler et al., 2001; Aarts et al., 2003; Wykes et al., 2007), proliferation and migration of osteoblasts (Abed and Moreau, 2007; Abed and Moreau, 2009), and volume regulation of cervical and renal epithelia (Numata et al., 2007). During embryogenesis, TRPM7 is required for normal development of melanoblasts, renal epithelia (Numata et al., 2007). During embryogenesis, TRPM7 is required for normal development of melanoblasts, osteoblasts and lymphocytes (Elizondo et al., 2005; McNeill et al., 2007; Jin et al., 2008). Electrophysiological studies suggest that Mg\(^{2+}\) influx through the TRPM7 channel leads to altered intracellular levels of Ca\(^{2+}\), which elicits the various cellular responses of TRPM7 (Aarts et al., 2003; Schmitz et al., 2003; Wei et al., 2009). However, the mechanisms that mediate the functions of TRPM7 in vertebrate organogenesis are poorly understood. The role of TRPM7 in the development of the exocrine pancreas has been unknown.

A variety of zebrafish mutations have identified roles of genes that regulate organogenesis, including that of the exocrine pancreas (Yee, 2010). The sweetbread (swd) mutation was recovered from an ethynitrosourea-induced genome-wide mutagenesis screen because the mutants had relatively small exocrine pancreas (Yee et al., 2005). The swd mutants develop hypoplasia of pancreatic acini and hypomorphic branching of pancreatic ducts, as well as skin hypopigmentation (Yee et al., 2005). Pancreatic cell fate specification and cytodifferentiation are unaffected by the swd mutations (Yee et al., 2005). These data suggest that the swd mutations primarily affect proliferation of exocrine pancreatic epithelia and, consequently, affect acinar and ductal morphogenesis. Identification of the gene affected by the swd mutation and the functional roles of the swd-locus-encoded protein is expected to advance our knowledge of the genetic regulation of exocrine pancreatic growth.

In this study, we present evidence that the swd locus encodes trpm7, the zebrafish ortholog of mammalian TRPM7. The swd mutation reduced exocrine pancreatic epithelial proliferation by impairing progression of cell division cycle and cell growth. The exocrine pancreatic phenotype of the swd and trpm7\(^{7508}\) mutants was partially rescued by supplementary Mg\(^{2+}\), with downregulation of p21\(^{\text{inducible}}\) and cyclin-G1 mRNA levels. Expression of suppressor of cytokine signaling 3a (socs3a) was elevated in the swd and trpm7\(^{7508}\) mutants, and repression of socs3a by supplementary Mg\(^{2+}\) or by antisense oligonucleotides improved exocrine pancreatic epithelial cell division and cell growth. TRPM7 was overexpressed in human pancreatic adenocarcinoma tissues and cell lines. RNA-interference-mediated silencing of TRPM7 impaired proliferation of pancreatic adenocarcinoma cells by arresting the cells in the G0-G1 phases of the cell cycle, and supplementary Mg\(^{2+}\) reversed these effects. These data indicate that Trpm7 plays a role in exocrine pancreatic proliferation and morphogenesis by the Mg\(^{2+}\)-sensitive pathways that involve Socs3a in zebrafish, and they support a contributory role of TRPM7 in the pathogenesis of human pancreatic adenocarcinoma.

**RESULTS**

The zebrafish swd mutations cause pancreatic acinar and ductal hypoplasia by impairing epithelial cell-cycle progression and growth

The two swd mutant lines (swd\(^{7508}\) and swd\(^{820}\)) develop relatively small exocrine pancreas and have hypophosphinated skin, and these two lines do not complement with each other, suggesting that they

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**Fig. 1.** The zebrafish swd\(^{7508}\) and swd\(^{820}\) mutations cause exocrine pancreatic hypoplasia and reduced skin pigmentation. (A-F) Bright-field images of the swd mutants and wild-type (wt) larvae. Green arrows point to pigmented skin. (G-I) Exocrine pancreas (red arrows) revealed by whole-mount in situ hybridization using anti-trypsin riboprobes. (J,K) Histological transverse sections of exocrine pancreas following immunohistochemistry using anti-Cpa antibodies. Staining with DAPI was used to visualize the nuclei. The histological sections are oriented as indicated: d, dorsal; v, ventral; r, right; l, left. (L,M) Exocrine pancreas (red arrows) revealed by whole-mount in situ hybridization using anti-ptfasa riboprobes. (N,O) Sagittal histological sections of larvae. e.p., exocrine pancreas; i, intestine; l, liver. (G-L) wt larvae were grown in E3 medium supplemented with PTU, which inhibits skin pigmentation, in order to facilitate visualization of the exocrine pancreas expressing trypsin or pfasa. Note that the larvae shown in A-O were analyzed on 5 dpf. (A-C,G-L-O) The larvae are positioned in the same orientation and viewed from the right lateral side. (D-F) The views are in the dorsoventral direction.
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as 682% (chymotrypsinogen B1) in the swdp75fm of exocrine pancreas and the skin hypopigmentation, the mRNA histological sections (Fig. 1N,O). Consistent with the growth defect (hypopigmentation), because the other digestive organs, including intestine and liver, of the swd mutants seem grossly normal on histological sections (Fig. 1N,O). Consistent with the growth defect of exocrine pancreas and the skin hypopigmentation, the mRNA levels of the exocrine pancreatic enzymes were reduced by as much as 682% (chymotrypsinogen B1) in the swdp75fm mutants; that of the melanin precursor, dopachrome tautomerase (dct), was reduced by 157%, as revealed by transcriptional profiling at 72 hpf (supplementary material Table S1).

The proliferative ability of exocrine pancreatic epithelial cells in the swd mutants was then examined for cell-cycle progression and cell growth. The proportion of epithelial cells in the S phase of the cell cycle was determined using 5-bromo-2'-deoxyuridine (BrdU) as a marker (Fig. 2A). Cell size as an indicator of growth was analyzed by morphometric determination of the surface area per cell (Fig. 2B). In the exocrine pancreases of the swdp75fm mutants, the mean proportion of nuclei that was immunoreactive for BrdU was significantly reduced, as compared with WT (28% vs 40%, respectively). This indicates that the ability of exocrine pancreatic epithelial cells to enter into S phase is impaired in swdp75fm mutants. Similarly, in swdp75fm mutants, the mean exocrine pancreatic epithelial cell growth at 3 dpf and 5 dpf (119 and 180 μm²/cell, respectively) was significantly lower than that in WT (176 and 456 μm²/cell, respectively). This suggests that exocrine pancreatic epithelial cell growth in the swd mutants is reduced and retarded. There was no increased apoptotic cell death in exocrine pancreas of either swd mutants or WT larvae at 3-5 dpf using the Apoptag assay (N.S.Y., unpublished). Taken together, these results suggest that the swd-locus-encoded protein is required for cell-cycle progression and growth of the exocrine pancreatic epithelia during morphogenesis.

