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Dynamic glucoregulation and mammalian-like responses to metabolic and developmental disruption in zebrafish

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Abstract

Zebrafish embryos are emerging as models of glucose metabolism. However, patterns of endogenous glucose levels, and the role of the islet in glucoregulation, are unknown. We measured absolute glucose levels in zebrafish and mouse embryos, and demonstrate similar, dynamic glucose fluctuations in both species. Further, we show that chemical and genetic perturbations elicit mammalian-like glycemic responses in zebrafish embryos. We show that glucose is undetectable in early zebrafish and mouse embryos, but increases in parallel with pancreatic islet formation in both species. In zebrafish, increasing glucose is associated with activation of gluconeogenic *phosphoenolpyruvate carboxykinase1* (*pck1*) transcription. Non-hepatic Pck1 protein is expressed in mouse embryos. We show, using RNA in situ hybridization, that zebrafish *pck1* mRNA is similarly expressed in multiple cell types prior to hepatogenesis. Further, we demonstrate that the Pck1 inhibitor 3-mercaptopicolinic acid suppresses normal glucose accumulation in early zebrafish embryos. This shows that pre- and extra-hepatic *pck1* is functional, and provides glucose locally to rapidly developing tissues. To determine if the primary islet is glucoregulatory in early fish embryos, we injected *pdx1*-specific morpholinos into transgenic embryos expressing GFP in beta cells. Most morphant islets were hypomorphic, not agenetic, but embryos still exhibited persistent hyperglycemia. We conclude from these data that the early zebrafish islet is functional, and regulates endogenous glucose. In summary, we identify mechanisms of glucoregulation in zebrafish embryos that are conserved with embryonic and adult mammals. These observations justify use of this model in mechanistic studies of human metabolic disease.

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Keywords

zebrafish; mouse; embryo; glucose; pck1; gluconeogenesis; islet; pdx1

1. Introduction

A balance between normal and embryotoxic glucose levels is essential for normal vertebrate development ([54] Riley and Moley, 2006). Exposure to hyperglycemia during embryogenesis causes glucose-associated neural tube closure defects ([39] Loeken, 2006; 51] Peng et al., 1994; 60] Stoate et al., 2008), increased oxidative stress ([39] Loeken, 2006; 65] Trocino et al., 1995; 70] Yang et al., 2008), reduced GLUT expression ([44] Moley, 1999; 45] Moley et al., 1998) and apoptosis ([44] Moley, 1999; 71] Zhao et al., 2009), as well as fetal islet hyperplasia and degranulation ([1] Aerts and Van Assche, 1981; 3] Aerts et al., 1997), and neonatal hyperinsulinemia ([64] Thureen et al., 2006) and macrosomia ([2] Aerts and Van Assche, 2001; 30] Henriksen, 2008; 60] Stoate et al., 2008). Intriguingly, mammalian embryos do not use glucose for energy until the 8-cell stage, after embryonic compaction. Instead, these early embryos depend on lactate and pyruvate for early growth ([9] Biggers et al., 1967).

Further insight into glucose's role in vertebrate embryonic development would benefit from *in vivo* mechanistic studies undertaken in an ancestral vertebrate, and could further our understanding of metabolic disease in humans. Unlike mammals, zebrafish are highly suited to forward genetic (e.g., ([5] Amsterdam et al., 1999)) and small molecule screens (e.g., ([41] Mathew et al., 2007; 46] Murphey et al., 2006; 56] Sachidanandan et al., 2008)) for identification of developmentally essential genes and pathways. Many of the molecular pathways governing axis formation and organogenesis identified by mutational analyses in zebrafish are conserved with mammals. While adult zebrafish regulate glucose similarly to mammals ([20] Eames et al., 2010; 21] Elo et al., 2007), the patterns of endogenous glucose accumulation and utilization in early embryos is unknown.

As a first step in developing this model we wanted to address two unresolved questions. First, do zebrafish embryos make and utilize glucose? Second, is the early zebrafish islet functional, and does it regulate glucose? Understanding the role of endogenous glucose in zebrafish embryogenesis, and how it might be regulated, would highlight differences and similarities with mammals. These data would provide a framework for interpreting transgenic and morpholino mediated knockdown analyses of glucose metabolism in zebrafish. Further, the capacity to manipulate endogenous glucose could circumvent the pleiotropic, osmotic effects of adding exogenous glucose ([27] Gleeson et al., 2007; 37] Liang et al.) to study diabetes in this model.

Here, we have adapted a fluorescent, dual enzyme assay for direct measurement of absolute glucose levels in zebrafish embryo lysates. Using this approach we document dynamic, developmental-stage specific changes in absolute glucose during normal zebrafish development. Early embryos contain no detectable glucose, but levels increase between 16 hours post-fertilization (hpf) and 24 hpf, with the peak in glucose occurring during early pancreatic endocrine cell differentiation and initial stages of islet morphogenesis ([7] Argenton et al., 1999; 8] Biemar et al., 2001). Surprisingly, we detected a very similar pattern in glucose abundance during mouse development. Embryonic day 9.5 (e9.5) isolated mouse embryos also contain undetectable amounts of glucose. This dramatically increases between e13.5 and e17.5, which also corresponds to the differentiation of insulin-expressing beta cells and initial stages of islet formation ([26] Gittes, 2009).

Further similarities between fish and mice were seen in the patterns of *pck1* mRNA and protein expression during embryogenesis. As for mouse Pck1 protein ([72] Zimmer and Magnuson, 1990), zebrafish *pck1* mRNA is expressed in the liver at 72- and 96-hpf, and in a number of non-hepatic tissues, such as nervous system, eye, and gut ([72] Zimmer and Magnuson, 1990). Inhibition of Pck1 enzyme activity with 3-mercaptopicolinic acid suppresses glucose accumulation at early, pre-hepatic stages, suggesting that localized gluconeogenesis provides glucose, anabolic precursors, or both, to rapidly developing tissues in non-placental and amniotic vertebrates alike. Finally, our data are the first to demonstrate that the early zebrafish islet is functional, as inhibition of normal islet development with *pdx1* morpholinos results in sustained hyperglycemia of zebrafish embryos.

Collectively, these studies reveal that zebrafish utilize both gluconeogenic and pancreatic islet-mediated mechanisms to modulate embryonic glucose levels. Further, our data demonstrate evolutionary conservation of key glucoregulatory mechanisms in zebrafish and mammals.

