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MOLECULAR AND EMBRYOLOGIC ANALYSES OF THE ER STRESS REGULATOR CHOP/GADD153 AND ITS RESPONSE TO GLUCOSE LEVELS IN ZEBRAFISH EMBRYOS

A Major Qualifying Project Report

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ABSTRACT

Type 1 and Type 2 diabetic patients experience prolonged hyperglycemia punctuated by short episodes of hypoglycemia. These patients develop diabetic complications, which involves loss of normal tissue function. Cell death via the endoplasmic reticulum (ER) stress pathway in response to nutrient availability is a potential mechanism underlying these complications. To understand glucose's role in ER stress *in vivo*, we induced hypo- and hyper-glycemia in zebrafish embryos, and analyzed normal development and CHOP/GADD153 expression, a major component of ER stress signaling.

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ACKNOWLEDGEMENTS

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BACKGROUND

Diabetes

Diabetes is a metabolic disorder that affects the body's ability to produce and use insulin, a hormone that is necessary to maintain normal levels of glucose in the circulation. Prolonged exposure to dysregulated glucose can lead to many complications including blindness, heart disease, kidney failure, cardiovascular disease, and degeneration of peripheral nerves. Diabetes is caused by impaired insulin secretion in pancreatic beta cells, and/or a reduced insulin efficacy (resistance) in muscle, adipose, and liver **(Figure-1)**.

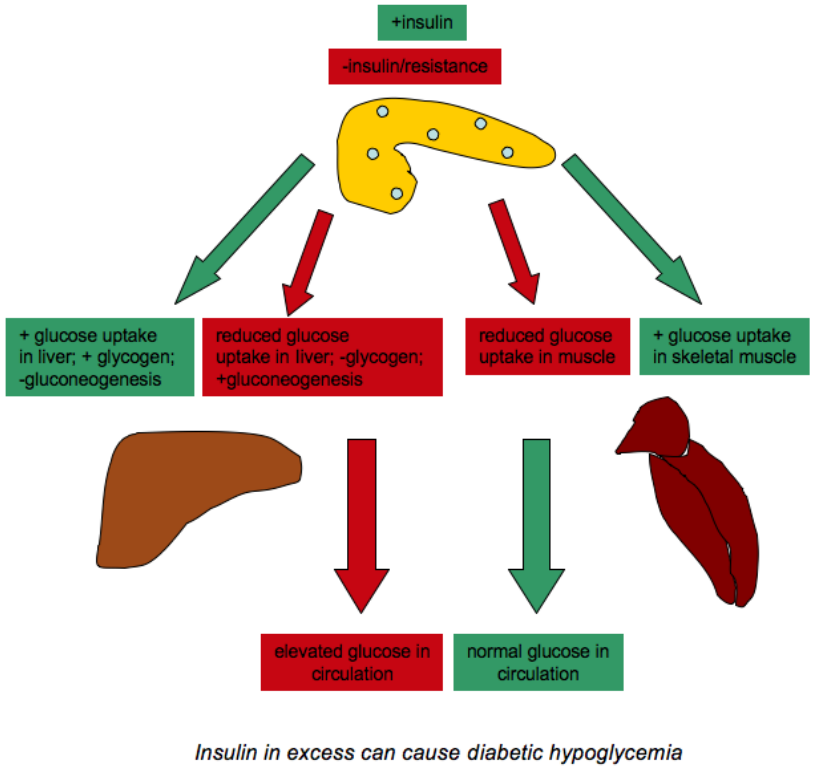

Figure 1: **Role of Pancreatic Insulin Regulating Blood Glucose Levels**. Diagram shows the pancreas (yellow) secreting insulin (green), which induces the uptake of glucose in the liver (diagram left) or skeletal muscle (diagram right). In the absence of insulin (diagram center) glucose uptake is reduced, increasing its levels in the blood (red, diagram lower).

Approximately 16 million Americans are affected by Type 1, or juvenile diabetes; it is the more severe form of this illness, and is caused by autoimmune destruction of one's insulin-producing beta cells. Type 1 diabetes is known as a complex trait disease as mutations in several different genes contribute to this disease. For example, because of the recent mapping of the genome, we know that the insulin-dependent diabetes mellitus (IDDM1) locus on chromosome 6 may hold at least one susceptibility gene for Type 1 diabetes (National Center for Biotechnology Information, 2007). It is unclear how a mutation at this locus adds to patient risk, although the region's gene map includes genes for antigens that normally tell the immune system not to attack itself. In Type 1 diabetes, the body's immune system recognizes its own pancreatic beta cells as foreign, and triggers an immunological assault on the insulin and the pancreatic cells that manufacture it. However, it is unsure how this mechanism actually works (National Center for Biotechnology Information, 2007). Currently, about 10 loci in the human genome have been correlated with Type 1 diabetes (National Center for Biotechnology Information, 2007). Among these include a gene at the locus IDDM2 on chromosome 11, and the gene for glucokinase (GCK) on chromosome 7, an enzyme that is important to glucose metabolism that helps modulate the secretion of insulin.

Type 2 diabetes, or adult onset/noninsulin-dependent diabetes, is the most common form of diabetes. In Type 2 patients, the defects in glucose homeostasis can exist within the pancreatic beta cell to cause it to not secrete sufficient insulin or the defect can exist in peripheral tissues, such as adipose tissue, liver, and muscle, which may not respond properly to insulin. This latter effect is termed "insulin resistance", and can result in from a disruption of the signal transduction pathway needed for insulin to upregulate glucose transporters on the cell surface (American Diabetes Association, 2010). Glucose transporters, such as GLUT-1 normally bind

glucose to transport it inside cells. If liver or muscle cells are defective, insulin cannot upregulate GLUT on the cell surface, so glucose does not enter cells for energy or storage, but remains in the blood stream causing hyperglycemia. Family history and genetics play a large role in type 2 diabetes (American Diabetes Association, 2010). Low physical activity, poor diet, and excess body weight (especially around the waist) significantly increase risk for type 2 diabetes.

