INVESTIGATING THE ROLE OF *foxm1*

IN ZEBRAFISH OTIC DEVELOPMENT

A Thesis

by

JAMES W. KUTLOWSKI

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Chair of Committee,	Bruce Riley
Committee Members,	Jennifer Dulin
	Vladislav Panin
Head of Department,	Thomas McKnight

December 2019

Major Subject: Biology

ABSTRACT

Preliminary data gathered by our lab suggests a requirement for lactate in the developing inner ear of zebrafish embryos, implicating the action of the Warburg effect in otic development. A potential regulator of this Warburg effect is the transcription factor *foxm1*, which may also be mediating the Wnt and Fgf signals known to pattern the inner ear. Studies of the effects of *foxm1* were performed with the heat-shock transgene *hs:foxm1*, morpholino oligomers for *foxm1* knockdown, and a TALEN-induced *foxm1* knockout line.

In transgenic *hs:foxm1* embryos the only observed effect of heat shocks was an increase *foxm1* mRNA abundance. Regardless of heat shock time point or BIO, IWR-1, or 2DG treatment status, heat shocks did not cause changes in morphology or gene expression. Several hypothetical explanations for the transgene's ineffectiveness were tested, with new experiments planned to continue the exploration of the effects of *foxm1* overexpression. Knockdown of *foxm1* by morpholino oligomers resulted in significant dorsalization, severely reduced Wnt signaling, altered Fgf target gene expression, and reduced *pgk1* expression. These results support the hypothesized role of *foxm1* as a Warburg regulator and a mediator of Wnt and Fgf signaling. A *foxm1* knockout allele was generated with the use of TALENs, and a *foxm1* knockout line was created. For unknown reasons, this knockout mutation was not observed to have any effect on morphology or gene expression. To test whether this was due to an early rescue effect, an attempt was made to generate maternal-zygotic mutants, but due to breeding issues the phenotype of MZ *foxm1* mutants remains unknown. Further experiments, possibly including the creation of another *foxm1* knockout allele, will be necessary to explore the true effects of *foxm1* knockout. It is our hypothesis that the *foxm1* knockout phenotype, in the absence of any genetic compensation effects, will resemble the *foxm1* morphant phenotype.

DEDICATION

To Mom, Dad, Leo, and Joe.

To Larry, Jeff, Meri, Louie, Bruce, and all teachers of science.

ACKNOWLEDGMENTS

I would like to thank Dr. Bruce Riley for allowing me to be a part of his lab, and for all of the mentoring and assistance he has provided to me during my time at Texas A&M University, especially in the face of the numerous difficulties encountered throughout the course of my research. I would also like to thank both Dr. Jennifer Dulin and Dr. Vladislav Panin for their time and contributions to my thesis committee, and their valuable questions which helped to guide my project. I would also like to extend further thanks to Dr. Christine Merlin, a former member of my thesis committee, and Dr. Wayne Versaw, a mentor during my time as a rotation student in his lab, for their assistance during my first year as a graduate student.

I would like to thank all of the members of the Riley Lab. I am thankful to Jennifer Dong for her training me in basic laboratory techniques and the handling and care of zebrafish, as well as all the work she does every day to keep the lab running. I am thankful to Dr. Jinbai Guo, Dr. Yunzi Gou, and Amy Tan for helping me through a number of complicated experiments and offering insights to improve my project. I am thankful to Jorden Holland and Whitney Roberson, as well as Nick Holloway of the MacKenzie lab, for their cooperation, collaboration, and commiseration.

I would like to thank Jennifer Bradford, our graduate program coordinator, for all of the work that she has done in helping myself and other graduate students through the sometimes chaotic and confusing administrative processes involved in graduate school.

Lastly, I would like to thank my friends and family for all of their support and encouragement, without which none of this would be possible.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a thesis committee consisting of Dr. Bruce Riley [advisor] and Dr. Jennifer Dulin of the Department of Biology, and Dr. Vladislav Panin of the Department of Biochemistry and Biophysics.

Preliminary experiments discussed in the Introduction section of this thesis were performed by past members of the Riley lab. Creation of the *hs:foxm1* transgene was also performed by past members of the Riley lab. Initial TALEN knockout of *foxm1* and initial knockout line establishment was managed by Dr. Jinbai Guo.

All other work conducted for the thesis was completed by the student independently.

Funding Sources

This work was supported by the National Institute on Deafness and Other Communication Disorders (project no. 2R01DC003806-21A1), a part of the National Institutes of Health. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Institute on Deafness and Other Communication Disorders or the National Institutes of Health.

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INTRODUCTION

The inner ear is responsible for both auditory and vestibular perception, and disruptions of its development can lead to serious sensory disorders such as deafness or vertigo. Some of these cases can be attributed to mutations that are well-studied, but for many cases the genetic cause remains unknown (Grindle *et al*. 2014, Eppsteiner & Smith 2011). Prior studies and preliminary experiments conducted by our lab have indicated that the transcription factor *foxm1* represents a promising research target for elucidating the genetic and cell signaling mechanisms which control otic development.

The process of selecting *foxm1* as a transcription factor of interest began with the identification of the mutation *sagd1*, which was generated by our lab in zebrafish via ENU mutagenesis. This mutation produced a phenotype with several otic development deficiencies. The *sagd1* mutants had reduced numbers of vestibular statoacoustic ganglion (SAG) neurons, half the normal number of hair cells, and reduced expression of several genes known to be targets of Fgf signaling, including *ngn1, etv5b,* and *pax5* (Millimaki & Sweet *et al.* 2007, Roehl & Nüsslein-Volhard 2001, Kwak *et al.* 2006). After several days, expression of these genes began to return to wild-type levels, but all homozygotes died between days 7 and 10 despite the absence of major defects outside of the ear. Whole genome sequencing revealed that the mutation was in the gene *pgk1* which encodes the glycolytic enzyme phosphoglycerate kinase 1. The identity of this mutation was confirmed by a CRISPR-generated *pgk1* knockout that was found to have an identical phenotype. Loss of this gene explained the embryos' eventual death, but it did not explain the specific developmental defects occurring in the inner ear.