2. Methods

2.1. Zebrafish

Wild type embryos expressing GFP under the control of the insulin promoter (ins:gfp) ([33] Huang et al., 2001) were collected from natural matings and reared in 1/3 Ringer's ([68] Westerfield, 2000) without phenylthiourea. Embryos were staged using morphological criteria up to 24 hpf and by time of development at 28.5 °C thereafter ([35] Kimmel et al., 1995).

In all experiments, embryos were screened and sorted under a Leica dissecting microscope. Wild type, morphant, and drug-treated embryos with normal somite shape, tail extension, eye development and (at later stages) pigment and motility were collected for microscopy and glucose measurement. Those exhibiting idiosyncratic deformities were not included.

2.2. Mice

Twelve week old BALB/c mice were purchased from Charles River Breeding Labs and housed in SPF conditions. Timed matings were used to obtain embryos at distinct gestational ages. Females were evaluated daily, and the presence of a plug was considered gestational day 0.5. At the appropriate times, females were sacrificed and uteri removed to cold phosphate buffered saline (PBS). Embryos were dissected from uterine and extra-embryonic tissues in several changes of cold PBS. Embryos were transferred to 1.5 ml or 50 ml tubes, excess liquid removed, and samples frozen on dry ice.

2.3. Comparative analyses of vertebrate Pck proteins and zebrafish pck mRNAs

Pck1 and Pck2 protein sequences (format: Pck1 NCBI accession, Pck2 NCBI accession) from zebrafish (NP_999916, NP_998357), *Xenopus laevis* (NP_001080152, AAH44042), chicken (NP_990802, NP_990801), mouse (NP_035174, NP_083270), and human (AAH23978, AAH01454) were aligned with ClustalW and Neighbor Joining trees generated (MacVector v. 9.5.2). Pck protein from Lactobacillus plantarum (YP_003064304) served as the outgroup to root the tree. To identify unique sites in *pck1* for gene-specific oligonucleotide design and probe synthesis we aligned the cDNA sequences of *pck1* (NM_214751) and *pck2* (NM_213192) using ClustalW.

2.4. Glucose measurements

Our study focuses on absolute, in vivo glucose levels, as this gives a more precise measure of the activity of glucose-production and utilization pathways. This information can be confounded and lost in relative measures of glucose (i.e., ratio of glucose to DNA content or body size). Pools of 20-25 screened zebrafish embryos were transferred to 10 mm plastic dishes, recounted, and transferred to 1.5 ml microcentrifuge tubes. Excess medium was removed and embryos were frozen on crushed dry ice. Samples were prepared by thawing samples on wet ice in the presence of a volume of assay buffer equivalent to 8 ul per embryo and homogenized. Lysates were cleared by centrifugation and stored at −80 °C.

Embryonic day (e) 8.5, 9.5, 13.5 and 17.5 mouse embryos were homogenized in 100, 100, 300 and 3 ml, respectively, of assay buffer on ice. Both 13.5 and 17.5 embryos were partially thawed and re-frozen to a slurry three times on dry ice to facilitate homogenization. Samples were cleared by centrifugation and supernatants stored at −80 °C. All zebrafish and mouse procedures were approved by the University of Massachusetts Medical School Institutional Animal Care and Use Committee (IACUC).

Assay reagents were purchased from Biovision. Reactions were assembled on ice in black, flat bottom 96-well plates (Costar). Standard curves were generated using glucose standard solution (according to instructions) and were included in each assay. To measure glucose in embryo extracts, 8 ul of sample was added to 42 ul assay buffer. To this, 50 ul of reaction mix containing 1 ul enzyme solution (glucose oxidase, horseradish peroxidase) and 0.4 ul glucose probe (Amplex Red) was added. Control reactions without sample lysate were included in each row. Reactions were incubated for 30 minutes at 37°C in the dark. Fluorescence (excitation 535 nm; emission, 590 nm) was measured using a Safire II plate reader equipped with XFLUOR4 software (v 4.51). This assay is specific for free glucose (BioVision).

Fluorescence values were corrected by subtracting measurements from control reactions without sample (Fig. 1). Glucose levels were interpolated from standard curves. Each sample was measured in triplicate and each experiment repeated a minimum of three times. We interpret our data with the understanding that we are not directly measuring gluconeogenic or glycolytic rates and that glucose values at any stage may reflect the net activities of these pathways.

2.5. Real time and Semi-quantitative RT-PCR

The oligonucleotides used in this study were designed using Oligo Primer Analysis Software version 6.89 (Molecular Biology Insights Inc.). Sequences are provided in Table 1. Total RNA was extracted using Trizol reagent in combination with PureLink columns (Invitrogen). Column-bound RNA was treated with DNAse prior to washing and elution (Invitrogen).

For quantitative RT-PCR, cDNA was synthesized from 1 ug of total RNA using oligo (dT) primers (Transcriptor High Fidelity cDNA Synthesis kit; Roche, Indianapolis, IN). Quantitative real-time PCR was performed in a Light Cycler (Roche) using the LightCycler DNA Master SYBR Green I PCR kit (Roche) and TaqStart Antibody (Clonetech, Mountain View, CA). Data represent results from at least two biological replicates. Each sample was run in duplicate.

For semi-quantitative RT-PCR, total RNA was purified as above and diluted to 50 ng/ul. Analyses were carried out on 50 ng of total RNA using the One-Step RT-PCR kit (Qiagen) according to the manufacturer's instructions. We limited the reactions by 1) using 1/2X dNTPs and enzyme mix and 2) reducing the number of amplification cycles to 30. For

detection of insulin receptors *a* and *b*, 75 ng of total RNA was used in endpoint PCR using 35 amplification cycles.

2.6. Morpholinos

The morpholino 5′GATAGTAATGCTCTTCCCGATTCAT3′ (GeneTools) targets the zebrafish *pdx1* translation start site. The standard control morpholino 5′CCTCTTACCTCAGTTACAATTTATA3′ was used as a control for toxicity. Single cell embryos were injected with 1-2 nl of 500 uM morpholino and allowed to develop to specific stages. Normal embryos were sorted, counted and processed for either glucose measurement, live imaging, or in situ hybridization.

2.7. 3-Mercaptopicolinic Acid

A stock solution of 100 mM 3-mercaptopicolinic acid (3-MPA, Toronto Research Chemicals) was made in embryo water containing 5mM HEPES buffer and the pH adjusted to 7.2 ([40] Makinen and Nowak, 1983). This was further diluted in embryo water to the indicated concentrations. Morphologically normal embryos were sorted, counted, and either processed for glucose assays or prepared for microscopy.