Hypoglycemia

Although the hallmark symptom of diabetes is hyperglycemia, diabetic patients also experience hypoglycemia, or low blood sugar, especially when too much insulin is injected. In studies performed by the American Diabetes Association, when type 1 diabetics and nondiabetics were normalized for hyperinsulenima, type 1 diabetic patients experienced more severe and prolonged hypoglycemia (Bolli, 1999). According to the study, rather than being insulin-sensitive, the subjects' hypoglycemia can better be explained by their compromised counterregulatory defenses (Bolli, 1999). The mechanism of the compromised counterregulatory defenses could be explained by two defects. The first defect, and most common, is the loss of glucagon response to hypoglycemia which is irreversible (Bolli, 1999). Glucagon is a hormone secreted by the pancreas which raises blood glucose levels, the opposite effect of insulin. When blood glucose levels fall low, the pancreas releases glucagon which causes the liver to convert glycogen into glucose. Glucose also stimulates the release of insulin, thus its loss of a response to it would result in a lack of productivity in the feedback system that keeps glucose levels at the right level (American Diabetes Association, 2010).

In the second defect, patients experience reduced responses to adrenaline, another counterregulatory hormone. This hormone normally acts to regulate the sympathetic nervous system, thus reduced responses to it would cause a lack of accuracy in response to "fight or flight" situations. This loss of adrenaline response is usually due to short-term antecedent "recurrent iatrogenic hypoglycemia", and is less often seen during long-term diabetes (Bolli, 1999).

Hyperglycemia

Hyperglycemia, the hallmark diabetic symptom, is defined as high blood sugar. During digestion, carbohydrates are broken down into sugar molecules. Glucose is the main energy source for the body, and it enters the blood stream immediately to travel to all parts of the body to provide an energy source. However, glucose cannot leave the bloodstream and enter tissues in need of energy without the help of insulin, which binds the insulin receptor to stimulate signal transduction pathways that upregulate glucose transporter molecules to the cell surface. Under conditions of insufficient insulin secretion, or of insufficient insulin-induced upregulation of GLUT, glucose remains in the blood resulting in hyperglycemia (Staff, 2010).

Normoglycemic blood sugar levels are approximately 110 milligrams per deciliter (mg/dL or 6 mmol/mL). Patients with hyperglycemia have blood sugar levels of 200 mg/dL, or 11 mmol/L (Staff, 2010). At this threshold, patients start to experience symptoms of hyperglycemia. Early signs of the condition are fatigue, frequent urination, increased thirst, headache, and blurred vision (Staff, 2010). Prolonged hyperglycemia can result in ketones (toxic acids) building up in the blood and urine, to cause nausea, vomiting, abdominal pain, weakness,

confusion, and coma (Staff, 2010). If untreated, hyperglycemia can create severe complications for a patient.

Diabetic Complications

When left untreated, or when undiagnosed, hyperglycemia has severe complications, possibly affecting every organ in the body. The eyes are often affected early in the disease. For example, patients can develop glaucoma or cataracts. Diabetics are 40% more likely to develop glaucoma than nondiabetics, as pressure builds in the anterior chamber to pinch blood vessels carrying blood to the retina and optic nerve (American Diabetes Association, 2010). The nerve damage causes loss of vision. Also, diabetics are sixty percent more likely to develop cataracts than nondiabetics; they develop them sooner in life, and they are more severe (American Diabetes Association, 2010). Additionally, diabetics are at risk for cardiovascular disease, neuropathy, nephropathy, and peripheral arterial disease (American Diabetes Association, 2010).

Some complications of hyperglycemia put diabetics at risk of death. When a patient develops diabetic ketoacidosis, there is not enough insulin in the bloodstream for the body to break down glucose for energy, so instead the body begins to break down adipose tissue, which releases ketone acids into the blood. This toxin floods the circulatory system, and eventually spills over into the urinary tract. If untreated, ketoacidosis can lead to a diabetic coma or death (Staff, 2010).

Another emergency complication of diabetes is diabetic hyperosmolar syndrome. In this condition, neither glucose nor adipose can be broken down for energy. Blood glucose levels become significantly increased, glucose is flushed into the urine, and the patient experiences

increased urination. Without treatment, the patient becomes extremely dehydrated which can be fatal, or else the patient may experience a diabetic coma (Staff, 2010).

Endoplasmic Reticulum Stress

Excessive loss of pancreatic beta cells, whether due to autoimmunity or to cell functional collapse, is a main cause of diabetes. Apoptosis is the main mode of pancreatic beta cell death (Oyadomari & Mori, 2004). One characteristic of pancreatic beta cells is a highly developed endoplasmic reticulum (ER) secretion capacity, especially involving insulin. The ER has many important functions, including post translational modification, folding, and assembly of newly synthesized secretory proteins. Its proper function is essential to the survival of cells (Araki et al., 2003). In order for proteins to perform proper cell functions, they need to be folded into their proper conformations. Unfolded or misfolded proteins are harmful to cell survival. Protein misfolding in the ER is termed "ER stress". ER stress is present in both physiological and pathological conditions. Physiological ER stress includes tissue ischemia and neuronal excitotoxicity. In natural cell injuries, ER stress is one of many components that disrupt the cell's homeostasis (Zinszner, et al., 1998). Increasing evidence indicates that pancreatic beta cell death in diabetes may result from increased ER stress (Harding and Ron, 2002).

In order for the cell to overcome this stress, the ER has a specific signaling pathway, known as the ER stress response pathway, or the unfolded protein response (UPR), which involves at least three different signaling responses (Wang et al., 2010).

Translational Attenuation

An early part of the UPR response is known as translational attenuation (**Figure-2,** upper diagram**)**. This response reduces the load of new protein synthesis in the ER to help prevent the accumulation of unfolded proteins in the lumen. In the event of ER Stress, PERK (one of the ER kinase sensors) is activated (not shown in the figure), which phosphorylates eukaryotic initiation factor-2 (eIF2) to halt the manufacture of new proteins (Wang et al., 2010). This PERK-mediated attenuation initiates a "survival signal" to stop the influx of newly synthesized proteins in the ER lumen to reduce the ER workload.

