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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.

1. Introduction

A balance between normal and embryotoxic glucose levels is essential for normal vertebrate development [54]. Exposure to hyperglycemia during embryogenesis causes glucose-associated neural tube closure defects [39,51,60] increased oxidative stress [39,65,70] reduced GLUT expression [44,45] and apoptosis [44,71] as well as fetal islet hyperplasia and degranulation [1,3] and neonatal hyperinsulinemia [64] and macrosomia [2,30,60]. 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].

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]) and small molecule screens (e.g., [41,46,56]) 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 [20,21] 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

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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 [27,37] 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 [7,8]. 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].

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], 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]. 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] were collected from natural matings and reared in 1/3 Ringer's [68] without phenylthiourea. Embryos were staged using morphological criteria up to 24 hpf and by time of development at 28.5 °C thereafter [35].

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 min 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).