2.8. In Situ Hybridization

We amplified a 366 bp fragment of zebrafish *pck1* from 24 hpf total RNA using the One-Step RT PCR kit (Qiagen). The T7 RNA polymerase promoter was incorporated into the reverse oligonucleotide for synthesis of antisense RNA probes. The identity of this transcription template was confirmed by cloning into standard pCRII (Invitrogen) and sequencing. Phylogenetic analysis of the protein encoded by this PCR product demonstrates that this gene represents the zebrafish homolog of cytosolic *pck1*. Digoxygenin-labeled RNA probes were synthesized from PCR products in vitro (Roche) and used in whole embryo in situ hybridization to identify *pck1*-expressing tissues ([18] diIorio et al., 2002).

2.9. Microscopy

Live, ins:gfp embryos were mounted in 1% methylcellulose, and GFP fluorescence visualized using a Nikon Eclipse E600 microscope equipped with an X-cite Series 120 metal halide fluorescent lamp, a Spot RT Color digital camera, and Spot image acquisition software. Brightfield, Nomarski images of live and in situ hybridized embryos were also acquired. For larger embryos, multiple images were taken along the anteroposterior axis and manually reassembled. In some cases, fluorescent and brightfield images were merged to provide anatomical detail.

3. Results

3.1. Assay for Direct Measurement of Glucose

To measure glucose we adapted a coupled-enzyme fluorescent assay from commercially available reagents (Biovision) (Fig. 1A). The conversion of glucose to D-glucono-1,5-lactone by glucose oxidase generates hydrogen peroxide. Peroxidase utilizes this peroxide to oxidize Amplex Red to a fluorescent product with excitation and emission peaks at 535nm and 590nm respectively.

To establish the proper baseline control for our studies, we examined the contributions of reaction components to fluorescence values read at emission 590 nm (Fig. 1B). Lysates do not autofluoresce at 590 nm. Further, embryonic peroxidases do not contribute to fluorescence in the absence of exogenous glucose oxidase. Non-specific fluorescence was generated in reactions containing buffer, AmplexRed probe, and the glucose oxidase and

peroxidase mixture (Fig. 1B). Multiple wells of control reactions containing buffer, enzyme and Amplex Red were included in each assay and used to correct sample measurements. Standard curves were included in every assay.

3.2. Glucose is dynamically regulated during zebrafish and mouse development

We measured glucose levels at developmental stages in zebrafish that represent significant changes in general embryo morphology as well as pancreatic islet development (Fig. 2A). Prominent, stage-specific features were used to assess the effects of drug treatments and *pdx-1* knockdown. At the 8-16 cell and 6 hpf stages, we examined the fidelity of cell division and progression of epiboly, respectively ([35] Kimmel et al., 1995). Embryos at 16 hpf have well-defined eyes and the brain has undergone significant regionalization ([35] Kimmel et al., 1995). By 24 hpf, embryos have functional circulatory systems, exhibit spontaneous twitching movements, and become pigmented.

The first appearance of mature hormone expressing, endocrine pancreas cells occurs by 14 hpf (12 somites) with the expression of insulin in a few scattered cells of the dorsal endoderm ([8] Biemar et al., 2001). This is followed by *somatostatin* at 16 hpf (16 somites), and *glucagon* in 20 hpf (24 somite) embryos (Fig. 2A) ([8] Biemar et al., 2001). Glucagon, which stimulates gluconeogenesis, is also expressed in enteroendocrine cells of the gut ([13] Chen et al., 2009), but this occurs at 96 hpf, well after the peak in glucose seen in this study. These can be visualized by fluorescence microscopy in ins:gfp fish ([8] Biemar et al., 2001; 33] Huang et al., 2001; 35] Kimmel et al., 1995). In 48 hpf wild type embryos, *insulin* expressing beta cells have condensed into an islet which has descended to a lateral position on the right side of the larva by 72 hpf ([8] Biemar et al., 2001) (Fig. 2A).

Normal embryos of discrete ages contained developmental stage-specific levels of absolute glucose, demonstrating that glucoregulatory mechanisms are active during embryogenesis (Fig. 2B). Surprisingly, very little glucose was detected in highly proliferative 8-16 cell embryos, suggesting either rapid utilization or minimal maternal provision of glycogen or glucose-6-phosphate. Glucose levels remain undetectable through 14-16 hpf, stages dominated by extensive cellular migration and reorganization to form the embryonic axis, and early stages of gut development. It is possible that glucose derived from glycogenolysis or gluconeogenesis supports early development. If this is the case, the extremely low levels of glucose we observe at these stages reflect its rapid utilization. However, it is also possible that early zebrafish embryos, like pre-implantation mammalian embryos, rely on alternative substrates, such as fatty acids, lactate and pyruvate, to drive early cell division ([57] Schlegel and Stainier, 2006).

Glucose increases to 60 pmoles per embryo at 14-16 hpf (Figure 2 B) when somite, eye, central nervous system and gut organ development are underway ([35] Kimmel et al., 1995). By 24 hpf, glucose peaks at 500 pmoles per embryo, a nearly 10-fold increase over levels seen at 14 hpf. Embryologically, 24 hpf embryos have scattered, insulin expressing cells that are arranged semi-contiguously within the dorsal endoderm during this early stage of islet formation ([8] Biemar et al., 2001; 18] diIorio et al., 2002). In 48 hpf embryos, concomitant with condensation of pancreatic beta cells into an islet (Fig. 2A) and a surge in pancreatic insulin expression, glucose levels drop by 50% to 250 pmoles. Glucose is further reduced to 100 pmoles per embryo by 72 hpf (Fig. 2B).

Many developmental pathways are evolutionarily conserved between fish and mammals, including the expression of insulin in early, prepancreatic embryos ([31] Hernandez-Sanchez et al., 2006; 38] Lighten et al., 1997). To determine if dynamic regulation of glucose is also a conserved feature of vertebrate development, we measured glucose in isolated mouse embryos derived from timed matings. Intriguingly, as for zebrafish, glucose was

undetectable in lysates from e8.5 and e9.5 mouse embryos (Fig. 2C), which represent early stages of murine pancreatic bud formation. Our data suggest that, like the zebrafish, either glucose utilization is rapid in mouse embryos, or they continue to use alternate substrates for growth ([9] Biggers et al., 1967).

At e13.5, mouse embryos began to accumulate measurable glucose (Fig. 2C). This corresponds to initial development, i.e. secondary transition ([26] Gittes, 2009), of mouse beta cells. Glucose levels subsequently increased approximately 12 fold to 86 nmoles in e14.5, and 40-fold to 400 nmoles by e17.5 (Fig. 2C), a stage characterized by islet morphogenesis ([69] Wilding Crawford et al., 2008). The pattern and relative developmental timing of glucose accumulation in mouse embryos was strikingly similar to that seen in zebrafish, and suggests that conserved mechanisms may underlie this aspect of glucose dynamics in both species.