To determine how a glycolysis deficiency specifically affects otic development, glycolysis and subsequent metabolic steps were inhibited in wild type embryos via treatment

with either the glycolysis inhibitor 2DG, the LDHA inhibitor galloflavin, or the lactate membrane transport inhibitor UK-5099 (Wick *et al.* 1957, Farabegoli *et al.* 2012, Corbet *et al.* 2018). All three treatments produced a phenotype identical to the *pgk1-/-* mutant, indicating that the ability to both produce and transport lactate is necessary for otic development. Additionally, treatment with exogenous lactate rescued *pgk1-/-* SAG neuron counts and Fgf target gene expression. Lactate treatment in wild type embryos led to increased SAG neuron counts and Fgf target gene expression compared to untreated embryos. These results indicate that lactate synthesis and secretion are required for normal otic development. Dependence on lactate is typical of a mode of metabolism historically associated with cancer cells, known as the Warburg Effect.

The Warburg Effect (a.k.a. “aerobic glycolysis”) was originally discovered in cancer cells and refers to a shift in metabolism in which these cells, regardless of oxygen availability, will convert the bulk of their pyruvate into lactate rather than using it in the citric acid cycle (Warburg 1956). In more recent years, it has been discovered that the Warburg Effect may not be solely a trait of cancer as it is also being utilized during development. The Warburg Effect has been observed to occur in the developing retina of frogs, in the posterior presomitic mesoderm of mice and zebrafish, and in human neural, hematopoietic, and pluripotent stem cells (Agathocleous *et al.* 2012, Bulusu *et al.* 2017, Gu *et al.* 2016, Oginuma *et al.* 2017, Sá *et al.* 2017, Wang *et al*. 2014, Zheng *et al.* 2016). These studies demonstrate the requirement for lactate in developing tissues, though there are multiple hypotheses as to why this is the case. One is that the rapid glycolysis generates sufficient ATP and the lactate that is subsequently produced serves as a pool of short carbon chains that the cell can then use for biosynthesis. Having this

resource available is important for a cell that is undergoing frequent division, but it is possible that lactate may have a cell signaling function as well (Liberti & Locasale, 2016).

It has been demonstrated that lactate can have a signaling role within the cell by binding and stabilizing the protein Ndrg3, thus preventing it from being degraded and allowing it to activate Raf-MAPK signaling (Lee *et al.* 2015). This is notable as it is the same pathway used to transmit Fgf signals, which are known to be required for the induction and maintenance of otic development (Ornitz & Itoh 2015, Phillips *et al.* 2004, Freter *et al.* 2008). As MAPK activity is induced by both Fgf signals and lactate, transcription of Fgf target genes is increased, including ETS factors such as *etv4* and *etv5b*, among others (Raible & Brand 2001, Roehl & Nüsslein-Volhard 2001). These genes are significant because their protein products are then phosphorylated and activated by MAPK and will act as transcription factors to mediate many of the downstream effects of Fgf. Thus, increased available lactate augments Fgf signaling by increasing the rate of transcription of ETS factors and allowing for their protein products to facilitate more rapid activation of downstream Fgf target genes. This would mean that for the *pgk1-/-* mutants generated in our lab, the otic development defects that were observed were likely due to insufficient Fgf activity caused by disrupted glycolysis and lack of functioning Warburg metabolism. All of this evidence supports a role for Warburg metabolism in otic development, but it is unclear how this process is regulated. A candidate gene for Warburg regulation in otic development is the transcription factor *foxm1*, the focus of this research project.

The gene *foxm1* belongs to the FOX (forkhead box) family of transcription factors. Members of the FOX family possess a forkhead DNA binding domain, a unique feature first discovered in the family's founding gene *fkh* in *Drosophila melanogaster* (Weigel *et al*. 1989). Since the discovery of *fkh*, numerous FOX genes have been identified in many different animals.

In humans, 50 FOX genes have been identified and categorized into 19 subgroups, designated FOXA through FOXS. FOX genes are assigned to these different subgroups based on their sequence similarity and the organization of their functional domains (Lam *et al.* 2013). FOXM1 is the sole member of the FOXM subgroup, and is highly conserved between humans and zebrafish.

FOXM1 has been mostly studied in the context of cancer biology, as it has a powerful effect on promoting cell division and has been observed to be upregulated in many metastatic tumors (Milewski *et al.* 2017, Chen *et al.* 2013). Among such studies, it has been observed that Foxm1 promotes Warburg metabolism by upregulating expression of both Pgk1 and LDHA in order to create abundant lactate. It has also been shown that Foxm1 is directly activated by MAP2K (a.k.a. MEK) (Ma et al 2005). Another detail which makes *foxm1* an attractive candidate for study is that there is evidence that it may mediate the effects of Wnt signaling. Wnt signals control cell fate decisions during otic development and maintain the integrity of the otic placode, a precursor structure to the inner ear (Phillips *et al.* 2004, Ohyama *et al.* 2006). Wnt signaling relies on the activity of β-catenin, a constitutively expressed protein in the cytoplasm that is normally quickly degraded by the APC complex. However, upon Wnt activation, this degradation is inhibited and β-catenin is translocated to the nucleus (MacDonald et al 2009, Kimelman & Xu 2006). The manner in which this nuclear translocation occurs has been a matter of ongoing investigation, as there may be more than one mechanism. One way in which this has been shown to occur is that cytoplasmic β-catenin binds to Foxm1 and then moves into the nucleus where these proteins can then bind to the transcription factors TCF and LEF. This protein complex then promotes transcription of Wnt target genes (Zhang *et al.* 2011).

The goal of this project is to investigate the role of the transcription factor *foxm1* during otic development. I hypothesize that Foxm1 mediates Fgf and Wnt signaling activity to levels sufficient for proper inner ear patterning and development. I further hypothesize that this system relies on Foxm1 modulating the activity of the Warburg effect, a mode of metabolism in which glycolysis and lactate production are highly elevated, despite the presence of oxygen. In this study, these hypotheses have been investigated through the use of zebrafish embryos containing heat shock inducible transgenes and knockout mutations, as well as with pharmacological activators and inhibitors and morpholino oligomers.