The swd locus encodes the zebrafish ortholog of mammalian TRPM7

Using positional cloning techniques, we identified trpm7 as a candidate gene being affected by the swd mutations. The zebrafish mutation touchtone (tct) affects trpm7 (Elizondo et al., 2005) and it has been shown to cause skin hypopigmentation with normal retinal pigment, which is similar to the swd mutants. The alleles of tct, trpm7j124e1 and trpm7j508c have premature stop codons that lead to deletion of the kinase domain in Trpm7 (Elizondo et al., 2005). To investigate whether exocrine pancreas is also affected, the trpm7j124e1 and trpm7j508c mutants were analyzed for immunoreactivity against Cpa or trypsin. Similar to swd mutants, the exocrine pancreases of the trpm7j124e1 mutant is relatively small, and the acinar cells expressed Cpa (Fig. 3A, upper panels). Morphometric analysis indicated that epithelial cell growth of exocrine pancreas is reduced in trpm7j124e1 mutants at 3 dpf and 5 dpf (Fig. 3B), and the pattern of the altered growth resembled that seen in swdp75fm mutants (Fig. 2B). Similarly, the exocrine pancreas of trpm7j508c mutants was relatively small (supplementary material Fig. S1A,B) and it exhibited reduced epithelial proliferation and growth (Table 1A).

A complementation test by crossing swdp75fm/+ and trpm7j124e1/+ showed that the phenotype of the swdp75fm/trpm7j124e1 mutants is indistinguishable from that of either swdp75fm or trpm7j124e1 mutants. About 25% of the progeny larvae (swdp75fm/trpm7j124e1) exhibited reduced skin pigment and a relatively small exocrine pancreas, as observed by using trypsin and Cpa as markers of acinar cells (Fig. 3A,C). The same results were obtained by crossing swdb82me1/+ and trpm7j124e1/+ (data not shown). The non-complementation between the swd/+ and trpm7j124e1/+ heterozygotes argues against the possibilities that the swd- and trpm7-encoded proteins are different effectors that act in the same signaling pathway, or that the swd and trpm7 loci encode different genes that are closely located near each other. Taken together, the data of genetic mapping, analysis of exocrine pancreas in the swd and trpm7 mutants, and complementation testing strongly support that the swdp75fm and swdb82me1 loci are allelic with trpm7j124e1 and trpm7j508c, and trpm7 is the gene affected by the swd mutants.

To verify that trpm7 is the gene responsible for the exocrine pancreatic defects of the swd mutants, a loss-of-function approach was employed by using morpholino antisense oligonucleotides (MOs) (trpm7-ATG-MO) to inhibit translation of trpm7 mRNA, and another MO directed against the junction between exon 12 and intron 12 of the trpm7 gene (trpm7-E1/MO) to interfere with

Fig. 2. The swdp75fm mutation impairs exocrine pancreatic epithelial cell division and cell growth. (A) Cell-cycle analysis of the exocrine pancreatic epithelia in swdp75fm mutants and WT siblings at 72 hpf. % BrdU+ nuclei represent the proportion of cells in the S phase of the cell cycle. (B) Morphometric analysis of cell growth (area, in μm², per cell) in the exocrine pancreatic epithelia of swdp75fm mutants and WT siblings on 3 and 5 dpf. Each value represents the mean ± s.e.m. Statistical analysis was performed using Student’s t-test, with *P<0.05 considered statistically significant.

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splicing of intron 12 and result in a frameshift and a premature stop codon (Elizondo et al., 2005). In the trpm7-atg-mo-injected larvae, both the size of exocrine pancreas and the area of skin pigmentation were reduced, resembling the phenotype of the swdp75fm mutant (Fig. 4A). Moreover, the pancreatic acini were relatively small and hypomorphic (Fig. 4B). The proportion of epithelia in the S phase of the cell cycle and the cell size of pancreatic epithelia were also significantly reduced (Fig. 4C,D). Similarly, injection of trpm7-E1-I-MO into WT embryos produced a relatively small exocrine pancreas resembling that in the swd and trpm7 mutants (supplementary material Fig. S2). These results further support that trpm7 is the gene responsible for the exocrine pancreas and skin pigment phenotype caused by the swd mutations, and Trpm7 is functionally required for epithelial proliferation that is coordinately regulated with morphogenesis and growth of exocrine pancreas.

**Supplementary Mg2+ partially rescues the exocrine pancreatic defects of trpm7 mutants by improving cell division and cell growth**

The role of Trpm7 in cellular Mg2+ homeostasis suggests that Mg2+ transport is impaired in swd mutants. This raises the possibility that the swd mutation disrupts the function of Trpm7 in controlling cellular influx of Mg2+, resulting in reduced exocrine pancreatic epithelial proliferation. To determine whether supplementary Mg2+ complements the exocrine pancreatic defect of the trpm7 mutants, the swdp75fm and trpm7j124e1 mutant embryos, as well as their WT siblings, were incuobated in medium with or without added MgCl2, and the exocrine pancreas was examined by in situ hybridization using anti-trypsin riboprobes. As shown in Fig. 5A and supplementary material Fig. S1C,D, respectively, there was partial improvement in the size of exocrine pancreas of the swdp75fm and trpm7j124e1 mutants when MgCl2 was present. There is no distinguishable difference in the size of exocrine pancreas of WT larvae grown in the presence or absence of supplementary Mg2+ (N.S.Y., unpublished). This suggests that the swdp75fm and trpm7j124e1 mutations impair the function of Trpm7 in controlling the cellular Mg2+ level, resulting in reduced size of exocrine pancreas.

The complementary effect of Mg2+ on the swdp75fm and trpm7j124e1 mutants was further evaluated for the proliferative ability of the exocrine pancreatic epithelia. Cell-cycle progression in the swdp75fm and trpm7j124e1 mutants was significantly improved when grown in the presence of supplementary Mg2+, as indicated by the proportion of cells in S phase [% BrDU]-positive (+) nuclei), as compared with those grown in E3 medium without supplementary Mg2+ (Fig. 5B; Table 1B). Supplementary Mg2+ had no significant effect on the proportion of cells in S phase in the exocrine pancreas of WT larvae. Besides, supplementary Mg2+ significantly improved epithelial growth in the exocrine pancreas of the swdp75fm and trpm7j124e1 mutants, relative to those grown without supplementary Mg2+ (Fig. 5C; Table 1B). Again, supplementary Mg2+ had no significant effect on the growth of the exocrine pancreatic epithelia in WT larvae. These data indicate that supplementary Mg2+ complements the exocrine pancreas defects of the swdp75fm and trpm7j124e1 mutants by improving cell-cycle progression and epithelial growth.