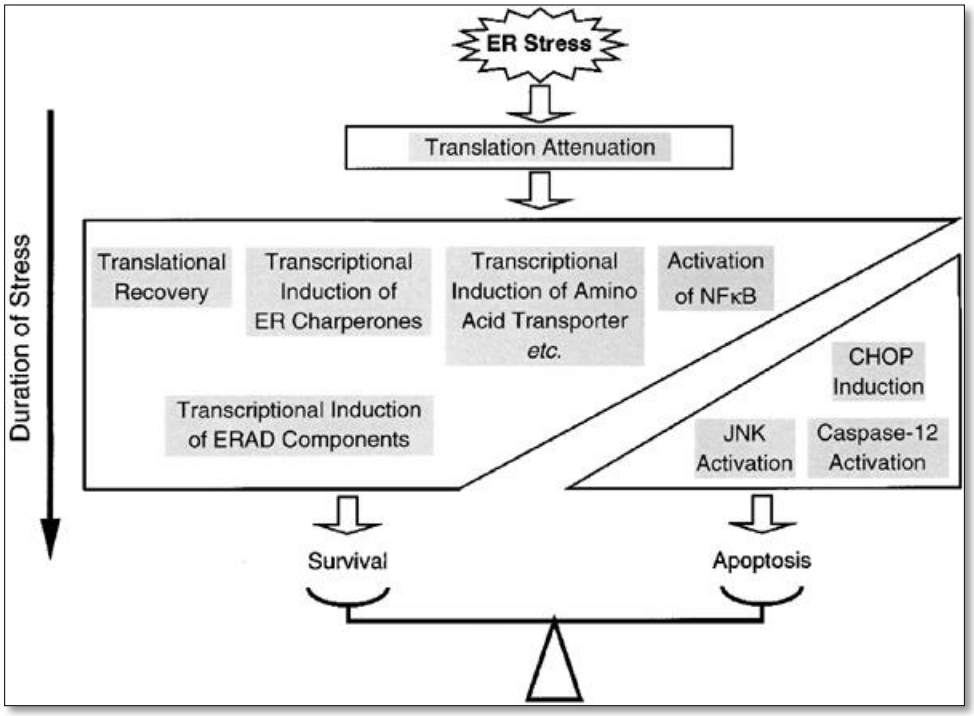

Figure 2: **ER Stress and Apoptosis.** Diagram shows the balance between various ER stress pathways that tend to increase cell survival versus apoptosis pathways that lead to cell death. These pathways are thought to be important in beta cell death in diabetes. (Oyadomari and Mori, 2004)

Induction of Stress Response Genes

In the next phase of the UPR, several groups of genes are transcriptionally induced to provide long-term adaptation to ER stress (diagram center). This new synthesis of stress-induced proteins escapes the general translational attenuation. In order for the ER to accommodate unfolded proteins, ER chaperones are induced to first try to refold them, and if this refolding response does not work then other components are introduced to degrade the unfolded or misfolded proteins. In addition, transcription factor NFκB is activated to induce an inflammation response to increase cell survival (diagram center right).

Considerable research has focused on the mechanism of chaperone production. IRE1α is self-activated by autophosphorylation. This endoribonuclease removes a 26 nucleotide intron from X-box binding protein-1 (XBP1) pre-mRNA. This splicing creates a frameshift to allow the translation of a functional bZIP transcription factor (Wang et al., 2010). The spliced bZIP transcription factor activates the expression of ER chaperones and enzymes which work to relieve the ER stress by facilitating protein folding, maturation, and secretion (Wang et al., 2010). Transcription factor ATF6 upregulates the expression of chaperones Bip/GRP78 and GRP94. ATF6 leaves the ER membrane to relocate to the Golgi apparatus. The Golgi processes ATF6 with proteases which produces and active bZIP transcription factor, which as described above activates expression of UPR target genes (Wang et al., 2010).

Cellular Apoptosis

If ER stress continues, eventually apoptosis signaling pathways are activated (diagram right side). At least three apoptosis pathways that are known to be involved, including the

activation of CHOP, JNK kinase, and caspase-12. At this point, the decision between survival and cell death depends on the balance between cell survival signaling versus apoptosis signaling.

CHOP and ER Stress

CHOP activation is of special interest to this MQP project. CHOP is a 29 kDa protein consisting of 169 amino acids in humans, and 168 residues in rodents. CHOP was the first protein to be identified as a dominant-negative inhibitor of the C/EBP family of proteins (Oyadomari & Mori, 2004). CHOP is also known as the growth arrest- and DNA damage inducible gene 153 (GADD153). GADD genes are a group of genes induced by toxic stress and growth arrest signals. These proteins form a family of transcription factors known to regulate a variety of genes involved in many physiological processes, including immune functions, cell differentiation, and proliferation (Oyadomari & Mori, 2004). CHOP is composed of an N-terminal transactivation domain **(Figure-3)** and a C-terminal bZIP domain, the latter contains a DNA-binding basic region and a leucine zipper dimerization region. The basic region contains conserved glycine (109) and proline (112) residues, which are essential for binding to the consensus C/EBP-binding site. Two serine residues (79 and 82) in the transactivation domain are phosphorylated by p38 MAP kinase, and this phosphorylation is required for the enhanced transcriptional activation of CHOP-induced genes.

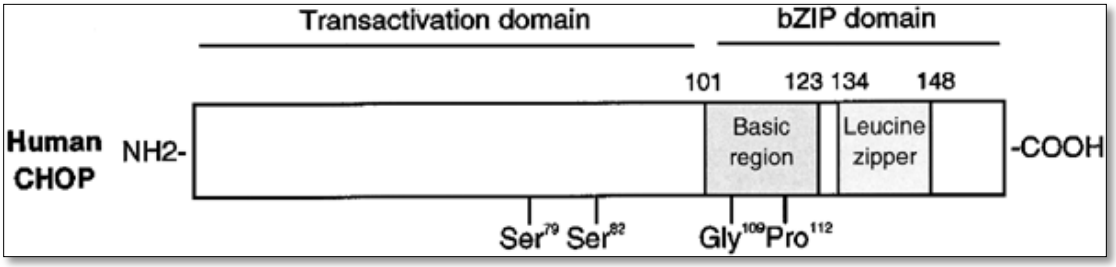

Figure 3: **Domain Structure of Human CHOP/GADD153 Protein.** CHOP consists of an N-terminal transactivation domain, and a C-terminal domain containing a C/EBP-binding basic domain and a leucine zipper dimerization domain. (Oyadomari and Mori, 2004)

Under ER stress conditions, chaperone BiP binds to unfolded proteins and activates stress transducers including PERK, ATF6, and Ire1 **(Figure-4)**. Activated PERK phosphorylates eIF2α, which results in translational induction of ATF4 (diagram left). During ER stress, the transcriptional induction of CHOP is regulated at least by four *cis*-acting elements, AARE1, AARE2, ERSE1 and ERSE2. ATF4, pATF6 (N) and XBP-1.