MATERIALS AND METHODS

Fish Strains and Developmental Conditions

All adult fish were maintained in a facility inspected and approved by the Institutional Animal Care and Use Committee (IACUC). Wild-type embryos were from the AB and TL lines. Transgenic lines used in this study included *Tg(hsp70:fgf8a)*^{x17} and *Tg(hsp70:foxm1)*. Transgenic lines are named in the text as *hs:fgf8* and *hs:foxm1*, respectively. Two *foxm1* mutant lines were created via TALEN-mediated gene targeting and were used for loss of function analysis. Mutants were identified using the screening methods described below. Embryos were maintained at 28.5°C (except where noted) and staged according to standard protocols (Kimmel *et al*. 1995). PTU (1-phenyl-2-thiourea, 0.3 mg/mL; Sigma) was added to block pigment formation where necessary.

Heterozygous and Homozygous *foxm1* Mutant Screening

Heterozygous and homozygous mutants were identified primarily through single embryo PCR genotyping. The fish being screened were each outcrossed with a wild type fish and eight embryos were collected. DNA was extracted from each embryo and the region of the *foxm1* sequence surrounding the TALEN-targeted region was amplified using PCR. This PCR product was then digested with a restriction enzyme and the restriction digest products were separated by gel electrophoresis. The TALEN edit disrupts a restriction enzyme cut site, and so the gel electrophoresis result allows for genotyping of the embryos. The genotypic ratio of the embryos allows for genotyping of the screened parent; a heterozygous parent outcrossed to wild type will

produce offspring that are 50% heterozygous and 50% wild type, while a homozygous parent outcrossed to wild type will produce offspring that are 100% heterozygous.

Some heterozygotes and homozygotes were alternatively identified by fin-clip genotyping. Adult fish were anesthetized in a tricaine solution and a small clipping of the anal fin was collecting using alcohol-sterilized microscissors, with the fish then being moved to individual recovery tanks. DNA was obtained from the fin clip tissue via proteinase K digestion. Screening was then carried out in the same way as the protocol described above, utilizing PCR, HpaII digestion, and gel electrophoresis to identify heterozygotes and homozygotes.

Heat Shock

Embryos were collected, transferred to 50 mm petri dishes, and incubated at 28°C. At the specified heat shock time point, the petri dishes were floated in a hot water bath at 37°C or 39°C for 30 to 60 minutes. The heat shock time point, water temperature, and duration varied between experiments. Following heat shock, the petri dishes were transferred to a 33°C incubator and allowed to recover for one hour before being transferred back to the 28.5°C incubator until ready for examination and/or subsequent experiments.

In Situ Hybridization

Whole-mount in situ hybridization and antibody labeling were performed as previously described (Phillips *et al.* 2001, Riley *et al.* 1999), with probes for *foxm1, pax5, pax2a, ngn1, top-gfp, sp5a, sp5l, gsc, pgk1,* and *etv5*.

Drug Treatment

The drugs used in this study were BIO, IWR-1, and 2DG (all from Sigma). Liquid stocks were created by dissolving each in DMSO. Treatments were performed by diluting the stock to the appropriate concentration in standard embryo incubation water ("fish water": distilled water with added salts and methylene blue). BIO and IWR-1 were diluted to a 10 mM concentration, and 2DG was diluted to a 250 µM concentration. At the appropriate time point, embryos were transferred to 50 mm petri dishes containing the drug solution. In experiments in which both heat shocks and drug treatments were applied, the embryos were transferred to the drug solution immediately after heat shock.

RT-PCR

Zebrafish mRNA was collected from embryos via a TRIzol and chloroform extraction. cDNA was synthesized through use of the SuperScript First-Strand Synthesis System RT-PCR kit from Invitrogen. PCR primers were designed based on *foxm1* sequence data available on ZFIN.

Morpholino Injection

All morpholino oligomers were obtained from Gene Tools, LLC. MOs were diluted in Danieux buffer (Nasevicius & Ekker, 2000) to a concentration of 5 µg/µL prior to injection. All embryos were injected at the one-cell stage with the use of a Narishige IM-300 microinjector. Following injection, all embryos were incubated under the same conditions as other embryos, as described above.

TALEN Design, Synthesis, and Screening

TALENs were designed with the assistance of the TAL Effector Nucleotide Targeter 2.0 online tool from Cornell University. TALENs were assembled through use of a modified version of the Golden Gate TALEN Assembly protocol originally described in Cermak *et al.* 2011. TALENs were injected into one-cell stage embryos with the use of a Narishige IM-300 microinjector. At 24 hours, DNA was extracted from a portion of the injected embryos. PCR was used to amplify the TALEN-targeted region, and the PCR product was subjected to a restriction enzyme digest (similar to methods described above) or a T7 digest to confirm the cutting ability of the TALEN. If the TALEN was identified to be an effective cutter, the remaining injected fish were raised to maturity for the origination of a new mutant line.

RESULTS

Use of a Heat-shock Inducible Transgene to Overexpress Foxm1

The *hs:foxm1* transgene consists of an *hsp70* heat-shock inducible promoter and the full cDNA of *foxm1*. This transgene was inserted into embryos using a meganuclease genome editing approach (Thermes *et al.* 2003). In situ hybridization (ISH) was used to identify *hs:foxm1* carriers, which were shown to have dramatically increased levels of *foxm1* mRNA after heat shock compared to wild type zebrafish (data not shown). Unexpectedly, despite the presence of this transgene and the significant increase in *foxm1* mRNA following a heat shock, little to no change was observed in either gene expression or embryonic morphology.

Embryos carrying *hs:foxm1* were heat shocked for one hour in a 39°C water bath at various time points after fertilization to determine if Foxm1 overexpression would alter the morphology of the embryos, and more specifically if it would alter the morphology of the otic vesicle, the precursor structure of the inner ear. Applying these heat shocks at 6, 9, 10, 12, and 18 hpf did not visibly alter the morphology of the embryos. In embryos heat shocked at 12 hpf and fixed at 24 hpf, ISH was used to evaluate the expression of genes known to be affected by Fgf or Wnt signals, including *pax2a, pax5, ngn1, gsc, sp5a, sp5l, etv5b,* and *top-gfp* (Ohyama *et al.* 2006, Kwak *et al.* 2006, Millimaki & Sweet *et al.* 2007, Kantarci *et al.* 2016, Weidinger *et al.* 2005, Roehl & Nüsslein-Volhard 2001, Horst *et al.* 2012). No significant differences in expression for any of these genes were observed when comparing heat-shocked *hs:foxm1* embryos to control embryos (Figure 1).