To further characterize the effect of supplementary Mg2+ on exocrine pancreatic epithelial proliferation in swdp75fm mutants, expression of the cyclin-dependent kinase inhibitor p21cikin4a and cyclin G1, which play crucial roles in the progression from G1 to S phase (el Diery et al., 1994; Zhao et al., 2003), was examined. Consistent with the defect in progression into S phase, the mRNA levels of both p21cikin4a and cyclin G1 were elevated in the swdp75fm mutants as compared with the WT siblings (at 0 mM supplementary Mg2+; Fig. 5D,E; supplementary material Table S1A,
Table 1. The exocrine pancreas of trpm7b508 mutants is relatively small, and the proliferative defect can be improved by supplementary Mg²⁺ or by antisense knockdown of socs3a

| Assay                  | trpm7b508 mutant | WT                          | t-test |
|------------------------|------------------|-----------------------------|--------|
| % BrdU+ nuclei         | 29.7±12.9        | 46.8±9.6                    | *P<0.05|
| Area (µm²)/cell        | 16.2±2.0         | 26.3±5.1                    | *P<0.01|

| Assay                  | trpm7b508 mutant + 40 mM Mg²⁺ | trpm7b508 mutant + socs3a-ATG-MO | t-test |
|------------------------|-------------------------------|----------------------------------|--------|
| % BrdU+ nuclei         | 22.5±11.1                     | 38.9±0.5                         | *P<0.05|
| Area (µm²)/cell        | 16.2±2.0                      | 22.6±0.5                         | *P<0.001|

A) trpm7b508 mutants and their WT siblings were incubated until 70 hpf, injected with BrdU and then processed by immunohistochemistry with anti-BrdU for the proportion of S-phase cells (% BrdU+ nuclei) or with anti-Cad antibodies for morphometric analysis of cell growth (area in µm²/cell). (B) trpm7b508 mutants and their WT siblings were incubated in E3 medium without or with supplementary 40 mM MgCl₂ until 70 hpf and then analyzed for % BrdU+ nuclei and area (µm²)/cell. (C) The embryos of trpm7b508 mutants were microinjected with socs3a-5-mispair-MO, incubated until 70 hpf, injected with BrdU and then analyzed for % BrdU+ nuclei and area (µm²)/cell. (B,C) trpm7b508 mutants were identified on the basis of their hypopigmented skin, which was not appreciably affected by supplementary MgCl₂ or injection of socs3a-ATG-MO. (A-C) Each value represents the mean ± s.d. *P indicates statistically significant difference.

Gene #25). In agreement with its complementary effect on the exocrine pancreas, addition of supplementary Mg²⁺ in the medium downregulated p21cdkn1a and cyclin G1 mRNA expression in the swdp75fm mutants to levels close to those of WT larvae (at 20 or 40 mM supplementary Mg²⁺; Fig. 5D,E). These data suggest that the swd mutation perturbs the function of Trpm7 in regulating cellular Mg²⁺ levels, causing upregulation of p21cdkn1a and cyclin G1 mRNA and leading to impaired growth of exocrine pancreas.

The Mg²⁺-sensitive Soc3a pathway mediates the proliferative role of Trpm7

To gain insight into the mechanism by which supplementary Mg²⁺ improves the exocrine pancreatic phenotype, the swdp75fm mutants and their WT siblings were incubated in the absence (−) or presence (+) of added 40 mM MgCl₂ and analyzed by transcriptional profiling. In the swdp75fm mutants (+Mg²⁺), expression of the negative modulator of epidermal growth factor (Egf)-induced proliferation, socs3a, was downregulated by 187% as compared with the swdp75fm mutants (−Mg²⁺) (supplementary material Table S2A, gene #5), and this was confirmed by real-time PCR (Fig. 6A). Similarly, the socs3a mRNA level was repressed by supplementary Mg²⁺ in the trpm7b508 mutants, as compared with that in the trpm7b508 mutants incubated without supplementary Mg²⁺ (supplementary material Fig. S1E). Expression of socs3a was verified to be upregulated in the swdp75fm mutants and trpm7b508 mutants relative to WT (all incubated in E3 medium without supplementary Mg²⁺), as indicated by real-time PCR (Fig. 6B; supplementary material Table S3, Fig. S1E). These data strongly suggest that Mg²⁺-modulated expression of socs3a mRNA is involved in the proliferative effect of Trpm7.

SOCS3 is a negative regulator of mitogenic signals, such as those induced by EGF (Gotoh, 2009), and of proinflammatory signals, such as those induced by interleukin 6 (IL-6), IL-1β, tumor necrosis factor-α and transforming growth factor-β1 (TGFβ1), in mammalian pancreatic epithelia (Vona-Davis et al., 2005; Yu et al., 2008). To
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To determine the role of Socs3a signaling in Trpm7-mediated epithelial proliferation in exocrine pancreas, the effect of antisense knockdown of socs3a was analyzed. Targeted depletion of Socs3a using MO directed against the translation start site of socs3a mRNA partially improved growth of exocrine pancreas in swdp75fm and trpm7b508 mutants. Relative to the control-MO-injected swdp75fm mutants, the exocrine pancreas of the swdp75fm mutants injected with socs3a MO was slightly increased in size (Fig. 6C). The proportion of cells in the S phase (% BrdU+ nuclei) and cell growth (area per cell) significantly increased in the socs3a-MO-injected swdp75fm and trpm7b508 mutants as compared with the control-MO-injected mutants (Fig. 6D,E; Table 1C). Therefore, Socs3a is involved in modulating the proliferative role of Trpm7 in exocrine pancreatic epithelia. Taken together, results of this study suggest that Trpm7 regulates epithelial proliferation and morphogenesis of exocrine pancreas via the Mg²⁺-sensitive, Socs3a-mediated signaling pathway.

TRPM7 is overexpressed in human pancreatic adenocarcinoma and is required for Mg²⁺-dependent cellular proliferation

Developmental regulators of exocrine pancreas have been shown to play important roles in pancreatic carcinogenesis (Yee and Pack, 2005; Yee, 2010). To test the hypothesis that TRPM7 plays a functional role in pancreatic adenocarcinoma, expression of TRPM7 was analyzed in human pancreatic adenocarcinoma tissues by immunohistochemistry. In contrast to the low level of immunoreactivity at the apical plasma membrane of pancreatic ductal epithelia, TRPM7 was diffusely expressed in the cytoplasm at elevated levels in pancreatic adenocarcinoma (Fig. 7A). Expression of TRPM7 mRNA was then quantified in a panel of established and characterized cell lines by real-time PCR. As compared with human pancreatic ductal epithelia, TRPM7 mRNA was expressed at elevated levels in five of seven human pancreatic adenocarcinoma cell lines examined (Fig. 7B). TRPM7 protein was similarly expressed at relatively high levels in those cell lines, as revealed by immunoblot analysis (N.S.Y., unpublished). Taken together, these data indicate that TRPM7 is overexpressed in human pancreatic adenocarcinoma and suggest a functional role of TRPM7 in the pathogenesis of pancreatic neoplasia.