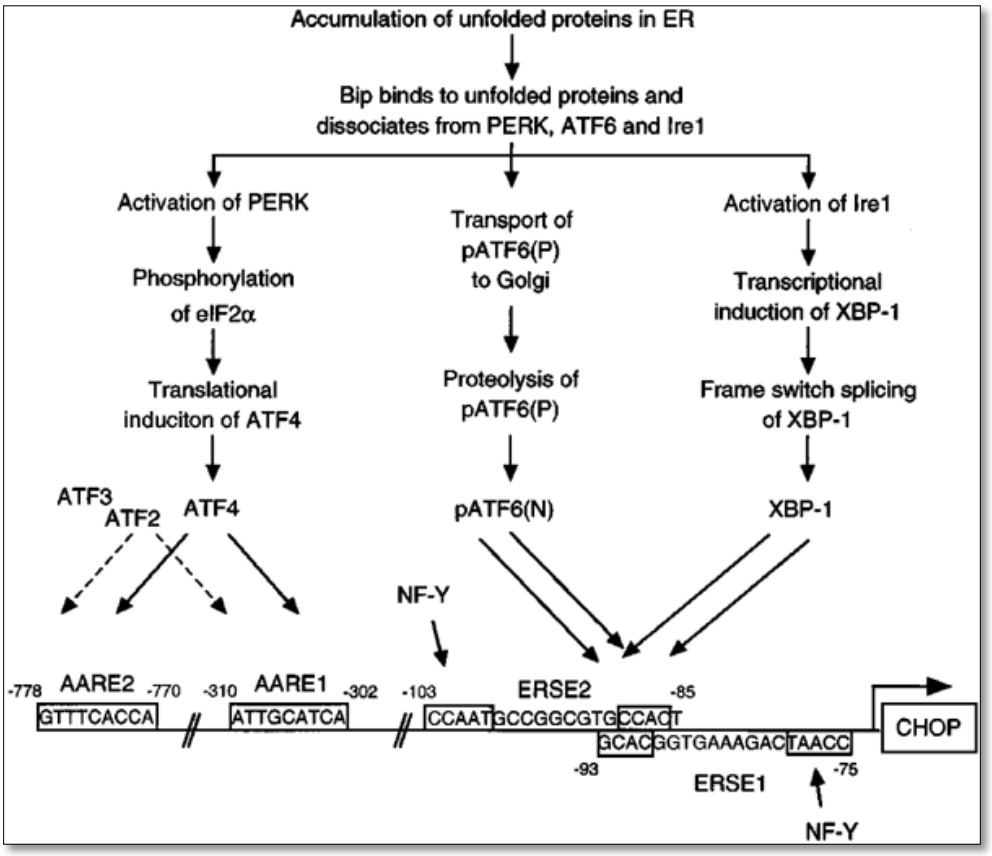

Figure 4: **Transcriptional Induction of CHOP during ER Stress.** ER stress is known to induce apoptotic CHOP expression via at least three known pathways, including PERK (diagram left), ATF6 (diagram center), and IRE1 (diagram right). Oyadomari and Mori, 2004)

Recent publications have tried to correlate the relationship of CHOP with ER stress. Since the precise mechanism by which ER stress induces CHOP is not known, studies have tried

to demonstrate that the CHOP induction signal originates from the ER itself, and is not just a downstream consequence of impaired ER function (Zinszner, et al., 1998). CHOP undergoes a stress-induced phosphorylation by members of the p38-MAP kinase family to activate the protein. CHOP has been shown to be nuclear, and able to form stable heterodimers with C/EBP relatives. The dimers are able to recognize DNA target sequences to alter gene expression.

Experiments have also shown that CHOP can be induced by specific pharmacological agents such as tunicamycin (an inhibitor of protein glycosylation) and thapsigargin (an ER-specific calcium ATPase inhibitor) (Zinszner, et al., 1998). Cells that lack CHOP (CHOP -/-) have a normal ER stress response. Normal cells, and cells with temperature sensitive mutations in essential components of the ER glycosylation apparatus, were cultured with tunicamycin. In normal cells treated with tunicamycin, the *chop* gene is induced to high levels, but is undetectable in chop-/- cells. It was also determined that induction of the ER chaperone Bip is indistinguishable in the two cell populations. This indicates that CHOP is not required for the initial ER stress response, so perhaps CHOP is required for the subsequent induction of apoptosis. These authors concluded that the *absence* of CHOP promotes *increased* survival of cells exposed to ER stress. These authors also concluded that CHOP does not appear to have an important role in cell-cycle arrest in response to tunicamycin (Zinszner, et al., 1998).

These findings are important to our project because it is necessary to determine how cells respond when ER stress is induced with a pharmacological agent.

PROJECT PURPOSE

Our lab is focused on the influence of the metabolic environment on pancreas and general embryo development. In mammals, both hypo- and hyper-glycemia are known to induce cellular stress responses and apoptosis, both of which are mediated by CHOP/GADD153. The purpose of this project was to investigate the developmental consequences of manipulating endogenous glucose levels in zebrafish embryos *in vivo*, and linking this to CHOP/GADD153 expression, as well as to embryonic growth and morphology.

METHODS

Semi-Quantitative RT-PCR

CHOP primers were designed to run with RNA samples from 10 different embryos. This RT-PCR test represented the embryos exposed to hypoglycemic conditions and their controls. The PCR machine was set to run at an annealing temperature of 57°C for 31 cycles, with a 32 second extension time. The resulting samples were run on a 1% agarose gel. Particularly of interest was the effect of treating 72 hour embryos with 3-mercaptopicolinic acid (MPA) versus the MPA control; this should demonstrate whether hypoglycemic conditions affected the expression of CHOP. MPA is an agent that blocks gluconeogenesis, thus inducing hypoglycemia.

In Situ Hybridization

Whole embryo *in situ* hybridizations were performed on 24.5 and 72 hour old embryos, with one embryo set that was treated with 2.0 mM MPA, and one set as the MPA control, to determine the expression of CHOP at each stage during development. Two probes designed against the CHOP gene were made and tested by using two different restriction enzymes: SpeI and Not1. SpeI restriction enzyme cut the RNA expression plasmid to express RNA from the T7 promoter; Not1 cuts the expression plasmid to express RNA from the Sp6 promoter. These "riboprobes" were digoxygenin-labeled synthesized from the expression plasmids *in vitro*, and were used to identify CHOP-expressing tissues under hypo- and hyper-glycemic conditions.