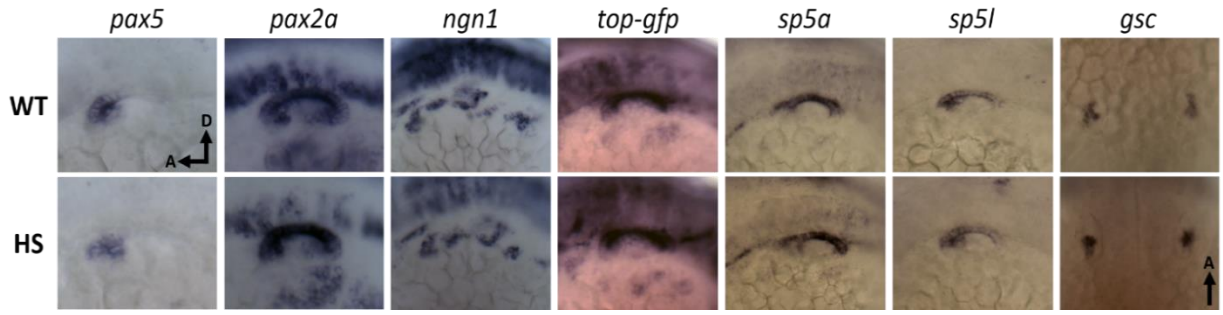

Figure 1. Effects of *hs:foxm1* on gene expression in the otic vesicle of zebrafish embryos at 24 hpf. All views are dorsolateral except *gsc* which is dorsal. All images taken at 200x magnification except *ngn1* which was taken at 100x.

The lack of perceptible effects in the initial heat shock experiments was unexpected, and indicated that some other factor may have been inhibiting the effects of overexpressed Foxm1. Two of the primary ways in which FoxM1 is regulated are by phosphorylation via MAPK, and degradation via the APC complex (Ma *et al.* 2005, Park *et al. 2008*). We hypothesized that it was possible that although Foxm1 expression had been significantly increased via the heat shock transgene, the effects of the Foxm1 protein were possibly being mitigated by one of these two regulatory mechanisms. It was possible that while Foxm1 levels were high, there was not a sufficient level of MAPK to activate the now-abundant Foxm1. It was also possible that the APC complex was able to degrade the excess Foxm1 protein before it could have an effect on development. To test these hypotheses, a series of experiments was performed utilizing the drugs BIO, IWR-1, and 2DG, as well as the heat shock transgene *hs:fgf8*. Contrary to our predictions, the results of these experiments also indicated that *hs:foxm1* did not have any effect on gene expression or morphology (Figure 2).

In the first of these experiments, the drugs BIO and IWR-1 were used. BIO prevents the APC complex from forming via inhibition of GSK3, and IWR-1 stabilizes the APC complex by

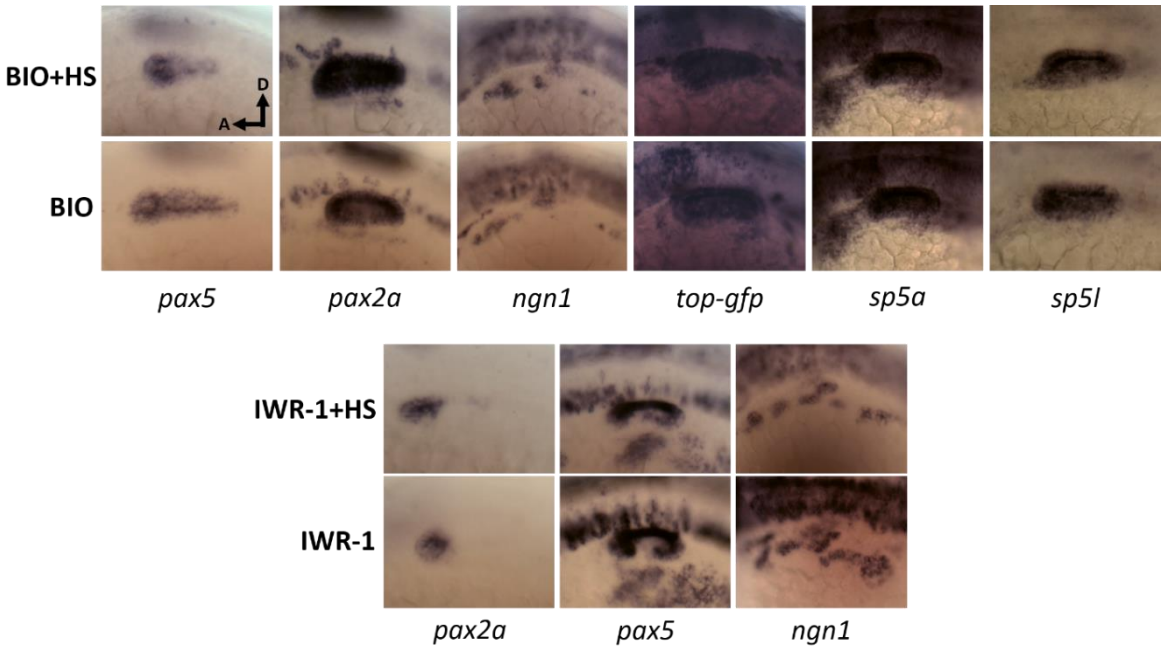

Figure 2. Effects of combining BIO or IWR-1 treatment with *hs:foxm1* on gene expression in the otic vesicle of zebrafish embryos. All views are dorsolateral, all images taken under 200x magnification.

binding to Axin. Control embryos were treated with either BIO or IWR-1 or a heat shock individually, while experimental embryos received either BIO or IWR-1 in addition to a heat shock. In all cases, BIO and IWR-1 were applied as 10 mM doses at 13 hpf immediately following heat shocks performed at 12 hpf. Gene expression did not appear to be significantly affected by overexpressed Foxm1 in these experiments, as *hs:foxm1* embryos receiving both a heat shock and drug treatment had the same levels of gene expression as the drug-only controls (Figure 2). These results indicate that activity of the APC complex is not the reason why *hs:foxm1* has no apparent effects.