To determine the functional role of TRPM7 in pancreatic cancer, the effect of small interfering RNA (siRNA)-induced silencing of TRPM7 on cellular proliferation, cell-cycle progression and apoptosis was determined. PANC-1 and BxPC-3 cells transfected

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with siRNA directed against TRPM7 expressed a 70% reduced level of TRPM7 mRNA as indicated by real-time PCR. TRPM7 protein levels were similarly reduced in the anti-TRPM7-siRNA-treated cells, as compared with that in the control-siRNA-treated cells, as shown by immunoblot analysis (N.S.Y., unpublished). Morphological examination revealed the presence of multinucleated cells and cytoplasmic vacuoles in both PANC-1 and BxPC-3 cells treated with anti-TRPM7 siRNA (Fig. 7C), and these features are consistent with arrest in cell division.

As compared with control-siRNA-treated cells, proliferation of the TRPM7-deficient cells was significantly reduced (by 32%) as measured by the MTS assay, and reduced by 36% as determined by counting viable cells (Fig. 8A). Flow cytometric analysis of cell cycle indicated an increase in the proportion of TRPM7-deficient cells in G0-G1 phases by 42% compared with controls, and a reduced proportion of cells in the S and G2-M phases by 32% and 17%, respectively (Fig. 8B). No significant change in the proportion of apoptotic cells or the level of the proapoptotic protein BAX by treatment of anti-TRPM7 siRNA was observed (N.S.Y., unpublished). Analysis of the cell-cycle regulators in the TRPM7-deficient BxPC-3 and PANC-1 cells by real-time PCR revealed an increase of p21CDKN1A mRNA levels, and a decrease of cyclin G1 and cyclin B1 mRNA levels (Fig. 8C). These findings indicate that TRPM7 is required for cellular proliferation of pancreatic adenocarcinoma by regulating transition from G1 to S and G2 to M in the cell division cycle.

The complementary effect of Mg2+ on the exocrine pancreatic defect of the zebrafish swd mutants is consistent with the role of TRPM7 in cellular Mg2+ homeostasis. To determine the role of Mg2+ in TRPM7-mediated proliferation of pancreatic cancer cells, TRPM7-deficient PANC-1 and BxPC-3 cells were incubated in culture medium in the presence or absence of 1 mM MgSO4, and then analyzed for proliferation. In the absence of supplementary Mg2+, proliferation was significantly reduced (by 37% and 35%) as indicated by the MTS assay and counting cells, respectively (Fig. 8D). In the presence of supplementary Mg2+, proliferation of the TRPM7-deficient cells was restored to the same levels as in the cells transfected with non-targeting control siRNA (Fig. 8D). These results indicate an essential role of Mg2+ in TRPM7-mediated control of proliferation in pancreatic adenocarcinoma cells, and they further support the idea that TRPM7 controls cellular influx of Mg2+ or, alternatively, that Mg2+ modulates TRPM7-mediated signaling.

**DISCUSSION**

The roles of ion channels in exocrine pancreatic development and oncogenesis are mostly unknown. Using zebrafish as a vertebrate model organism, we have presented evidence that the ion channel...
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kinase Trpm7 controls the size of exocrine pancreas during embryogenesis by regulating cell division and cell growth. Results of this study reveal that Mg2+-sensitive Socs3a signaling modulates the proliferative role of Trpm7 in exocrine pancreas. The findings in zebrafish can be translated into human pancreatic adenocarcinoma, in which TRPM7 is overexpressed and required for Mg2+-dependent proliferation of the cancer cells. We hypothesize that Trpm7 contributes to the homeostatic mechanism that controls the size of exocrine pancreas in vertebrates, and suggest that TRPM7-mediated signaling can be further evaluated as diagnostic and/or prognostic biomarkers and therapeutic targets in human pancreatic adenocarcinoma.

Trpm7 regulates exocrine pancreatic epithelial proliferation and morphogenesis

TRPM7 is ubiquitously expressed in mammals (Fonfria et al., 2006) and zebrafish (http://zfin.org), yet loss-of-function mutations in trpm7 produce organ-specific phenotypes. In zebrafish, Trpm7 is functionally required for survival of melanophores: disruption of trpm7 by the trpm7j124e1, trpm7b508 or swd mutations, or by targeted knockdown using MO, diminishes skin pigmentation and depletes melanophores (Rawls et al., 2003; Cornell et al., 2004; Arduini and Henion, 2004; Elizondo et al., 2005; Yee et al., 2005; McNeill et al., 2007) (this study). Furthermore, it has been shown that the zebrafish tct/trpm7j124e1 and nutria/trpm7j124e2 mutations impair skeletal development, which is associated with nephrolithiasis and impeded growth (Elizondo et al., 2005). Although global deletion of the Trpm7 gene in mouse is embryonically lethal, selective disruption of Trpm7 in developing thymocytes causes loss of thymic architecture and a reduced number of T cells (Jin et al., 2008). In this study and our previous report (Yee et al., 2005), analyses of the swd, trpm7j124e1 and trpm7b508 mutants and the morphants with disrupted expression of trpm7 support the functional role of Trpm7 in regulating epithelial cell-cycle progression and growth and, consequently, acinar and ductal morphogenesis. It is interesting to note that global analysis reveals elevated expression of p21cdkn1a, cyclin G1 and socs3a in swd mutants. The relatively specific phenotype in the exocrine pancreas and skin pigmentation might be related to the specific requirement of different tissues, timing of expression and molecular context. However, recent evidence suggests that a developmentally established intrinsic program limits the number of embryonic progenitor cells in the mouse pancreas and determines the organ size (Stanger et al., 2007). In light of those findings, we hypothesize that Trpm7 is involved in the
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homeostatic mechanism that regulates proliferation of exocrine pancreatic progenitor cells and controls the growth and thus the size of exocrine pancreas during embryonic development.

The molecular nature of the swd mutations in the trpm7 gene remains to be identified; however, this study provides evidence that the kinase domain and the C-terminal sequence of Trpm7 is required for normal growth of exocrine pancreas. This is supported by the data showing the relatively small exocrine pancreas and the reduced epithelial cell division and growth in trpm7j124e1 and trpm7b508 mutants. Electrophysiological studies in mammalian cells with heterologously expressed kinase-deficient TRPM7 suggest that TRPM7 kinase activity is not necessary for channel activation but is important for sensitivity of the channel to Mg2+ (Schmitz et al., 2003). Results of another study indicate that the channel function of TRPM7 is not dependent on its kinase activity or autophosphorylation, and they suggest that the kinase domain is structurally necessary for the channel activity of TRPM7 (Matsushita et al., 2005). In light of those studies and the defects observed in the trpm7j124e1 and trpm7b508 mutants (Elizondo et al., 2005; McNeill et al., 2007) (this study), we hypothesize that the kinase domain of Trpm7 is required for its functional role in normal development. Whether the swd mutations produce the skeletal defects of the trpm7j124e1 adult mutants remains to be determined. Further studies should directly determine the role of channel activation and kinase activity of Trpm7 in pancreatic epithelial proliferation and growth of exocrine pancreas. Moreover, future gain-of-function studies by overexpressing trpm7 under an exocrine-pancreas-specific promoter such as that of the ptf1a or elastase gene in WT or a zebrafish line with a mutation in K-ras or p53 are expected to help define the role of Trpm7 in pancreatic growth.