3.3. Phylogenetic analysis of vertebrate Pck proteins

We hypothesized that increasing glucose in zebrafish embryos was due to activation of gluconeogenesis. In mammals, *pck1* but not *pck2*, is regulated by insulin ([47] O'Brien et al., 1995). Our first goal, therefore, was to develop reagents that were unequivocally specific to zebrafish *pck1* or *pck2* and did not cross-hybridize. To this end, we carried out phylogenetic comparisons among vertebrate Pck proteins that, for the first time, included the zebrafish putative mitochondrial Pck2 (Fig. 3A). Our analysis (Fig. 3A) identifies distinct Pck1 and Pck*2* lineages and confirms the identity of zebrafish Pck1. Intriguingly, zebrafish Pck2 resides at the base of the Pck1 clade, suggesting that this protein represents a transitional isoform of vertebrate Pcks. In contrast to previous studies ([21] Elo et al., 2007), for which the zebrafish *pck2* cDNA sequence was unavailable, we used cDNA sequence alignments to identify *pck1*-specific regions for designing RNA in situ hybridization probes and oligonucleotides for PCR analyses (Table 1).

3.4. Developmental stage-specific expression of pck1 and pck2

In mice, Pck1 is expressed in diverse tissues during early development ([72] Zimmer and Magnuson, 1990). To determine if this pre-hepatic, pck1 expression also occurs in zebrafish, we used semi-quantitative RT-PCR. Using gene-specific oligonucleotides for zebrafish *pck1* and *pck2*, we detect low levels of maternally provided pck1 mRNA in 16 cell embryos. Zygotic *pck2* expression is detectable at 6h of development, while *pck1* mRNA is expressed between 6- and 16- hpf, which closely corresponds to the initial increases in free glucose seen in 14-16 hpf embryos (Fig. 3B). We found that, like mouse embryos, zebrafish *pck1* mRNA is expressed prior to overt liver development. Finally, our data revealed an increase in *pck1* expression during development, whereas *pck2* appeared to decrease after 24 hpf. Whether this decline in *pck2* expression reflects a reduced reliance on Pck2-mediated gluconeogenesis remains to be determined.

3.5. Zebrafish pck1 is expressed in multiple cell types prior to hepatogenesis

We focused our in situ analyses on zebrafish *pck1* because this gene is expressed in multiple, pre-hepatic cell types in mouse embryos ([72] Zimmer and Magnuson, 1990) and is insulin-responsive in mammals ([47] O'Brien et al., 1995). In contrast, *pck2* appears to be constitutively expressed and not responsive to insulin ([11] Caton et al., 2009; 66] Watford et al., 1981). To identify the cells of the pre-hepatic zebrafish embryo that express pck1 mRNA, we employed RNA in situ hybridization.

Zebrafish *pck1* was previously shown to be insulin-responsive in larvae and adults ([21] Elo et al., 2007), but its endogenous expression pattern during pre-hepatic developmental stages is unknown. We show that *pck1* is prominently expressed in the yolk syncytial layer (YSL),

as well as the developing brain and tail bud of 11 hpf embryos (Fig. 3C). Expression in the YSL is consistent with its role in regulating nutrient transfer from yolk to the embryo proper ([35] Kimmel et al., 1995), and the timing of expression is consistent with our RT-PCR results showing activation of *pck1* mRNA expression between 6- and 16-hpf. By 24 hpf, *pck1* mRNA is regionally expressed in the caudal-most region of the tail, where there is active cell division, as well as the developing eye and midbrain (Fig. 3C). By 48 hpf, YSL expression of pck1 occurs in discrete cell clusters residing at the interface of the yolk and embryo proper (Fig. 3C). At this stage, expression is also seen in the developing fin buds and pharyngeal arches. By 72 hpf, in addition to continued YSL expression, *pck1* mRNA is expressed in the liver, the major source of expression in adult mammals (Fig. 3C). Expression of *pck1* mRNA in 96 hpf embryos expands to include developing neuromasts and cells at the terminal tip of the gastrointestinal tract (Fig. 3C). This regional expression of *pck1* suggested to us that developing tissues may have autonomous control over gluconeogenesis, especially at stages prior to islet, liver, and vasculature development.

3.6. Quantitative insulin A, insulin B and pck1 gene expression during development

Previous work demonstrated high levels of *insb* expression in early embryos, while in later embryos expression of pancreatic *insa* predominates ([50] Papasani et al., 2006). For a better understanding of how insulin levels are associated with *pck1* expression and glucose abundance, we quantified these variables at identical developmental stages.

In zebrafish, prepancreatic *insb* mRNA was abundant between 16 cells and 16 hpf and this was associated with undetectable levels of *pck1*, pancreatic *insa*, and glucose (Fig. 4A and B; Fig. 2B). Reduction of *insb* between 16- and 24 hpf is associated with increased *pck1* expression, onset of glucose accumulation and low, but increasing, levels of *insa* transcription (Fig. 4A and B; Fig. 2B). While our measures are associative, they are strikingly similar to the mechanisms of hepatic *pck1* suppression by insulin in adult mammals ([47] O'Brien et al., 1995; 63] Sutherland et al., 1995).

Intriguingly, pancreatic *insa* peaks in zebrafish at 48 hpf, subsequent to the endogenous glucose surge at 24 hpf (Fig. 2B). Subsequent to islet formation, *insa* expression decreases to steady-state levels through 120 hpf (Fig. 4A). During this same time frame (48- to 96-hpf), *pck1* expression continues to increase (Fig. 4B). At 120 hpf, however, *pck1* mRNA dramatically increases, even as *insa* levels remain steady (Fig. 4B). This likely reflects the development and maturation of pro-gluconeogenic alpha cells and glucocorticoid synthesis in the interrenal organ ([12] Chai et al., 2003) of older zebrafish embryos.

We hypothesized that *insb* is physiologically active between 16 cells and 16 hpf, and represses zebrafish *pck1* expression during early development. To address this, we analyzed early embryos for expression of *insulin receptors a* and *b*. In a manner similar to that seen in mammals ([38] Lighten et al., 1997; 58] Schultz et al., 1992), both zebrafish insulin receptor genes were expressed in 16 cell, 6 hpf and 16 hpf embryos (Fig. 4C), suggesting that insulin signaling via *insb* may occur prior to islet development.