Another experiment to assess the impact of the *hs:foxm1* transgene was performed in which *hs:foxm1* heterozygotes were bred with *hs:fgf8* homozygotes. It was hypothesized that if insufficient levels of MAPK activity were responsible for the lack of an effect by *hs:foxm1*, an

increase in MAPK activity caused by Fgf8 overexpression would allow overexpressed Foxm1 to become activated and have a significant effect on embryo morphology. At 6 hpf, some of the embryos received a mild heat shock, some received a dose of BIO, and some received both treatments. Due to the genotypes of the parents, all of the embryos would possess *hs:fgf8* while only half would also possess *hs:foxm1*. It was expected that combining BIO treatment with *hs:fgf8* activity would cause both APC complex inhibition and increased MAPK activation, allowing Foxm1 to have the strongest possible effect. Despite the combination of all of these factors, no difference was seen between the embryos containing both transgenes and the ones containing only *hs:fgf8*. In early trials it was observed that there was some variation in phenotype with the morphological effects on some embryos appearing milder and some more severe, but genotyping the embryos via PCR revealed that these differences were not correlated with *hs:foxm1* status. Under these extreme conditions, *hs:foxm1* still did not appear to have any discernible impact (Figure 3).

A final drug treatment experiment was performed with the glycolysis inhibitor 2DG to test the ability of the *hs:foxm1* transgene to influence Warburg metabolism. It was hypothesized that 2DG-treated embryos would perform glycolysis at a slower rate and generate less lactate for Warburg metabolism, but that overexpression of Foxm1 could allow compensation to occur by increasing the expression of Pgk1 and LDHA, thereby increasing the impact of each round of glycolysis able to be completed in the presence of 2DG. Embryos received a dose of 2DG at 14 hpf and a heat shock at 16 hpf. Embryos were fixed and imaged at 18 or 20 hpf to observe the effects of the treatments at differing time points. The genes *pax5* and *etv5b* were used as markers for changes in Warburg metabolism because both are known to be affected by Fgf signals (Kwak

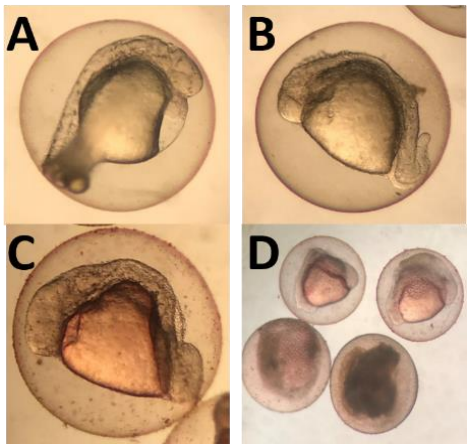

Figure 3. Effects of heat shock and BIO treatments on *hs:foxm1*(het);*hs:fgf8*(homo) zebrafish embryos. (A) HS only, a very mildly affected embryo, similar phenotype to wild type. (B) HS only, a severely affected embryo, showing a phenotype characteristic of hs:fgf8 activity. (C) HS and BIO, a severely affected embryo, similar to B. (D) HS and BIO, several dead and dying embryos as a result of combined BIO and hs:fgf8 activity.

et al. 2006, Roehl & Nüsslein-Volhard 2001). In the case of both *pax5* and *etv5b*, 2DG appeared to have fairly mild effects, with expression of both genes in control embryos returning to normal by 20 hpf. In both 2DG-treated embryos and heat shock control embryos, heat shock treatment did not appear to significantly affect the total level of gene expression for either *pax5* or *etv5b*, but it did appear to alter the pattern of *etv5b* expression; the *etv5b* staining pattern in heat-shocked embryos appeared more diffuse than in control embryos, with the three bands between the otic vesicles becoming less well-defined (data not shown).This data indicates that *hs:foxm1* does not rescue embryos from the effects of 2DG.

All of these data illustrated an apparent absence of effect from *hs:foxm1* and prompted an investigation of the transgene. Sequencing data revealed that the transgene sequence was identical to the native zebrafish *foxm1* cDNA, which shares a high degree of sequence identity with the FOXM1c splice isoform found in mammals.

Mammals possess three splice isoforms produced from *FOXM1*, which are FOXM1a, FOXM1b, and FOXM1c. They differ from each other by their possession of two additional exons, A1 and A2 (Figure 4) (Wierstra & Alves 2007, Kong *et al.* 2013). These exons encode autoinhibitory protein domains which reduce FOXM1's ability to act as a transcription factor. FOXM1a contains both A1 and A2, and has been found to be fully transcriptionally inactive. FOXM1b contains neither A1 nor A2, and has the greatest amount of transcriptional activity. FOXM1c contains only A1, and is transcriptionally active but has a much weaker effect than FOXM1b due to the autoinhibitory effects of its A1 domain. In zebrafish, the only reported Foxm1 cDNA sequence most closely matches the mammalian FOXM1c isoform. It was then hypothesized that the autoinhibitory nature of the A1 domain of FOXM1c may be why overexpressing zebrafish Foxm1 had a minimal effect, and that if a Foxm1 isoform equivalent to FOXM1b exists in zebrafish, this could be used to create a much stronger transgene.

An RT-PCR approach was used to attempt to identify the splice isoforms of Foxm1 in zebrafish. PCR primers were designed to target areas near the beginning and end of the *foxm1* cDNA (Figure 4), and thus would produce three different products if all three isoforms were

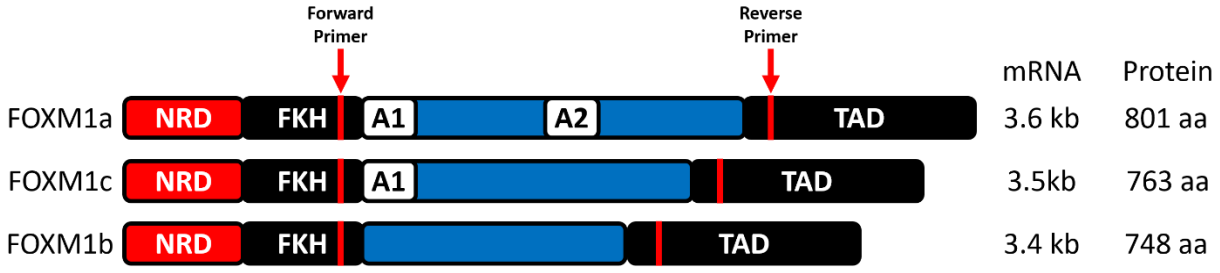

Figure 4. The three splice isoforms of FOXM1 known to exist in mammals and hypothesized to exist in zebrafish. Marked on each isoform's diagram in red is the target site of the PCR primers developed to amplify the different isoforms from zebrafish cDNA. A1 and A2: The A1 and A2 autorepression domains, NRD: N-terminal repressor domain, FKH: Forkhead DNA-binding domain, TAD: transactivation domain.

present. Gel electrophoresis following PCR revealed only one product, and this was of the expected size for the FOXM1c isoform. This result combined with the lack of any other zebrafish Foxm1 splice isoform reports in zebrafish genetic databases suggests that the FOXM1c-like isoform is likely the only one present in zebrafish.