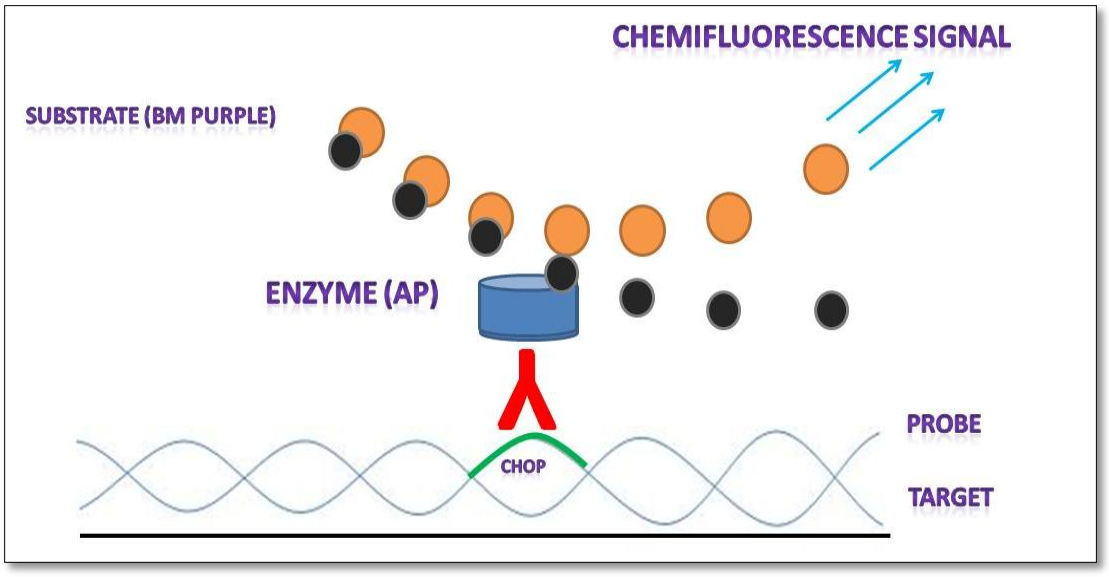

Figure 5: *In Situ* Hybridization Schematic. This figure depicts the process of *in situ* hybridization. The substrate, BM purple, is dephosphorylated by the enzyme alkaline phosphatase present in the antibody detecting the digoxigenin-labeled RNA probe, allowing the chemifluorescence to show where the CHOP gene is expressed in the embryo.

Glucose Measurements

Groups of 20-25 screened embryos were recounted and transferred to 1.5ml microcentrifuge tubes. Embryo lysates were cleared by centrifugations, and stored at -80°C. Reactions were assembled on ice with reagents purchased from Biovision. The standard curves for glucose were generated using the Biovision standard solution, according to the instructions provided. Glucose measurements in embryo extracts were performed by adding 8 µl of sample to 42 µl of assay buffer. 50 µl of reaction mix containing 1 µl enzyme solution, and 0.4 µl glucose probe were added. Reactions were incubated at 37°C for 30 min in the dark, and fluorescence was measured using a Safire II plate reader equipped with ZFLUOR4 software (v 4.51), specific for free glucose. Glucose levels were estimated from standard curves, and measured in triplicate.

RT-PCR, Ligation, Transformation

Sample RNA was measured in the treated and control samples on a nanodrop spectrophotometer. Our control sample measured 273.43 ng/µl, and our treated sample measured 188.26 ng/µl. We used these measurements to determine the amount of RNA needed to run our RT-PCR.

For CHOP RT-PCR, the oligonucleotides used were ZfchopF1qRT102809 with the sequence AGTTGGAGGCGTGGTATGA, and ZfchopR1qRT102809 with the sequence AGATCTCCGGATGAGGTGTT. The optimal annealing temperature for these oligonucleotides was 57.4°C with a length of 256 bp. We ran the RT-PCR program in the thermocycler for 35 cycles, and stored the amplified samples in a -20°C freezer.

We then used our amplified CHOP RT-PCR amplicons to clone into plasmid PCRII (Invitrogen) using TA cloning in which the 3' A tail on the amplicon is annealed to the 3' T-tail on the plasmid (**Figure-6**). The RT-PCR amplicon, PCR II vector, ligation buffer, and DNA ligase were combined to run in the thermocycler on the Ligation program, which ran overnight.

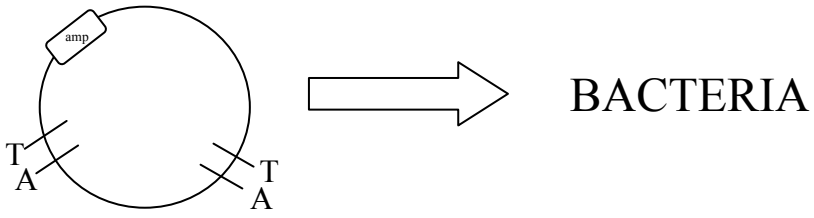

Figure 6: Cloning of CHOP into Plasmid PCRII. This figure shows the construction of the plasmid that was used in order to successfully transform our cloned products into the competent *E. coli* cells. The 3' A-tail on the amplicon is directly annealed to the 3' T-tail on the vector. Nucleotides thymine and adenine are represented because it accurately depicts how our cloned product was inserted into the vector.

In order to transform our cloned products we performed the following steps. We thawed the competent cells (One Shot® TOP10 Chemically Competent *E. coli*, also Invitrogen) and added an aliquot of the ligation reaction to the cells, while gently swirling to mix the two. After the cells were incubated on ice for 30 minutes, we added 0.5 mL of SOC medium and allowed the cells to grow at 37°C for 1 hour. After the cells incubated, we spun them down and removed the medium, leaving about 30 µl so we could resuspend the cells in the remaining medium. We then aseptically transferred the cells to an amp+ plate, spread the cells with a glass spreader, and allowed the plate to incubate overnight at 37°C.

After the cells had incubated overnight, we isolated and grew four bacterial colonies in 50mL falcon tubes. We aseptically selected four different colonies that were large and isolated to avoid satellite colonies, and put them in the falcon tubes containing 10 mL of Lennox broth. These four samples were then put in the incubator at 37°C for one hour, after which we performed a midi-plasmid preparation using the protocol from *Midi Plasmid Preparation Kit (UM-100)* from *Boston BioProducts, Inc.*

After the plasmid preparation was complete, we measured all four samples in the nanodrop spectrophotometer to see which colony had the highest yield. Colony 1 yielded 336.86 ng/µl, Colony 2 yielded 290.25 ng/µl, Colony 3 yielded 320.74 ng/µl, and Colony 4 yielded 334.75 ng/µl. Using colony 1, we split the sample into two equal aliquots, and digested the plasmid with two restriction enzymes, SpeI and NotI that would allow us to access the promoters of interest, T7 and Sp6.