Mg²⁺-sensitive Socs3a pathway links Trpm7 to growth-factor and cytokine-induced signaling

This study reveals a mechanism that modulates the proliferative role of Trpm7 in exocrine pancreas by the Mg²⁺-sensitive Socs3a...
pathway. Mg^{2+} is considered a key factor that coordinates control of cell proliferation (Rubin, 1975; Walker and Duffus, 1980) and is involved in receptor-mediated mitogenic signals of growth factors (Wolf and Trapani, 2008). Consistent with the role of Trpm7 in cellular Mg^{2+} homeostasis, the exocrine pancreatic defect of the swd and trpm7b508 mutants can be partially rescued by supplementary Mg^{2+}. Similarly, the rescue effect of supplementary Mg^{2+} on melanophores has also been demonstrated in tct/trpm7m124e1 mutants (Elizondo et al., 2005; McNeill et al., 2007). These findings suggest that the swd and trpm7b124e1 mutations perturb cellular Mg^{2+} homeostasis, such that supplementary Mg^{2+} restores the cellular balance of Mg^{2+}. Alternatively, supplementary Mg^{2+} might activate the signaling pathways downstream of Trpm7 in the mutants. However, Mg^{2+}-mediated rescue is partial, possibly because a high concentration of Mg^{2+} in the medium is cardiotoxic. Evidence for this is that, in the larvae incubated at 50 mM and 100 mM MgCl2, generalized edema is observed beginning at 4 dpf and it becomes prominent at 5 dpf and afterwards (N.S.Y., unpublished).

Transcriptional profiling of swd mutants incubated in the absence or presence of supplementary Mg^{2+} has begun to elucidate the signaling pathways that mediate the developmental role of Trpm7 in exocrine pancreas. As shown in this study, the negative regulator of growth-factor and cytokine signaling, Socs3a, is involved in the Mg^{2+}-sensitive proliferative action of Trpm7. Recent evidence from targeted deletion of Trpm7 in mice has indicated that signal transducers and activators of transcription 3 (STAT3), which is negatively regulated by Socs3, is involved in TRPM7-mediated signaling during lymphocytic development (Jin et al., 2008). In agreement with the elevated level of socs3a mRNA in the swd/trpm7 mutants in our study, disrupted expression of Trpm7 in mice causes downregulated expression of Stat3 mRNA (Jin et al., 2008). Indeed, repression of STAT3 decreases proliferation of a variety of cell types, including in pancreatic cancer cells (Kotha et al., 2006; Sahu and Srivastava, 2009). Thus, the involvement of Socs3a (this study) and STAT3 (Jin et al., 2008) links TRPM7 to the signaling pathways that mediate the mitogenic effects of growth factors and the proinflammatory effects of cytokines. Besides, expression of the developmental pathways known to play important roles in exocrine pancreas, including Hedgehog, Notch, Wnt, Tgfβ, and Fgf, is altered in swdp75fm mutants (supplementary material Table S3). Ongoing studies are focused on determining how these developmental pathways influence expression of socs3a and the other proinflammatory genes in the swd/trpm7 mutants as well as in human pancreatic adenocarcinoma cells incubated in the absence or presence of supplementary Mg^{2+}. These data are expected to help understand the mechanism that mediates the functional roles of Trpm7 and Mg^{2+} in exocrine pancreatic epithelial proliferation and morphogenesis.

Potential role of TRPM7 in the pathogenesis of pancreatic cancer

In our attempt to translate the developmental studies of Trpm7 in zebrafish to human disease, we identified a functional role of TRPM7 in human pancreatic cancer. This finding is supported by the global genomic analysis of gene expression in human pancreatic adenocarcinoma tissues and cell lines (Jones et al., 2008). Detailed examination of the level and pattern of TRPM7 expression in normal pancreatic ductal and acinar epithelia, intraepithelial neoplasia, and invasive adenocarcinoma is ongoing and is expected to provide further insights into its role in the initiation and progression of pancreatic tumors.

Moreover, TRPM7 is required for proliferation of the cancer cells as evidenced by the cellular assays and modulation of the cell-cycle regulators, and TRPM7-mediated proliferation of pancreatic adenocarcinoma cells is Mg^{2+}-dependent. These findings are consistent with and complementary to the zebrafish loss-of-function studies of Trpm7 and Mg^{2+}-mediated rescue in the developing exocrine pancreas. The upregulated expression of p21^CDKN1A and repression of cyclin B1 in TRPM7-deficient pancreatic adenocarcinoma cells are consistent with the observed reduction of the proportions of cells in the S and G2-M phases. It is interesting to note that cyclin G1 expression is downregulated in the pancreatic cancer cells that had reduced expression of TRPM7, suggesting a growth-promoting activity of cyclin G1. By contrast, supplementary Mg^{2+}-mediated partial rescue of the exocrine pancreatic defect in the zebrafish swd mutants is associated with repression of cyclin G1, suggesting a growth-inhibitory activity of cyclin G1. The apparently conflicting roles of cyclin G1 in growth control have also been noted by others and might be at least partially explained by the intrinsic growth-suppressing activity of cyclin G1 being dependent on its expression level (Zhao et al., 2003). However, the findings of this study together with the established role of TRPM7 in cation transport strongly indicate a physiological role of TRPM7 and Mg^{2+} and Ca^{2+} in pancreatic ductal epithelia, and suggest that aberrant TRPM7-mediated signals contribute to uncontrolled proliferation of neoplastic cells.

Conclusion and prospective

Genetic studies of the zebrafish swd and trpm7 mutants, and expression analysis of TRPM7 in human pancreatic adenocarcinoma – as well as experiments of silencing the trpm7 gene in zebrafish larvae and in human pancreatic cancer cells – provide evidence that TRPM7 is functionally required for pancreatic epithelial proliferation. These findings suggest that Trpm7 and/or Mg^{2+} and the associated Socs3a signaling play an important role in the homeostatic mechanisms that control the number of pancreatic progenitors and determine the size of exocrine pancreas during development. Moreover, aberrant TRPM7-mediated signaling might be involved in the uncontrolled cellular proliferation during formation of pancreatic neoplasia. Ongoing studies focus on determining the signaling mechanisms that mediate the functional role of TRPM7 in exocrine pancreatic growth during vertebrate morphogenesis and the tumorigenic process, and on exploring TRPM7 as a diagnostic and/or prognostic biomarker and a therapeutic target in human pancreatic adenocarcinoma.