One final future direction exists for the *hs:foxm1* transgene, which is to remake the transgene with a different genetic editing system. Our current transgene was created by using a meganuclease system, an older and less reliable method than the more efficient Tol2 system. Previous meganuclease-generated heat shock transgenes in our lab have been observed to be similar to *hs:foxm1* in that a heat shock dramatically increases transcript abundance, but causes little to no effect on gene expression. It is not clear why this occurs, but it is possible that remaking *hs:foxm1* with the Tol2 system would produce a more functional transgene with which all of the *hs:foxm1* experiments could be repeated.

Knockdown of Zebrafish Foxm1 by Morpholino Oligomers

Morpholino oligomers (MOs) are synthetic oligonucleotides that have a methylenemorpholine backbone linked by phosphorodiamidate groups rather than a ribose backbone linked by phosphate groups. They contain a sequence of RNA bases which allows them to bind to a matching pre-mRNA or mRNA molecule, and their synthetic backbone prevents splicing or translation, respectively. This backbone also makes them difficult for cellular machinery to remove, and so they are able to effectively inhibit expression of a target gene for an extended period of time (Bill *et al* 2009). A Foxm1 translation blocker MO was developed by our lab to knock down Foxm1 expression and study the effects on zebrafish embryos (Figure 5, Morpholino B), and two additional MOs were later created to verify the results of experiments performed with the initial MO (Figure 5, Morpholinos A and C).

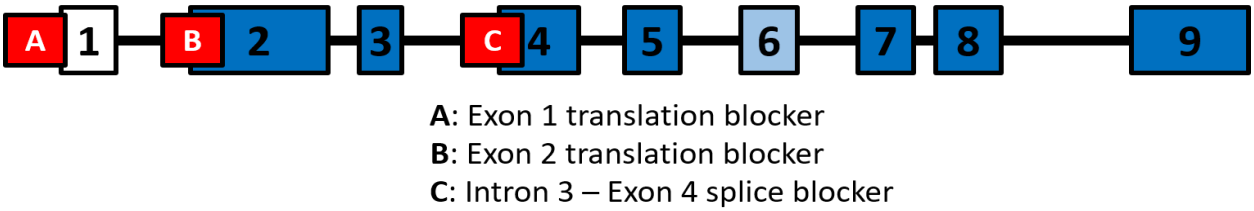

Figure 5. Morpholino oligomer target sites on *foxm1* mRNA. Morpholino B is the original morpholino used for all of the preliminary experiments. Morpholinos A and C were later used to validate the results of Morpholino B. In the *foxm1* mRNA diagram, coding exons are shown in blue, *foxm1*'s initial noncoding exon is shown in white, and exon 6 is shown in light blue to denote its status as encoding the A1 autorepression domain.

Zebrafish embryos were injected with Foxm1 MO at the one-cell stage and were evaluated for morphological changes at 24 hpf. Many of the morphants did not survive to 24 hpf, and those that did were severely dorsalized, possessing a head, an extremely shortened trunk, and a shrunken and curled tail (Figure 6). This phenotype is identical to that of zebrafish *wnt8* mutants, indicating that the likely reason for this extreme morphological effect is a disruption in Wnt signaling caused by an inability of β-catenin to translocate to the nucleus in the absence of Foxm1 (Lekven *et al.* 2001). ISH was used to examine levels of expression of *pax2a*, *pax5*, *ngn1*, *gsc*, and *pgk1* in Foxm1 morphants at 24 hpf. Expression of *pax2a*, *pax5*, *ngn1*, and *gsc* were all expressed similarly to wild type embryos aside from disruptions of their total area of expression caused by the severe morphological disruptions of the morphant. However, *pgk1* expression at 24 hpf appeared to be significantly lower than in wild type embryos, and appeared nearly absent in several of the morphants (Figure 6).

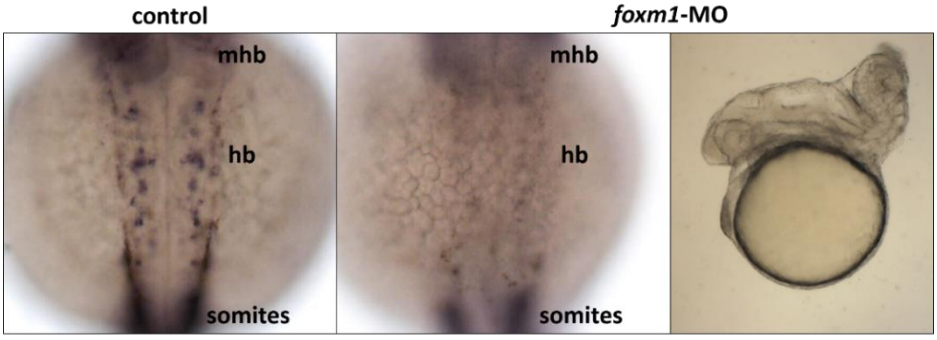

Figure 6. *pgk1* expression is dramatically reduced in *foxm1* morphants. Expression of *pgk1* was reduced or completely depleted specifically in the hindbrain region of zebrafish embryos. Morphants display similar morphological issues to *wnt8* mutants (right). ISH images taken at 24 hpf at 100x magnification. Whole-embryos image taken at 40x magnification. mhb = midbrain-hindbrain border, hb = hindbrain.