RESULTS

This project focused on the effects of hyper- and hypoglycemic conditions in developing zebrafish embryos. Both types of glycemic conditions trigger an ER stress response and apoptosis, which is mediated by CHOP/GADD153. The project monitored the expression of CHOP/GADD153 in response to manipulation of varying glucose environments. In **Figure 7**, a CHOP RT-PCR was performed on RNA samples from wild type embryos ranging from 16 to 96 hours post fertilization. The RT-PCR CHOP oligonucleotides used were 270 bp in length. The CHOP amplicon is represented by the lowest (and strongest) of the three bands shown. It is evident that CHOP is upregulated at each stage of development assayed. The band increases in intensity from left to right, allowing us to deduce that CHOP is expressed at each developmental checkpoint.

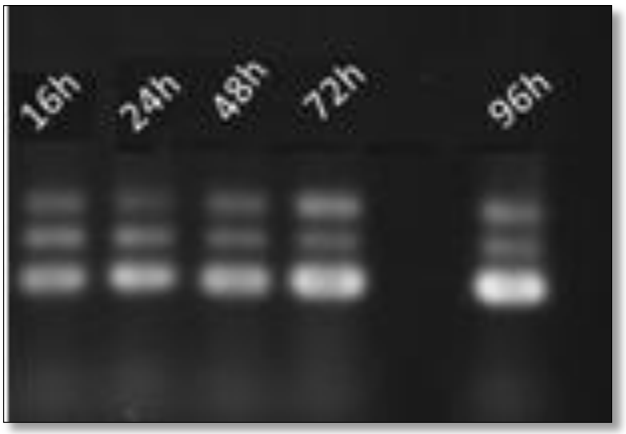

Figure 7: Expression of CHOP at Various Stages of Development. Semi-quantitative RT-PCR was performed on 16, 24, 48, 72, and 96 hour samples wild type for CHOP expression. They were run against a 100bp ladder (not shown, see next figure) to demonstrate that they were in fact CHOP. The CHOP amplicon is represented by the strong lowest band in each lane. The figure shows that at each stage of development, CHOP is upregulated because of the increasing intensity of the bands.

Following this experiment we ran another RT-PCR monitoring the effects of a gluconeogenesis inhibitor, MPA, on CHOP expression in the 72 hour embryo. As seen in **Figure 8**, lane-1 shows CHOP in the control embryo, while lane-2 shows CHOP levels in embryos treated with MPA. It appears that CHOP expression is slightly increased in the MPA-treated RNA sample, although not by a significant amount.

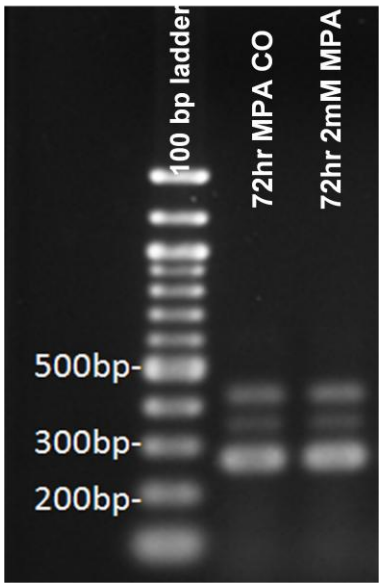

Figure 8: RT-PCR Analysis of CHOP Expression at 72 Hours in the Presence of a Gluconeogenesis Inhibitor (MPA). Semi-quantitative RT-PCR was performed on 2 mM MPA-treated and MPA-control embryos. This figure shows that CHOP expression was slightly upregulated in the MPA-treated samples, though it was still present in control samples. The CHOP amplicon was expected at 270 bp, corresponding to the lowest of the 3 bands, and the presence of two other larger bands was unexpected and considered "junk" bands.

Figure 9 shows the *in situ* hybridization procedure performed on the 72 hour MPA control embryos (riboprobe prepared from the T7 promoter). CHOP expression is seen below the eyes (arrows). There was no inhibition of gluconeogenesis in this control embryo, yet there

is still ample expression of CHOP. Thus, even in normoglycemia, cells are being sent down the apoptosis pathway, mediated by CHOP.

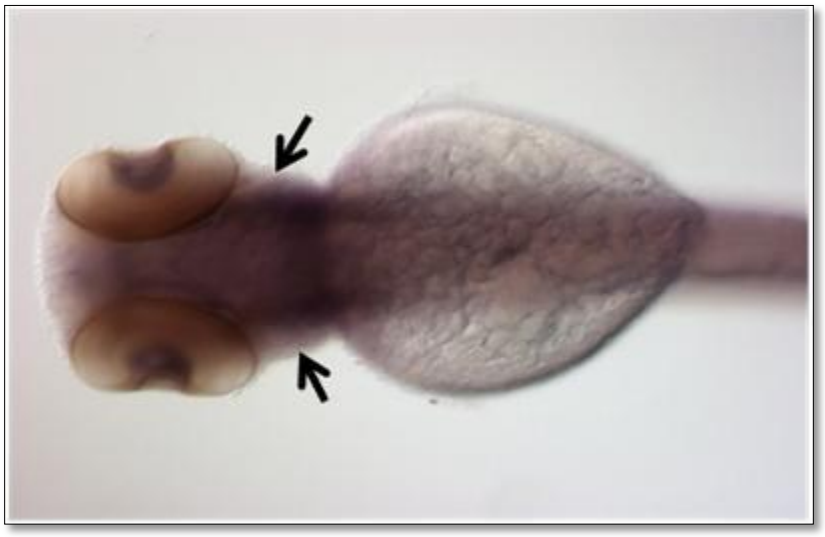

Figure 9: CHOP *In Situ* Hybridization, Dorsal View of 72 Hour MPA-Control. The probe used was CHOPT7.

Figure 10 shows a dorsal view of two 72 hour embryos probed for CHOP mRNA through *in situ* hybridization under control and MPA conditions. One embryo is treated with MPA to block gluconeogenesis (right panel), while the control was not treated (left panel). Both the control and MPA treated samples showed patterns of CHOP expression beneath the eyes, and surprisingly the intensity of the staining was similar, implying similar amounts of CHOP expression.

Figure 10: CHOP *In Situ* Hybridization, Lateral View of 72 Hour Embryos. Probe was CHOPT7**.** (Left) Control embryo. This image shows CHOP expression below the eyes even when gluconeogenesis is not inhibited. When gluconeogenesis is blocked by MPA (Right), the BM purple *in situ* probe should dye darker in the embryo to indicate more CHOP expression in these stressful conditions. However, this was not the case as the 2mM treated embryo showed similar staining patterns and intensity.