METHODS

Zebrafish stock

Zebrafish husbandry is described in detail elsewhere (Brand et al., 2002). The swdp75fm and swg82mf mutant lines were described previously (Yee et al., 2005). WT zebrafish of the AB strain and the trpm7b124e1 mutant line were obtained from the Zebrafish International Resource Center (Portland, OR). The trpm7b508 mutants were provided by Gregory Bonde (Laboratory of Robert Cornell) at the University of Iowa. The E3 embryo medium
contains 5 mM NaCl, 0.17 mM KCl and 1.65 mM MgSO₄. To facilitate visualization of exocrine pancreas in the larvae, 1-phenyl-2-thiourea (PTU; Sigma) was added to E3 medium at 0.003% to inhibit skin pigmentation as indicated. For supplementary Mg²⁺, a stock solution of 1 M MgCl₂ hexahydrate (Sigma-Aldrich) was prepared in purified water (Milli-Q Synthesis, Millipore Corporation, Billerica, MA) and diluted at the indicated concentrations with E3 medium.

**Immunohistochemistry, in situ hybridization, and histological analysis**

These procedures are essentially as described (Yee and Pack, 2005; Yee et al., 2005). For immunohistochemistry, rabbit anti-Cpa antibodies (Rockland Immunocchemicals), mouse anti-BrdU antibodies (Roche), guinea pig anti-insulin and anti-glucacon antibodies (Linco/Millipore), and rabbit anti-pan-cadherin antibodies (Sigma) were used at 1:100 dilution in 5% goat serum (Gibco/Invitrogen). For in situ hybridization, trypsin and ptf1a antisense riboprobes were prepared (Yee et al., 2005; Lin et al., 2004). Zebrafish larvae were examined under a stereo-microscope (Leica MZ16F, Plymouth, MN) with bright light. Images were captured using QImaging digital camera and Q-Capture software (Leica MZ16F, Plymouth, MN) with bright light. Images were captured using QImaging digital camera and Q-Capture application, and constructed using Adobe Photoshop 7.0. For histological analysis, zebrafish larvae were embedded in JB-4 Plus solution (Polysciences) and sectioned at 5-μm intervals using a semi-automatic microtome (Leica RM2255, Plymouth, MN). The immunostained sections were mounted with VECTASHIELD with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories) and examined under a compound microscope (Olympus BX51, Tokyo, Japan) using fluorescence. The non-immunoassayed sections were stained with methylene blue (Acros)-azure II (Sigma) solution, mounted with Permount (Sigma) and examined under a compound microscope (Olympus BX51) using bright light. The images were acquired using a digital camera (Olympus DP71), analyzed using DP Manager software (Olympus) and constructed using Adobe Photoshop 7.0.

**Exocrine pancreatic epithelial cell cycle**

BrdU (Sigma) was used as a cellular marker for the S phase of the cell cycle, detected by immunohistochemistry, followed by histological examination, and analysis was performed by cell counting as described (Yee and Pack, 2005). Briefly, each larva at 72 hpf was injected with 30 nl of 30 mM BrdU into the yolk sac under a stereo-dissecting microscope (Leica MZ16F, Plymouth, MN), followed by incubation at 28.5°C for 1 hour, and then fixed with 4% paraformaldehyde. For immunohistochemistry, the larvae were permeabilized with proteinase K, treated with 3 N hydrochloric acid to denature the double strands of DNA, and washed with phosphate buffered saline, pH 7.0, with 0.1% Tween 20 (PBS-T). Mouse anti-BrdU antibodies were added at 1:100 dilution for incubation at 4°C overnight. Guinea pig anti-insulin and anti-glucacon antibodies (Linco/Millipore) were also added at 1:100 dilution as markers of pancreatic islet cells. Following incubation, the larvae were washed with PBS-T, and then incubated with Alexa-Fluor-488-conjugated goat anti-mouse IgG and Alexa-Fluor-594-conjugated goat anti-guinea-pig IgG (Invitrogen). Following incubation at 4°C overnight, the larvae were washed with PBS-T, serially dehydrated with ethanol and embedded with JB-4 plastic solution (Polysciences). The embedded larvae were cross-sectioned at 5-μm intervals and analyzed. Five larvae in each group were evaluated, and for each larva three sections containing exocrine pancreas posterior to the last section of islet were analyzed. For each larva, the number of DAPI-positive nuclei, number of BrdU-positive nuclei, and proportion of DAPI-positive nuclei being BrdU-positive and representing the percentage of cells in the S phase, were determined.

**Exocrine pancreatic epithelial growth**

Morphometric analysis of cell growth was performed using cadherin as a marker of intercellular junctions. Larvae were fixed with methanol/dimethyl sulfoxide (80:20) as described (Yee et al., 2005).

Cadherin was detected by immunohistochemistry using rabbit anti-pan-cadherin antibodies (Sigma) at 1:100 dilution. Guinea pig anti-glucacon antibodies (Linco/Millipore) were also added at 1:100 dilution as markers of pancreatic islet cells. The immunoassayed larvae were embedded and cross-sectioned at 5-μm intervals. Five larvae in each group were evaluated, and for each larva three sections containing exocrine pancreas posterior to the last section of islet were analyzed. The surface area of exocrine pancreas on each of three sections posterior to the last section of islet, the number of DAPI-positive nuclei, and the surface area per cell, which indicates cell growth, were determined.

**Positional cloning and complementation test**

To identify the gene affected by the swd mutation, we collected 2500 swdp85fm mutants and isolated genomic DNA for bulked segregant analysis and high-resolution meiotic mapping as described (Yee et al., 2007). Using simple sequence repeats as genomic markers, available at http://zfrhmaps.tch.harvard.edu/ ZonRHmapper/RHLg/lg18.htm, we have located the swd mutation on chromosome 18 (Manimegalai Muthumani, Michael Pack, and N.S.Y., unpublished). On the basis of four recombination events in 1222 meioses, we identified a genomic marker z25105 within 0.3 cm (estimated to be 180 kb, assuming an average of 60 kb per 0.1 cm in zebrafish) from the swd mutation. In this region, a gene that corresponds to the zebrafish ortholog of mammalian TRPM7 is affected by the zebrafish mutation tct (Rawls et al., 2003; Cornell et al., 2004; Arduini and Henion, 2004; Elizondo et al., 2005). The alleles of tct are also known as trpm712461 and trpm712088. A complementation test performed by crossing swdp85fm+/+ or swdp82mf/+ with trpm712461/+ produced 25% of mutant larvae with indistinguishable phenotype of exocrine pancreas and skin pigmentation as the swdp85fm, swdp82mf and trpm712461 mutants.