This supports prior studies which have shown Foxm1 to regulate Pgk1 expression and further supports Foxm1 knockdown as being the cause of the extreme morphological changes observed in the morphant embryos, rather than unintended off-target effects from the morpholino.

When examined at the earlier stage of 10.5 hpf, the effects of the Foxm1 MO were even more striking. Morphant embryos appeared to become ovular and elongated, which is indicative of dorsalization. ISH revealed that expression of *top-gfp*, a marker of Wnt signaling, is nearly completely absent in morphants, indicating that Foxm1 knockdown is very strongly inhibiting Wnt signals (Figure 7). The expression domain of *etv5b*, and indicator of Fgf signaling and dorsal and posterior identity, is dramatically expanded throughout the embryo, with the hindbrain expression of *etv5b* expanding fully into the ventral region, indicating that nearly the entire embryo has obtained a dorsal identity (Figure 7). This extreme early dorsalization is likely why so many of the morphant embryos do not survive to 24 hpf.

One potential issue when using morpholinos is that they will sometimes bind non-specifically to a different mRNA than that of the intended target, thus causing a misleading phenotype. For this reason, we created two additional morpholinos to validate the morpholino results we had obtained thus far (Figure 5). Both of these morpholinos targeted different regions of the *foxm1* mRNA, yet the resulting phenotypes from each of them were essentially identical to the original morphant phenotype, although there was a slightly more pronounced continuum of severity with less lethality observed. These results indicate that the phenotype is a specific effect of Foxm1 knockdown and not the result of any off-target effects.

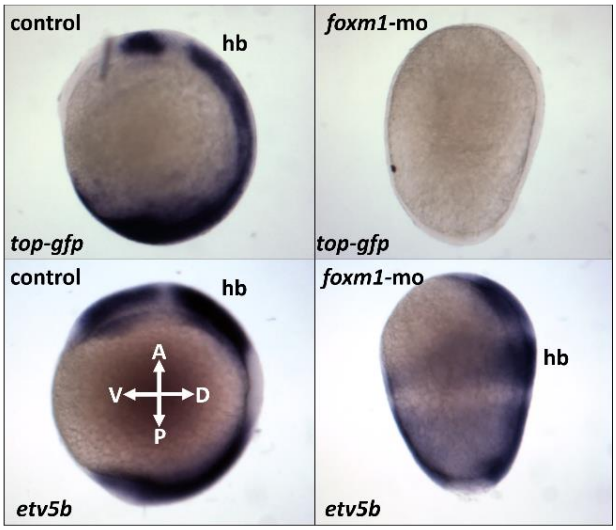

Figure 7. *top-gfp* and *etv5b* expression in *foxm1* morphants at 10.5 hpf. The elongated, ovular shape of morphants indicates dorsalization. In most morphants, top-gfp expression is entirely absent, and *etv5b* expression reveals the level of dorsalization to be so severe that hindbrain identity extends into the ventral region of the embryo. A = anterior, D = dorsal, P = posterior, V = ventral, hb = hindbrain.

Knocking Out *foxm1*

A TALEN was designed to knock out *foxm1* and eventually create a zebrafish *foxm1 -/-* mutant line. TALEN-injected founders were screened via an HpaII restriction enzyme digest assay and *foxm1 +/-* embryos were obtained. DNA sequencing revealed that the knockout allele contained a 5 bp deletion in the first coding exon of *foxm1*, predicted to cause a desired premature stop following 175 nucleotides of nonsense. When the *foxm1 +/-* fish reached maturity, they were bred to obtain *foxm1 -/-* embryos. It was predicted that the homozygous mutants would resemble the Foxm1 morphants, but these embryos appeared fully wild type both morphologically and in the expression of several genes including *pax2a, pax5, ngn1,* and *gsc* (data not shown). Through development and into maturity, these fish lacked visible defects and were not discernible from other fish except through use of the HpaII screening assay.

One potential reason for the lack of an observable *foxm1 -/-* phenotype is a maternal effect caused by the presence of maternal *foxm1* mRNA in the egg originating from the *foxm1 +/-* parent.

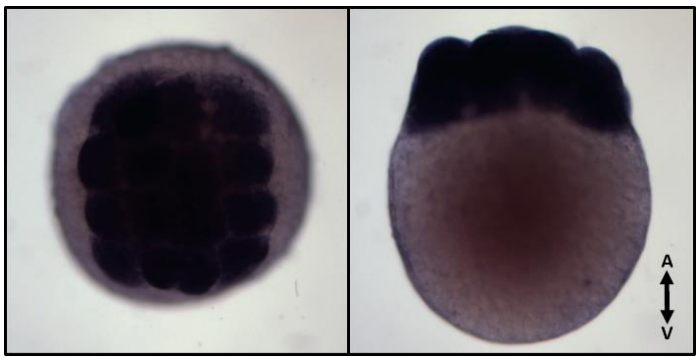

Figure 8. Maternal *foxm1* mRNA in 16-cell stage zebrafish embryos. Left: animal pole view, 40x. Right: lateral view, 40x

ISH revealed that at the 16-cell stage (prior to activation of the zygotic genome, which occurs at the 1,000-cell stage in zebrafish), zebrafish embryos contain very high levels of *foxm1* mRNA (Figure 8).

In order to eliminate this maternal mRNA as a factor that may limit the effect of the *foxm1* mutation, the progeny of a *foxm1* +/- intercross were raised to maturity and several *foxm1* -/- fish were identified. Breeding these fish should create maternal-zygotic mutants lacking any *foxm1* maternal effects. Several rounds of breeding produced many eggs, all of which failed to develop. These results are inconclusive, as it is not clear if this was due to a lack of *foxm1* or another factor. Unfortunately, the *foxm1* -/- parents in these crosses were lost, and more *foxm1* -/- fish will need to be obtained to pursue a true *foxm1* maternal-zygotic mutant.