Figure-11 shows photographs of general embryo morphology after incubation with 3-MPA between the two to eight cell stage for 24 hours. The MPA treatment (right panel) does not appear to affect the rate of growth or morphological development compared to control (left panel). This figure shows us that there is no obvious phenotypic difference in early development when exposed to different glycemic environments.

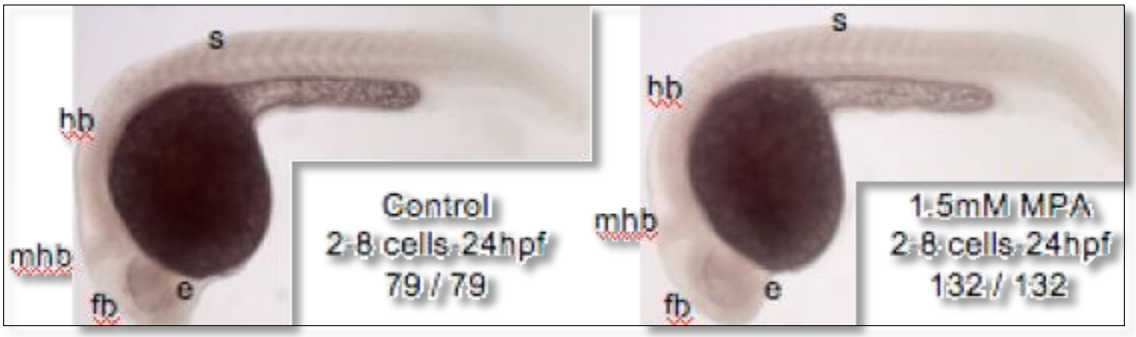

Figure 11: Photography of Zebrafish Embryos To Assay General Morphology Affected by MPA. There is no obvious morphological effect of the MPA treatment (right panel) compared to control embryos (left panel). e, eye; fb, forebrain; hb, hindbrain; mhb, midbrain-hindbrain boundary; s, somite.

Figure 12 shows that 3-MPA causes a dose-dependent decrease in total embryonic glucose for embryos analyzed at 22 hours post fertilization. This is significant because it confirms that MPA is indeed creating a hypoglycemic environment.

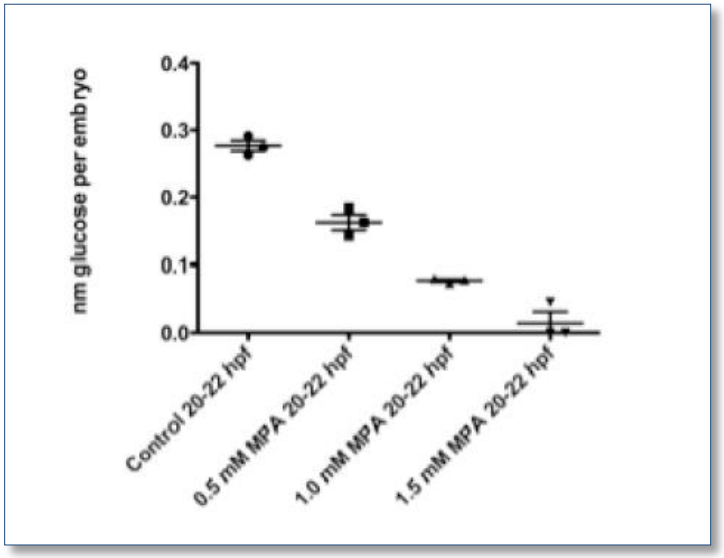

Figure 12: Analysis of Normal and MPA-Treated Embryo Glucose Levels. 3-MPA causes a dose-dependent reduction in total embryonic glucose. Embryos were analyzed at 22 hours post-fertilization.

Additional experiments involving more stages of embryo development showed that the treatment with 1.5 mM 3-MPA (the highest dose used in the previous figure) caused a 10-fold reduction in free glucose back to levels normally seen in 8-cell embryos (**Figure-13**). This suggests that pathways other than gluconeogenesis do not compensate for the loss of normal, embryonic glucose accumulation at this stage (22 hours post-fertilization).

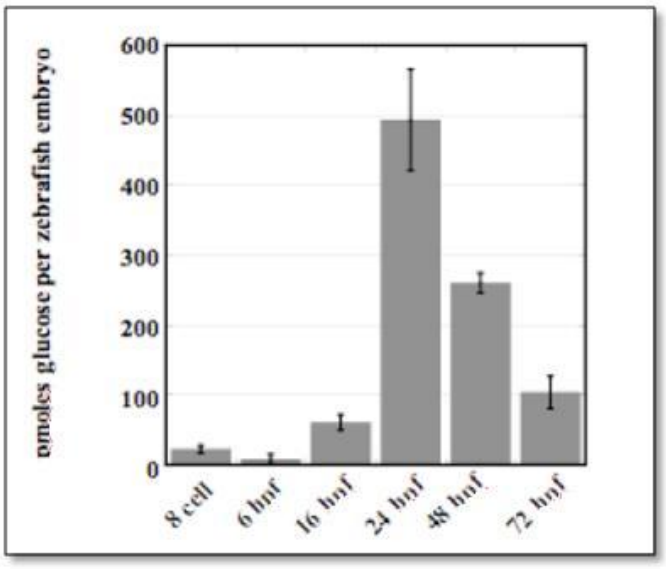

Figure 13: Assay of Picomoles of Glucose per Zebrafish Embryo at Different Developmental Stages Under Normal Conditions. Note the high levels of glucose present in 24 hr embryos.

Pck1 is a regulator of gluconeogenesis so we also analyzed its expression in early embryos (**Figure 14)**. At 24 hour post fertilization (left image), *pck1* is expressed in the developing eye and tail. At 72 hours (right image), expression in the liver is seen (see lowercase letter "l" in right figure below). This *Pck1* expression is very similar to the pattern of expression of CHOP, seen in previous *in situ* hybridization images. These images show the effect of a normal hyperglycemic environment on *pck1* in the embryos at 24 and 72 hours post fertilization.