3.7. Inhibition of pck1 activity prevents glucose accumulation in zebrafish embryos

The onset of zygotic *pck1* expression and corresponding reduction of *insb* mRNA between 16- and 24- hpf prompted us to analyze the contribution of gluconeogenesis to free glucose in early embryos. 3-MPA is a well-documented inhibitor of mammalian PCK1 ([40] Makinen and Nowak, 1983). We hypothesized that if zebrafish PCK1 function is evolutionarily conserved and sensitive to 3-MPA, we should observe suppression of glucose accumulation in drug treated embryos at 24 hpf.

In preliminary experiments, we determined an effective dose of 1.5mM 3-MPA to be non-toxic to overall development, but effectively blocks glucose accumulation. Because glucose levels drop normally between 24- and 48- hpf, we treated ins:gfp embryos with 1.5mM 3-MPA between 16- and 22-hpf. At this dose, 3-MPA was not overtly toxic. Somite shape, brain regionalization, eye formation and tail extension of 3-MPA-treated embryos were all comparable to controls. Further, the gross morphology of the 22 hpf pancreatic islet was also similar to that seen in controls, (Fig. 5A). This excludes the possibility that the observed hypoglycemia was due to 3-MPA-induced expansion of normal beta cells. However, 3-MPA caused a dose-dependent reduction in normal glucose accumulation compared to controls (Fig. 5B), suggesting that this drug specifically inhibits Pck1 activity. Treatment of embryos with 1.5mM 3-MPA from 16- to 22-hpf caused a 10-fold reduction in free glucose back to levels normally seen in 8-cell embryos (compare to Fig. 2B). This latter observation demonstrates that glucose production in the zebrafish embryo occurs almost exclusively through Pck1 activity, and glycogenolysis does not compensate for loss of normal, embryonic glucose accumulation at this stage.

Having established that 3-MPA inhibits normal glucose accumulation at 24 hpf, we examined the possibility that maternally provided PCK1 protein provides glucose prior to the onset of zygotic *pck1* gene expression at 16 hpf. To do this, we incubated embryos in 1.5mM 3-MPA beginning at the 2-8 cell stage and examined them microscopically at 24 hpf (Fig. 5C). 3-MPA treated embryos developed at the same rate as controls, and exhibited normal brain compartmentalization, somite shape, and yolk utilization (Fig. 5C). This demonstrates that glucose production through Pck1 is not essential for normal, morphological development in early zebrafish embryos. The significance of increased endogenous glucose on beta cell development, maturation, and insulin content remains unknown. Insulin-mediated repression of *pck1* expression in adult, mammalian liver is well-documented ([47] O'Brien et al., 1995). Given the extremely low glucose and lack of *pck1* expression early zebrafish embryos, it seems possible that *insb* may function to suppress *pck1*, and minimize embryonic exposure to the potentially teratogenic effects of glucose ([4] Akazawa, 2005; 22] Eriksson and Borg, 1991; 23] Eriksson et al., 1991; 24] Freinkel, 1988; 44] Moley, 1999).

3.8. The early zebrafish islet is functional and regulates glucose

The endogenous reduction in glucose between 24- and 48- hpf is temporally associated with formation of the primary islet. To directly address the functionality of this early islet, we employed an antisense morpholino approach to suppress pancreatic development. We injected standard control and *pdx1*-specific morpholinos into single cell embryos and harvested them at 48- and 72- hpf for live imaging, in situ hybridization, and glucose measurements. Our rationale for targeting *pdx1*, and not *insa* and *insb* directly was that 1) *pdx1* is essential for mammalian islet development ([34] Jonsson et al., 1994; 48] Offield et al., 1996), 2) this approach has been used successfully to abrogate zebrafish islet development in other studies ([32] Huang et al., 2001), 3) *pdx1* is tissue restricted to the developing duodenum and pancreas ([34] Jonsson et al., 1994; 48] Offield et al., 1996) and 4) *pdx1* is expressed at much lower levels than pancreatic insulin, so it can be manipulated with lower, non-toxic concentrations of morpholino. Our expectation was that if the 48 hpf islet regulates glucose, abrogation of its development should result in embryonic hyperglycemia.

Injection of 1-2nl of 500uM *pdx1*-specific morpholino into ins:GFP embryos caused reduction in the number of GFP-positive cells constituting the 48 hpf islet (Fig. 6A). This was not seen in embryos injected with the standard control morpholino. Despite the predominance of embryos with hypomorphic islets, we measured significant differences in glucose between control- and *pdx-1*- morpholino injected embryos (Fig. 6 B). Glucose was

reduced in 48 hpf embryos injected with the standard control morpholino in a manner similar to that seen during normal development (Fig. 6B). In contrast, glucose remained high in 48 hpf *pdx1* morphants, and this hyperglycemia persisted through 72 hpf (Fig. 6B). Because *pdx1* expression is specific to pancreas and duodenal progenitors, we conclude that the reduction in glucose normally seen in 48 hpf embryos is mediated by the primary, 48 hpf islet.

4. Discussion

4.1. Roles for Glucose in Zebrafish Embryogenesis

Further insight into mechanisms of glucose regulation and toxicity, as they pertain to human disease, may be provided by mechanistic studies in alternate biological models. We were prompted to investigate glucose regulation in the zebrafish embryo model because they are amenable to genetic and small molecule screens that may identify genes and pathways with relevance to human disease. However, prior to undertaking such studies we wanted to determine if zebrafish embryos use mammalian like mechanisms of glucose regulation.

Our data demonstrate that zebrafish embryos actively regulate glucose during development. Glucose was undetectable in 16 cell embryos but was made at measurable levels beginning at 14-16 hpf. Glucose levels in both mouse and zebrafish embryos increased substantially through early stages of pancreatic beta cell differentiation and islet formation. Surprisingly, we were unable to find any references in which endogenous glucose was measured in fetal mice, making ours the first to report direct measurement of dynamic glucose levels in placental embryos.

Given the low levels of glucose in early embryos of both mice and zebrafish, it is likely that maternally provided lipids and yolk proteins such as Vitellogenin provide most of the energy required for early migration and proliferation of embryonic blastomeres. As seen in human embryos ([36] Leese et al., 1993), the increased glucose seen between 16- and 24-hpf may signify depletion of maternal energy stores and a switch from non-glucose metabolism early in development to glucose oxidation. Alternatively, newly generated glucose may serve to 1) recycle lactate and 2) provide energy and/or ribose-5-phosphate for nucleotide synthesis in later organogenesis.