DISCUSSION AND CONCLUSIONS

The goal of this project was to investigate the role of the transcription factor *foxm1* in otic development, including its effects on mediating Fgf and Wnt signaling during otic development as well as its function as a regulator of the Warburg effect. This study began with the use of the *hs:foxm1* transgene with the aim of observing the effects of *foxm1* overexpression on otic development, embryo morphology, and expression of Fgf and Wnt target genes. Given that *foxm1* is known to be a powerful promoter of cell division (Chen *et al.* 2013), it was expected that heat shocks of these transgenic embryos would cause profound alterations not just in otic development but also possibly in overall embryo morphology. Surprisingly, despite *foxm1* mRNA expression being observed to significantly increase, heat shock treatments of *hs:foxm1* fish did not appear to alter development in any way, regardless of heat shock time point or duration. This remained true even when other heat shock transgenes were present, such as *hs:fgf8*, or the drugs BIO, IWR-1, and 2DG were applied; the effects of *hs:fgf8* and the drug treatments were evident, but the added presence of *hs:foxm1* did not appear to have any effect. After collecting these data, the presence of *hs:foxm1* in the transgenic line was checked and confirmed by PCR and sequencing which verified that it was indeed present.

Together, the data obtained throughout experiments with the *hs:foxm1* line suggest that something must be preventing the heat-shock overexpression of this particular transgene from having an effect on development. One possibility was that the zebrafish *foxm1* cDNA used to create the transgene was of a splice isoform which contained one or more exons encoding autoinhibitory domains which would cause the Foxm1 protein to inhibit its own function. Sequencing of the *hs:foxm1* transgene revealed that it was identical in sequence to the only

previously reported zebrafish *foxm1* cDNA sequence. This cDNA sequence has a high degree of identity with the human FOXM1c splice isoform, which does indeed encode an autoinhibitory domain in its A1 exon. The human FOXM1b splice isoform contains no such autoinhibitory domains and so an equivalent isoform in zebrafish would potentially allow for creation of a far stronger heat shock-inducible transgene. An RT-PCR experiment was conducted to find and identify other splice isoforms of Foxm1 in zebrafish, but unfortunately none besides the FOXM1c-like isoform were found.

Based on these comparisons to human data, it may be possible that the lack of effect from *hs:foxm1* can be explained by the presence of an autoinhibitory domain. However, while the zebrafish *foxm1* and human FOXM1c sequences have a high degree of identity, they are not totally identical, and they have a number of differences in sequence content and length in the region of this A1 exon/autoinhibitory domain. It is possible then that perhaps zebrafish *foxm1* does not actually exert an autoinhibitory effect or that the effect is not strong enough to suppress the effects of heat-shock overexpression. It may be the case that the failure of *hs:foxm1* to elicit developmental changes may be for another reason, such as the nature of the meganuclease system by which this transgene was created. It is unclear how this would be occurring, but other transgenes created by our lab with this system in the past have demonstrated similar issues to those of *hs:foxm1*, with mRNA overexpression being visible while all other expected effects are weak or absent. When new versions of these transgenes were later made using the newer and more efficient Tol2 system, their effects were immediately visible and the issues were resolved. In continuing the investigation of the effects of *foxm1* overexpression, remaking the transgene with the Tol2 system or another newer genetic editing tool will likely be necessary.

In addition to attempts to overexpress *foxm1*, knockdown experiments were performed through use of morpholino oligomers. The results of these experiments support many of the hypotheses made about the role of *foxm1*. The dorsalized morphology of the morphants (resembling *wnt8* mutants) and the changes in *top-gfp* and *etv5b* expression demonstrate that *foxm1* is a mediator of Wnt and Fgf signaling. The reduction in *pgk1* expression in the area closest to the otic vesicle but not in the somites indicates not only that *foxm1* is regulating the Warburg effect, but that this is occurring in a specific manner and in a specific area, which is supportive of our hypotheses about the role of *foxm1* in relation to otic development. To verify that these observations were due specifically to knockdown of *foxm1* and not unintended off-target effects, two additional MOs were tested and found to have nearly identical effects to the first MO. These results provide strong support for the observed morphant phenotype being a specific consequence of *foxm1* knockdown.

Although matching results were obtained with three different MOs, further verification of the observed phenotype by use of a mutant line was necessary due to the troubling number of instances in which morphant and mutant phenotypes do not resemble one another (Kok *et al.* 2015). A TALEN was created to induce a knockout mutation in *foxm1*, and the presence of this mutation in embryos was confirmed by both a restriction enzyme assay and sequencing. Despite the fact that this mutation (a 5 bp deletion) caused a frame shift and should have led to a fairly early premature stop in translation, embryos homozygous for the mutation did not appear to have any developmental defects whatsoever. This was unexpected, and it was hypothesized that this may be due to a possible rescue effect from the high amount of *foxm1* maternal mRNA that was observed to be present in early stage embryos. Crosses of *foxm1* -/- fish were performed in an effort to obtain maternal-zygotic mutants, but all of the embryos from these crosses failed to

develop. This failure of development may have been due to the lack of maternal *foxm1* or some other unknown factor, but the parent fish were lost before this could be investigated further.

In order to continue this study of *foxm1* and its role in otic development, several projects mentioned previously must be pursued. The first of these is that a new heat shock transgene must be made with a newer, more efficient genetic editing tool. This new transgene will reveal whether the issues with *hs:foxm1* encountered in this study were due to the effects of the autoinhibitory domain of the Foxm1 protein or due to unknown issues with the insertion of the transgene. More importantly, this new transgene will allow for the true effects of *foxm1* overexpression to be investigated.

In regard to the *foxm1* mutant line, the first task will be to obtain more *foxm1* -/- fish with which to produce more maternal-zygotic mutants. If the developmental issues of these mutants are observed throughout these new crosses, a different approach may be needed to observe the effects of *foxm1* knockout. One such approach would be the creation of a floxed allele for *foxm1*. A floxed allele, by allowing for temporally-controlled knockout of *foxm1*, would allow embryos to survive past the earliest and most critical stages of development before losing the gene.

Despite sequencing data for the TALEN-generated *foxm1* mutation indicating a frame shift and premature stop, the mutation is not causing the developmental defects that would be expected. It is unclear why this is occurring, but one possibility is that there is another transcription factor present which may be compensating for the loss of *foxm1*, activated either by signaling changes that emerge as a consequence of a loss of *foxm1* or by the effects of nonsense-mediated decay of the mutant *foxm1* mRNA. It is also possible that this lack of an effect from the *foxm1* mutation is due to some other unknown reason, and the creation of a new knockout mutation for comparison may aid in clarifying the puzzling nature of the current knockout.

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