**Targeted gene knockdown with morpholino anti-sense oligos**

MO was microinjected into the embryos as described (Yee and Pack, 2005). Briefly, embryos were microinjected at the one-cell stage with MO (0.45 pmole in 1 nl per embryo) into the yolk sac and incubated at 28.5°C until analysis. The MOs were directed against the translation start site of zebrafish trpm7 mRNA (trpm7-ATG-MO: 5'-CCAGGACTTCTGGGAGATTCTCTTC-3' based on trpm7 cDNA sequence; GenBank accession number AY860421); the junction between exon 12 and intron 12 of the trpm7 gene (trpm7-12-MO: 5'-GTTGTTGAGATTCTCTGTGCAC-3'), which interferes with splicing of intron 12 and results in a frameshift and stop codons.
a premature stop codon as described by Elizondo et al. (Elizondo et al., 2005); the translation start site of zebrafish socs3a mRNA (socs3a-ATG-MO: 5'-TGTTGGGTACATCGGTCATACCA-3', based on socs3a cDNA sequence; GenBank accession number BC049326). Gene-specific mismatch oligos including trpm7-5-mispair-MO (5'-CCAGCTTGCATTGACTTCTTGGTTTCC-3') and socs3a-5-mispair-MO (5'-TGTTGCGTTATGATGCGAGAACAC-3') (lowercase indicates mismatched bases), as well as standard control oligos (5'-CCTTTACCTCAGTTCAATTATA-3') that have been shown not to be directed against any zebrafish gene sequence, were used as controls. The trpm7-ATG-MO, trpm7-E1-MO, trpm7-5-mispair-MO, socs3a-ATG-MO, socs3a-5-mispair-MO and control MO were designed and produced by Gene Tools, LLC (Philomath, OR).

**Semi-quantitative real-time PCR**

Total RNA was isolated from 20 swdp75fm mutants or their WT siblings using TRIzol (Invitrogen) and RNeasy Mini Kit (Qiagen) according to the manufacturers’ instructions. First-strand cDNA was generated using SuperScript reverse transcriptase and random primers (Invitrogen) and amplified with SYBR Green (Applied Biosystems) using ABI Prism 7700 real-time PCR system (Applied Biosystems, Foster City, CA). The sequences of the primers used for analysis of expression of p21\_cdkina, cyclin G1 and socs3a are based on zebrafish cDNA sequences (GenBank accession numbers: AL912410, ATG-MO, and BC049326, respectively) as follows: p21\_cdkina: 5'-ATGCACTTCGACAGATA-3', 5'-CGCAA-CAGACCAACATCC-3'; cyclin G1: 5'-TAAGATCGCCCA-CTTACCTA-3', 5'-ACCATAGTCTGAACGCAGGAG-3'; socs3a: 5'-GGGGGAGAAAAATTCCCTTG-3', 5'-GCCCAAAAACGT-AAGCCATA-3'.

**Glyceraldehyde-3-phosphate dehydrogenase** (gapdh) was used as internal control to normalize for the amount of RNA in each sample. The primers used for amplification are based on zebrafish gapdh cDNA sequence (GenBank accession number BC095386): gapdh: 5'-GATACAGGGAGAGCCAGTCTT-3', 5'-CTGTCAGGA-GAAATACCAGCA-3'.

The conditions for quantitative PCR are as follows: 2 minutes at 50°C, 10 minutes at 95°C, followed by 40 cycles: 15 seconds at 95°C, 1 minute at 60°C, and 10 minutes at 72°C. Each sample was analyzed in triplicate, and each experiment performed three times. The mRNA levels were analyzed, and the mean ratio of p21\_cdkina, cyclin G1 and socs3a mRNA to gapdh mRNA was determined using the Comparative Ct Method (Applied Biosystems). Samples of PCR using primers directed against p21\_cdkina, cyclin G1, socs3a and gapdh were analyzed by agarose gel electrophoresis to validate specificity of amplification products.

**Transcriptional profiling**

Twenty swdp75fm mutants or their WT siblings were incubated at 28.5°C in E3 medium with or without supplementary 40 mM MgCl2 for 72 hours. Total RNA was extracted using TRIzol (Invitrogen) and RNeasy Mini Kit (Qiagen) from each group of larvae. Each sample of RNA (50 ng) was converted by single primer isothermal amplification (SPIA) to cDNA using the Ovation RNA Amplification System (NuGEN Technologies). The amplified cDNA product was purified, fragmented, biotin-labeled, mixed with hybridization buffer, placed onto Affymetrix Zebrafish arrays (containing 14,900 transcripts) and incubated at 45°C for 18 hours with 60 rpm rotation. Following hybridization, the arrays were washed, stained with streptavidin-phycocerythin (Molecular Probes/Invitrogen) and signal-amplified with anti-streptavidin antibodies (Vector Laboratories). Arrays were scanned with the Affymetrix Model 3000 scanner (Santa Clara, CA), and data were collected using the GeneChip operating software (Affymetrix). The microarray data were analyzed using analysis of variance (ANOVA) with the Partek Genomic Suite Software.

**Tissues and cell cultures**

Human pancreatic adenocarcinoma and matched normal tissues embedded in paraffin were obtained from the Tissue Procurement Core of the University of Iowa (with Institutional Review Board approval). The human pancreatic adenocarcinoma cell lines BxPC-3, Capan-1, HPAF-II, Mia PaCa-2, Panc 02.03, PANc-1 and PL45 were obtained from the American Type Culture Collection (ATCC, Manassas, VA), and maintained according to the ATCC instructions. The human pancreatic ductal epithelial cell line H6c7 was described previously (Furukawa et al., 1996). Cell culture media were supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone), 100 U/ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco). Cells were grown in a humidified atmosphere containing 5% CO2 at 37°C. All experiments were performed using culture medium unless otherwise specified. The cells were used within 20 passages of the stocks frozen in liquid nitrogen.

**Immunohistochemistry of TRPM7**

The paraffin-embedded tissues were sectioned at 4-μm intervals using a microtome (Leica Microm HM355S, Plymouth, MN), deparaffinized in xylene and treated with citrate buffer, pH 6, pre-heated in a steamer to unmask antigens. The sections were treated with 3% hydrogen peroxide to quench endogenous peroxidase activity, and then incubated with goat anti-TRPM7 antibodies (1:25; Abcam) for 60 minutes, rinsed with Tris-buffered saline and incubated with horseradish-peroxidase-conjugated anti-rabbit IgG (EnVision+ System; Dako) for 30 minutes. The signals were detected by color reaction using 3,3’-diaminobenzidine (Dako) as substrate for 5 minutes. The sections were counterstained with hematoxylin (Richard-Allan Scientific) and mounted using Permount (Sigma). Negative controls were incubated without primary antibody to rule out non-specific binding by the secondary antibody and to confirm the efficiency of blocking. The expression of TRPM7 was examined under a compound microscope (Olympus BX51). The images were captured using a digital camera (Olympus DP71), analyzed using DP Manager software (Olympus) and constructed using Adobe Photoshop 7. The images shown are representative of matched specimens from five patients.