The conserved timing of increased glucose abundance with pancreatic islet maturation in both mouse and zebrafish suggests another possible, perhaps complementary, role for glucose in these embryos. In both species, glucose is tightly regulated: levels are suppressed during early development, increase through stages of early endocrine cell differentiation and proliferation, and reach peak levels as the islet matures. Treatment with 3-MPA deprives zebrafish embryos of glucose between 16- and 24- hpf, demonstrating that zebrafish and mammalian Pcks are functionally conserved. However, continuous 3-MPA treatment from the 2-8 cell stage to 24 hpf does not adversely affect either beta cell development or gross embryonic morphology, indicating that embryonic cell survival and normal proliferation are not impaired. This favors the hypothesis that glucose might drive the functional maturation of beta cells in zebrafish. In mice, embryonic glucose is required for maturation of beta cell insulin content ([10] Cao et al., 2004; 19] Dudek et al., 1984) and for coupling of glucose to regulated insulin secretion ([29] Hellerstrom and Swenne, 1991).

4.2. Regional Pck1 Expression Associates with Non-Hepatic Whole Embryo Glucose Production

Using quantitative RT-PCR, we demonstrate that *Pck1* transcripts are rare during early development. In 16 hpf embryos,, perhaps as lipid components of the yolk are consumed and they embryo begins using yolk proteins for energy, low level expression of *Pck1* in the YSL

is activated and gluconeogenic conversion of amino acids to glucose may proceed. Intriguingly, as for the mouse ([72] Zimmer and Magnuson, 1990), *pck1* is dynamically expressed in a number of tissues at discrete stages of embryogenesis. In addition to the YSL, *Pck1* transcripts were detected in the tail bud, brain, eye, pectoral fins, neuromasts and liver at stages when these tissues are proliferating and terminally differentiating. We postulate that extra-hepatic expression of *Pck1* permits local or autonomous regulation of glucose synthesis, to provide energy or anabolic precursors, to rapidly developing tissues. This may be especially important in regions of the embryo where vasculature has not developed or is functionally immature.

4.3 Insulin Expression Levels Suggest Mammalian-like Regulation of Pck1 and Glucose

Zebrafish express insulin from two distinct loci, *insb* and pancreatic *insa*, with distinct patterns of transcription during embryogenesis. While *insa* is expressed solely in the pancreas, *insb* is abundantly expressed in blastomeres during early development, and at low levels in the pancreas and brain at later stages ([50] Papasani et al., 2006). Rodents also have two insulin genes, but these do not represent a true tandem duplication. Instead, the rodent insulin I gene represents a retrotransposed, partially spliced cDNA with only a partial promoter that restricts expression to the pancreatic beta cells ([59] Soares et al., 1985; 67] Wentworth et al., 1986). In contrast, the ancestral rodent insulin II gene is expressed in the thymic epithelium ([52] Pugliese et al., 1997), pancreatic beta cells, and choroid plexus ([17] Deltour et al., 1993). Both of these models differ from humans, which possess only a single insulin gene. However, in all of these species, including humans ([38] Lighten et al., 1997) *insulin* is expressed during early pre-pancreatic development. This suggests that vertebrates have conserved requirements for insulin in early development, and that this has been met either through gene duplication and promoter divergence, or through increased complexity of the single insulin promoter as in humans.

In zebrafish, *pck1* mRNA levels are inversely related to prepancreatic *insb* and pancreatic *insa* expression through 96 hpf. This suggests that early *insb* may function through the expressed insulin receptors to suppress *pck1* expression and glucose accumulation in the early zebrafish embryo. Based on our zebrafish and mouse data we hypothesize that one conserved function for pre-pancreatic insulin expression may be to suppress glucose production, as it can be teratogenic to embryos ([4] Akazawa, 2005; 23] Eriksson et al., 1991; 24] Freinkel, 1988; 44] Moley, 1999). Significantly, human, rodent, and apparently zebrafish preferentially utilize non-glucose substrates at early stages ([9] Biggers et al., 1967), further supporting this idea.

The rapid decline in *insb* mRNA between 16- and 24-hpf in zebrafish embryos, coupled with low levels of pancreatic *insa* at these stages, may permit *pck1* expression and glucose accumulation for later development. As development proceeds, the relationship between insulin and *pck1* becomes less clear. The dramatic increase in *pck1* mRNA at 120 hpf, even in the context of constant *insa* levels, is likely due to functional maturity of pro-gluconeogenic factors such as glucagon in the islet and glucocorticoid expression in the interrenal organ ([12] Chai et al., 2003).

While our data correlate insulin expression levels with *pck1* expression and glucose abundance, it is also likely that IGF and Growth Hormone (GH) signaling play important roles ([53] Reinecke, 2010). IGF1 is a major regulator of embryonic growth in mammals ([15] DeChiara et al., 1990) and fish ([43] McCormick et al., 1992) and is capable, like insulin, of stimulating glycogen synthesis in the liver ([16] Degger et al., 2000) and glucose up-take by cultured cells ([49] Pantaleon and Kaye, 1996). IGF ligands and receptors are expressed in early zebrafish embryos ([42] Maures et al., 2002), and hybrid insulin/IGF

receptors expressed in the chick ([25] Garcia-de Lacoba et al., 1999), may also have the potential for regulating *pck1* expression.

4.4. The early zebrafish islet is functional and regulates glucose

We were able to demonstrate that 3-MPA inhibit induces hypoglycemia in the zebrafish embryo, and transcript levels of *insa*, *insb*, and *pck1* mRNA levels suggest a mammalian-like glucoregulatory cascade. To further define the mechanistic relationship among these factors in the zebrafish embryo, we sought to ablate the developing pancreas using morpholinos specific for *Pdx1*, a transcription factor expressed in pancreatic progenitor cells and in the duodenum. In humans, reduced *pdx1* activity causes hypomorphic pancreas development ([14] Clocquet et al., 2000; 28] Hani et al., 1999) and pancreatic agenesis ([61] Stoffers et al., 1998; 62] Stoffers et al., 1997). Mice homozygous for targeted *pdx1* deletion exhibit pancreatic agenesis ([34] Jonsson et al., 1994; 48] Offield et al., 1996) and are severely hyperglycemic at birth ([48] Offield et al., 1996). In our experiments, *pdx1* morpholino caused islet hypoplasia, and complete agenesis in only a few cases. Even so, we measured significant and persistent hyperglycemia in *pdx1* morphant embryos. In addition to demonstrating a functional glucoregulatory role for the primary zebrafish islet even though it may not contribute substantially to mature beta cell mass ([6] Anderson et al., 2009). The expression of *insb* in the brain ([50] Papasani et al., 2006) also suggests a possible role for CNS regulation of glucose, though its dependence on *Pdx1* has not been determined.