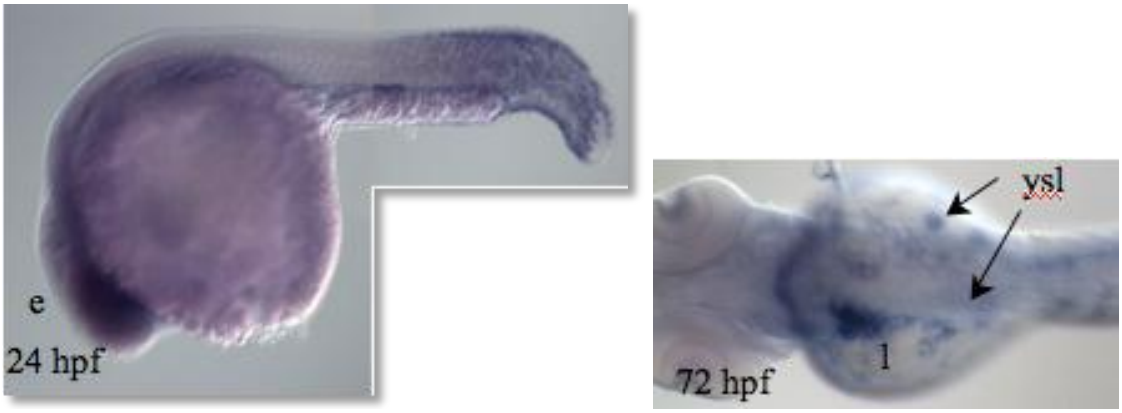

Figure 14: PCK1 *In Situ* Hybridization. Shown are 24 hr (left) and 72 hr (right) embryos, demonstrating pck1 expression at each stage. Arrows in the right image denote expression in the yolk syncytial layer.

The levels of *pck1* were also quantitated at different stages of development (**Figure-15**). This analysis shows that at developmental stages from 16-cells to 16 hours post-fertilization (first 3 histobars), when embryo glucose levels are low and *insulin b* levels are high, *pck1* levels are low. At 24 hours post fertilization, when glucose levels are high and there are low levels of *insulin b* while the levels of *insulin a* increase, *pck1* levels begin increasing. Finally, between 48 and 120 hours post-fertilization, when glucose levels are low and insulin a levels are high, *pck1* levels are also high. Thus, at very early stages of Zebrafish development, there is an inverse correlation between *pck1* and insulin levels, but a direct correlation later in development. This differs from adult mammals where *pck1* expression is inhibited by insulin (Quinn & Yeagley, 2005). In the zebrafish embryo, *pck1* is not expressed at early stages, when non-pancreatic *insb* mRNA is abundant and glucose is very low.

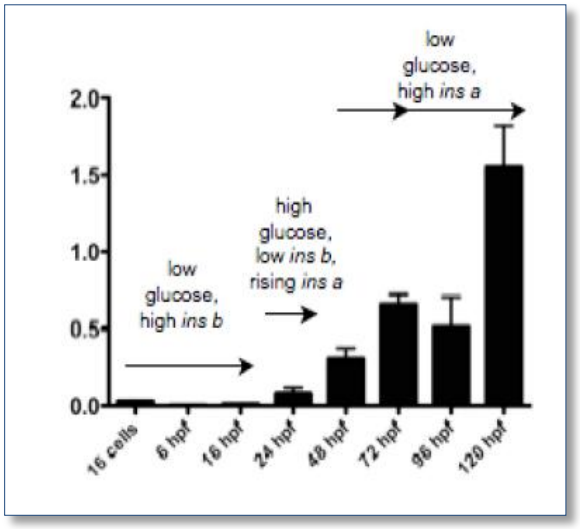

Figure 15: Levels of *pck1* mRNA During Zebrafish Development. Y-axis denotes levels of *pck1* mRNA. Histobars denote means of 3 independent experiments. Error bars denote standard error.

DISCUSSION

In our lab, the focus is on monitoring the effects of the metabolic environment on Zebrafish embryos during development. In this project, embryos were exposed to MPA to induce hypoglycemic conditions, and the effects on ER stress marker CHOP/GADD153 expression were observed. It was our hypothesis that CHOP would be upregulated in both hypoglycemic and hyperglycemic conditions (as in diabetes) and down regulated in normal conditions, because the apoptosis pathway would be necessary to destroy stressed cells. We observed CHOP expression below the eyes in normal embryos, and when gluconeogenesis was blocked, CHOP expression slightly increased to initiate the apoptosis pathway of diabetic cells, as expected. Thus, even when gluconeogenesis is not blocked, the CHOP apoptosis pathway is still being used by the embryo. This implies that glucose does not regulate CHOP because it is seen in similar pattern and intensity regardless of glycemic conditions.

We also assayed the levels of embryonic *pck1*. *Pck1* is an enzyme that stimulates gluconeogenesis (to elevate blood glucose), which is the opposite of MPA which creates a hypoglycemic environment. *Pck1* mRNA expression was seen in the eye, tail, and liver. An *in situ* hybridization (see **Figure 14**) shows the expression of *pck1* at different stages of development. The data indicated that the pattern of *pck1* regulation, as well as intensity of its expression, is similar to the pattern and intensity of expression of CHOP. Again, this implies that glucose may not regulate CHOP. It was expected that production of glycogen and the apoptosis pathway would not be seen in the same cells. As stated by Wang, under ER stress, there is translational attenuation so that more proteins are not produced incorrectly; eventually CHOP is regulated to destroy cells under ER stress. The presence of *pck1* while there is also ER

stress (CHOP) implies that glucose is not the effector causing ER stress and apoptosis; otherwise, *pck1* production would be stopped to cease ER stress.

General embryo morphology was also analyzed under control and hypoglycemic conditions (MPA). We hypothesized that treatment of zebrafish embryos with 3-mercaptopicolinic acid (3-MPA) would show phenotypic abnormalities, as being deprived of a main energy source during the first 24 hours of development should theoretically be detrimental to the embryo. However, we observed no obvious phenotypic differences. This implies that glucose, besides not being important for CHOP regulation, may also not be important for early development.

A study performed by Carayannopoulos et al. (2004) also studied the effects of compromising glucose metabolism on mouse development. Their data showed that the glucose transporter GLUT9 is crucial in preimplantation development in mice. Mice without GLUT9, or with defective transporters, had increased pregnancy loss (Carayannopoulos et al., 2004). So if in mice glucose transporters are critical for development, this implies glucose is important in mouse development, so why the difference with Zebrafish. Importantly, Caryannopoulos noted that blocking GLUT9 did not kill embryos until transfer back into the womb. Since zebrafish embryos are never transported back to the womb, perhaps a similar glucose-dependent survival phenomenon is not witnessed in zebrafish.

For future studies, the effects of lack of glucose in embryos need to be examined on long term Zebrafish development. While we did not see any obvious phenotypic differences at 24 hours, it is crucial to determine whether differences appear later in development, and if so what is their severity. Also, it is critical to see *when* the embryo starts to depend on the presence of

glucose; thus it should be examined whether the lack or surplus of glucose too early or too late in development puts the embryo at risk for diabetes.

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