**Absolute quantification of mRNA in human cells by real-time PCR**

Total RNA extracted from each cell line was analyzed using ABI Prism 7500 real-time PCR system (Applied Biosystems, Foster City, CA) essentially as described (Yee et al., 2010). The sequences of the primers used were designed based on human cDNA sequences (GenBank accession numbers: TRPM7, NM_000389; cyclin G1, 5'-CATTTTAAGAGT-
GTGGCAGT-3', 5'-AGTGCCAGGAAAAACACTA-3'; cyclin G1: 5'-GCGTGGAGGAACTCTTCTG-3', 5'-AAGCTGTGGGAAAGCTGATA-3'; cyclin B1: 5'-TCTCCATTATGTAGGTTTC-3', 5'-CAGTTCACAAAGCAGTGCA-3'. The mRNA levels were determined with a standard curve using known concentrations of mRNA of each gene.

RNA-interference-mediated gene silencing
PANC-1 and BxPC-3 were grown to 70-80% confluency, trypsinized and resuspended at 10^6 cells in 100 μl of Nucleofector Solution (Amaxa/Lonza) containing 600 nM siRNA directed against human TRPM7 (sc-42662; pre-made and validated by Santa Cruz Biotechnology). Transfection was based on electroporation using Nucleofector II (Amaxa/Lonza, Cologne, Germany) and performed under the optimal conditions already established for the cell line by the manufacturer and confirmed in our laboratory. For controls done in parallel, 10^6 cells were transfected with 600 nM non-targeting control siRNA (sc-37007; Santa Cruz Biotechnology) or no siRNA. Immediately following transfection, 500 μl of pre-warmed (37°C) culture medium was added to each cuvette, and the cells transferred to a six-well plate containing 1.5 ml pre-warmed (37°C) culture medium. The siRNA-treated cells and controls were incubated at 37°C with 5% CO2 for 48 or 72 hours following transfection, the cells were assayed for proliferation, morphology, cell cycle, apoptosis, mRNA and protein levels.

Cellular morphology
PANC-1 and BxPC-3 cells were transfected with anti-TRPM7 siRNA, non-targeting control siRNA or no siRNA, plated in six-well tissue-culture dishes at a density of 5×10^5 per well, and incubated for 24 hours. Images of the cells were captured under an inverted light microscope with phase contrast (Olympus IX81, Tokyo, Japan).

Proliferation assay
The effects of siRNA directed against TRPM7 on cellular proliferation were quantified using the CellTiter 96 AQUGENE One Solution Cell Proliferation Assay (Promega) using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonophenyl)-2H-tetrazolium (MTS) as described (Chun et al., 2009). In this study, PANC-1 and BxPC-3 cells were transfected with anti-TRPM7 siRNA or non-targeting control siRNA. The transfected cells were seeded at 2×10^4 in 100 μl medium per well of a 96-well plate (Corning Incorporated) for MTS assay, or at 5×10^5 in 4 ml medium per well of a six-well plate for cell counting. The cells were incubated for a total of 72 hours with fresh culture medium being replaced at the final 24 hours. Proliferation was analyzed using the MTS assay or by counting cells using trypan blue for exclusion of dead cells.

Flow cytometric analysis of cell cycle
PANC-1 and BxPC-3 cells were deprived of serum for 24 hours, then transfected with anti-TRPM7 siRNA or non-targeting control siRNA, seeded at 5×10^5 in 4 ml medium in each well of a six-well plate, and incubated for 72 hours. The DNA contents were analyzed by flow cytometry using a FACScan (Becton, Dickinson and Company, Franklin Lakes, NJ) using propidium iodide (Invitrogen) as described (Chun et al., 2009). Non-specific sub-G0-G1 events were gated out of analyses, allowing for more accurate analysis of cell-cycle distribution curves in the viable cells.

Flow cytometric analysis of apoptosis
PANC-1 and BxPC-3 transfected with anti-TRPM7 or non-targeting control siRNA were incubated with fluorescein-isothiocyanate-conjugated annexin V (Invitrogen) and propidium iodide (Invitrogen) at 72 hours post-transfection, and analyzed for apoptosis by flow cytometry as described (Chun et al., 2009).

Statistical analysis
The mean, s.d., s.e.m. and P-values were analyzed using the Student’s t-test. Statistical significance was considered at a P-value <0.05.

**TRANSLATIONAL IMPACT**

**Clinical issue**
Pancreatic adenocarcinoma, the most common pancreatic cancer, is almost always fatal because it is typically diagnosed at a late stage and is generally resistant to conventional therapies. The genetic alterations that occur during pancreatic carcinogenesis are a subject of intense study, but little is known about the molecular regulation of normal exocrine pancreatic development. A comprehensive understanding of both normal and neoplastic development will allow the identification of biomarkers and targets of this disease, and facilitate the design of strategies to improve the prevention, early detection and treatment of this disease.

**Results**
In this paper, the authors identify the ion channel kinase Trpm7 as a regulator of exocrine pancreatic development. Using zebrafish with a mutant trpm7 gene, they show that Trpm7 is required for epithelial proliferation in the exocrine pancreas: in the absence of normal Trpm7, cell-cycle progression and cell growth during morphogenesis are inhibited. Trpm7 regulates cellular Mg2+ and Ca2+ homeostasis, and adding supplementary Mg2+ partially rescues the exocrine pancreatic defects of the zebrafish trpm7 mutants. Addition of Mg2+ also represses the suppressor of cytokine signaling 3a (socs3a) gene, and the authors go on to show that simply inhibiting socs3a expression improves trpm7 mutant exocrine pancreatic growth. The proliferative role of Trpm7 is therefore sensitive to Mg2+ and also involves Socs3a-mediated signaling. These findings are shown to have relevance to pancreatic adenocarcinoma, because human TRPM7 is overexpressed in pancreatic adenocarcinoma tissues and several pancreatic cancer cell lines. Partial silencing of TRPM7 reduces proliferation in the cell lines by impairing cell-cycle progression and inducing non-apoptotic cell death. As in the zebrafish trpm7 mutants, supplementary Mg2+ rescues the proliferative defect of TRPM7-deficient pancreatic cancer cells.

**Implications and future directions**
This work demonstrates a growth-regulatory role for TRPM7 in the pancreas during development and in cancer. To determine its precise function, however, it will be necessary to define how signaling from TRPM7 is integrated with established signaling pathways in pancreatic epithelia and cancer cells. Given that TRPM7 is a potential biomarker for pancreatic adenocarcinoma, its expression in the development and progression of pancreatic neoplasia, and the relationship between the expression level of TRPM7 and the prognosis and treatment response of patients warrants investigation. Finally, this work also suggests that TRPM7 is a possible therapeutic target. The effects of modulating TRPM7 expression and/or activity using small molecules, either in isolation or in combination with cytotoxic chemotherapeutic agents and other targeted therapeutics, should be evaluated in appropriate tumor models.

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COMPETING INTERESTS
The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS
N.S.Y. conceived, designed and directed this study; he supervised conduction of the experiments, analyzed and interpreted the data, and wrote and prepared the manuscript. W.Z. and I.C.L. designed and conducted the experiments and analyzed the data.

SUPPLEMENTARY MATERIAL
Supplementary material for this article is available at http://dmm.biologists.orglookup/suppl?doi=10.1242/dmm.004564/DC1

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