Taken together, our data suggest that the early zebrafish embryo utilizes mammalian-like mechanisms to regulate glucose abundance during development. Given the success of forward genetic and chemical screens in early embryos for identifying genes, developmental pathways, and bioactive compounds, we believe that this model will be a valuable tool for further investigation of human glucose regulation and therapies for diabetes.

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B

	lysate buffer	lysate buffer AR	buffer enzymes AR	lysate buffer enzymes AR
Mouse embryonic day 13.5	3	201	2625	25450
Mouse embryonic day 17.5	3	277	2864	45176
	Lysates do not autofluoresce at 530nm	Low level of AR oxidation by lysate	*Non-specific oxidation of AR by enzymes	

Fig. 1.

Methodology for glucose quantitation. (A) Production of hydrogen peroxide from glucose by glucose oxidase is coupled to peroxidase mediated oxidation of Amplex Red (AR) to a fluorescent probe. Peak excitation and emission of oxidized probe occurs at 535-and 590 nm, respectively. (B) Various combinations of sample lysate, reaction buffer, enzymes and fluorescent substrate were used to determine contributions of individual reaction components to total fluorescence. Reactions containing buffer (star), enzymes and fluorescent probe were included in each assay and were subtracted from total fluorescence to determine glucose-specific values. Sample A, e13.5 mouse embryo. Sample B e17.5 mouse embryo. Zebrafish lysates showed identical properties (not shown).

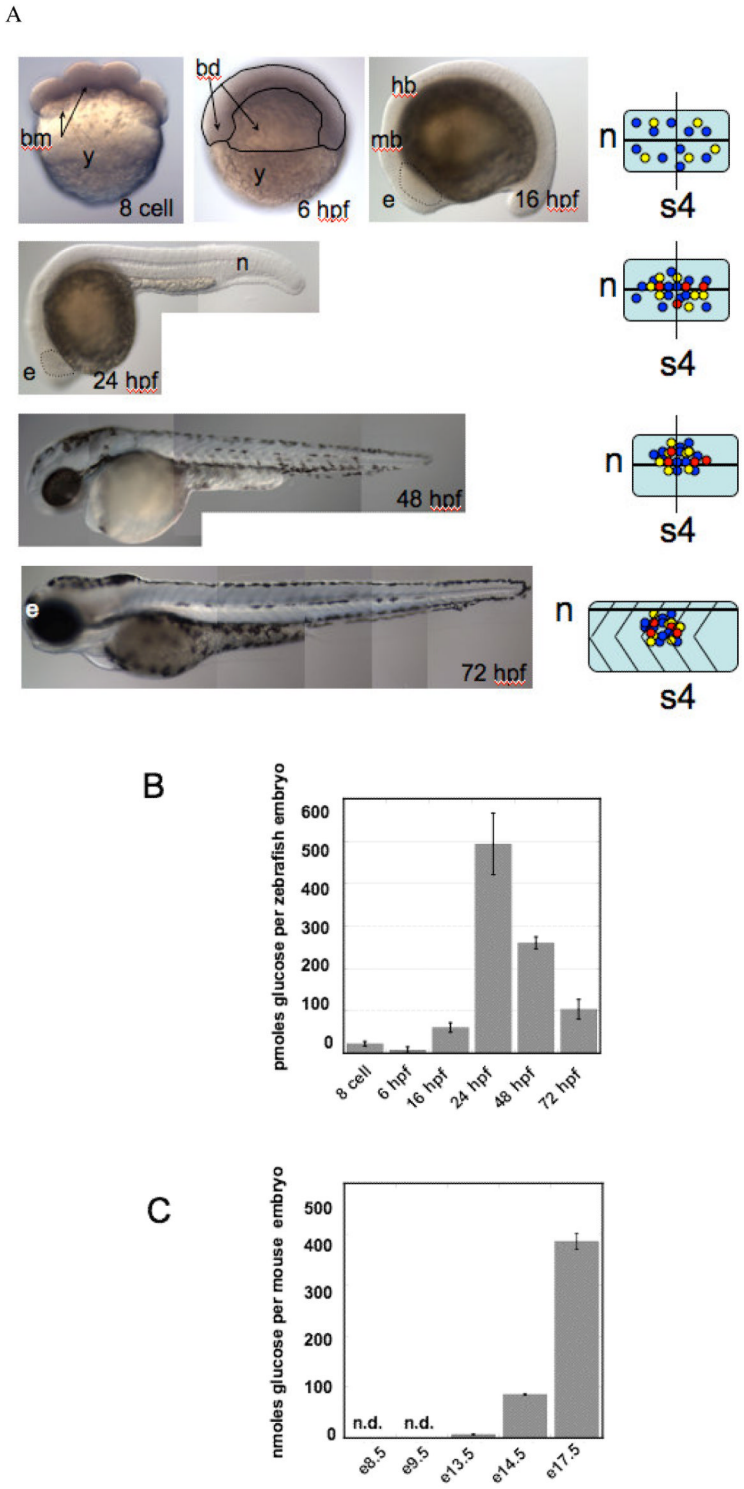


Fig. 2.

Absolute glucose measures in zebrafish and isolated mouse embryos. (A) Zebrafish embryo morphology at stages examined. Blue box denotes the field of endocrine cell development within the endoderm at the level of somite four (s4). Anterior is to the left; n, notochord;

blue, beta cells; yellow, delta cells; red, alpha cells. Adapted from ([7] Argenton et al., 1999; 8] Biemar et al., 2001; 18] diIorio et al., 2002; 55] Roy et al., 2001). (B) Picomoles of glucose per zebrafish embryo and (C) nanomoles of glucose per mouse embryo at discrete developmental stages. Note the similarity in glucose dynamics, but not absolute levels, between mouse and zebrafish. Means and standard deviations are provided in b, c. bd, blastoderm; bm, blastomeres; e, eye; h, heart; hb, hindbrain; mb, midbrain; n, notochord; y, yolk.

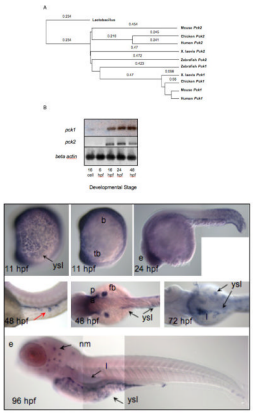


Fig. 3.

Temporal and spatial expression of *pck1* during zebrafish development. (A) Phylogenetic analysis distinguishes Pck1- from Pck2-related proteins. (B) Semiquantitative RT-PCR showing onset of *pck1* and *pck2* gene expression. Note that a small amount of maternally provided *pck1* is present in 16 cell embryos. Low-level zygotic expression of *pck2* is first detectable at 6 hpf. (C) RNA:RNA in situ hybridization demonstrates *pck1* expression in the 11 hpf YSL, brain, and tail. In 24 hpf embryos, *pck1* is expressed in the eye and tail. By 48 hpf *pck1* is expressed at the margin between the yolk extension and the embryo proper (red arrow). Expression in discrete YSL clusters (black arrows) as well as fin buds and pharyngeal arches is also seen. At 72 and 96 hpf, *pck1* expression is seen in the liver, YSL, and cranial neuromasts. b, brain; e, eye; fb, fin bud; nm, neuromasts; pa, pharyngeal arch; tb, and tail bud.

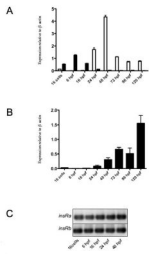


Fig. 4.

Gene expression during zebrafish development. (A) Relative, quantitative expression of *insa* (open bars) and *insb* (black bars) during development. (B). Relative, quantitative expression of *pck1* during development. (C) Non-quantitative RT-PCR demonstrates early expression of insulin receptors *a* and *b*.

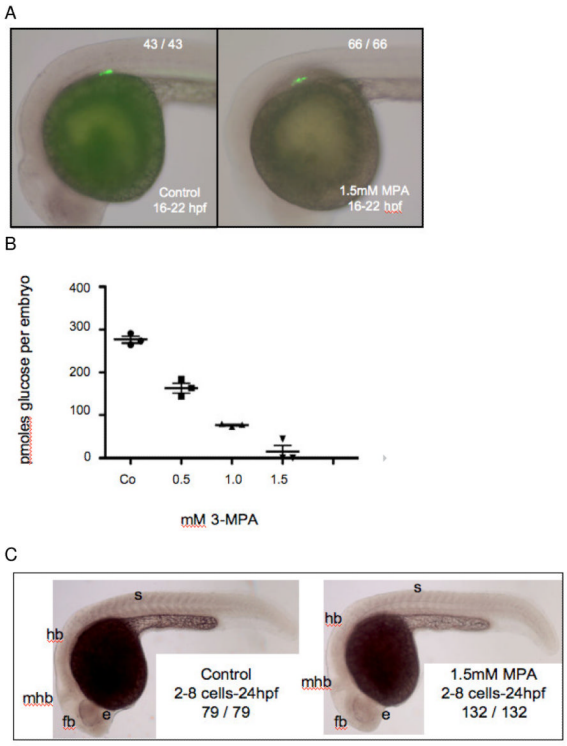
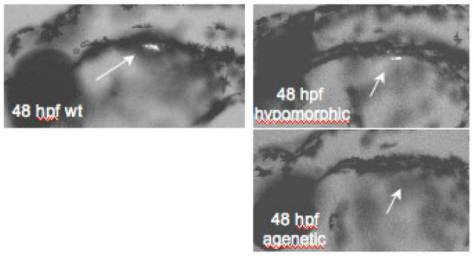


Fig. 5.

Treatment of zebrafish embryos with 3-mercaptopicolinic acid (3-MPA). (A) 1.5mM 3-MPA is not toxic to beta cell development and does not induce islet hyperplasia. (B) 3-MPA causes a dose-dependent reduction in total embryonic glucose in 22 hpf embryos. (C) Long-term incubation with 3-MPA between the 2-8 cells and 24 hpf stages is not toxic, and does not perturb somite shape, brain regionalization, eye development or tail extension as compared to controls. Ratio in bottom left denotes the number of normal embryos/total embryos examined. e, eye; fb, forebrain; hb, hindbrain; mhb, midbrain-hindbrain boundary; s, somite.

A



	Wt	Hypomorphic	Agenetic
500uM std control replicate a	37 (100)	0	0
500uM std control replicate b	31 (100)	0	0
500uM pdx1mo replicate a	4 (14.8)	22	1
500uM pdx1mo replicate b	4 (11.8)	28	2

B

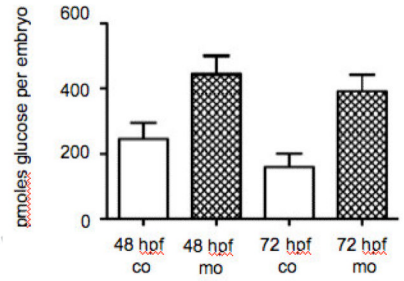


Fig. 6.

Morpholino-mediated suppression of *pdx1* causes islet hypoplasia and elevated glucose in zebrafish embryos. (A) Live images and phenotypic frequencies of 48 hpf ins:gfp embryos injected with standard control or *pdx1*-specific morpholinos at the single cell stage. Arrows indicate anatomical site of normal islet development. (B) Glucose levels in lysates of embryos at 48- and 72- hpf after microinjection of standard control morpholino (white) or *pdx-1* morpholino (hatched). Data are presented as means and standard deviations.

Table 1

Oligonucleotide sequences used to generate ISH RNA probes and in quantitative (qRT-PCR) and semi-quantitative RT-PCR (sqRT-PCR). For added specificity, at least one of the *pck*1 and *pck2* oligonucleotides were anchored in either the 5′ or 3′ untranslated region (UTR). In *pck1* Forward, 10 of 21 total nucleotides (underscored) are unique to *pck1*. In *pck1* Reverse, 14 of 20 total (underscored) are unique to *pck1*. The *pck1* primers were used in PCR and to generate gene-specific, digoxigenin-labeled RNA probes for in situ hybridization.

Gene	Forward	Reverse
pck 1	5′TCTCCATCCCTCCGCTCATCA3′ (5′URT)	5′ GGCCCAGCTGACTGCTCCT3′ (Exon 2)
pck2	5′ CAAACACTGGCATCTGCAT3′ (3′URT)	5′ TGGCCAGCTCAGGTA3′ (3′ UTR)
insa	5′GTTGGTCGTGTCCAGTGTAAGCACTAA3′	5′CCACCTCAGTTTCCTGGGCAGATTTA3′
insa	5′TCTCTGCCTGGATCGCAGTCTTCT3′	5′GGATCCGCATCTGCTGCCTCATAA3′
β actin	5′CCCCTCCATTGTTGGACGAC3′	5′TAGCCACGCTCGGTCAGGAT3′
insRa	5′CATAACCTGATGCAGATGTGCTGGCAT3′	5′TGCGTGTACGGCACATGCTCCTCAT3′
insRb	5′TTGCCGAGCCAGTGACTCACGAA3′	5′ACCAGTAAGAAAATGCAGATGGCTA3′