INVESTIGATING DIFFERING DEGREES OF FOXO3A EXPRESSION IN ADULT NEURAL STEM CELLS BETWEEN AGE GROUPS IN ZEBRAFISH

By

Francis Griffin Bacik

A Thesis Presented to

The Faculty of Humboldt State University

In Partial Fulfillment of the Requirements for the Degree

Master of Arts in Psychology: Academic Research

Committee Membership

Dr. Ethan B. Gahtan, Committee Chair

Dr. Amanda Hahn, Committee Member

Dr. Andrew Kinziger, Committee Member

Dr. Chris Aberson, Program Graduate Coordinator

May 2018

Abstract

INVESTIGATING DIFFERING DEGREES OF FOXO3A EXPRESSION IN ADULT NEURAL STEM CELLS BETWEEN AGE GROUPS IN ZEBRAFISH

Francis Griffin Bacik

One factor influencing organismal longevity is the activity of transcription factors of the "fork head domain" family, otherwise known as "Forkhead box" (Fox) proteins. Studies of the four isoforms of the "O" subclass of Fox proteins found in human genes have revealed a direct relationship between FoxO3a-dependent gene expression and the conservation of neural stem cell (NSC) in the adult brain, specifically in the subgranular zone of the dentate gyrus in the hippocampus and the subventricular zone of the cortex. This transcription factor has also been shown to regulate apoptosis in nervous tissue during embryonic development in zebrafish. The current study aimed to investigate the differences in FoxO3a expression in adult zebrafish across two different age groups. The primary hypothesis of this study was that a significant positive correlation would be found between expression of FoxO3a in the medial pallium of the brain and progressive organismal aging. Tissue samples from the MP of one- and two-year-old zebrafish were dissected and cDNA was isolated for analyses via qPCR and Western Blot. The resulting data showed no significant difference in FoxO3a expression between younger and older fish.

Acknowledgements

First and foremost, I would like to thank Dr. Ethan Gahtan for serving as my research supervisor and graduate advisor throughout the course of my time in the Psychology Department's Academic Research Master's Program. His feedback on the various early drafts of this paper and honest critiques of the proposed experimental designs which preceded those drafts have proven to be invaluable to the development of the current proposal.

I also wish to extend my gratitude towards the faculty of the Biology Department's Core Facilities and its director Dr. David Baston for providing the necessary funds and resources for this study. I sincerely hope the findings of my work prove to have been worth their much appreciated investment.

Keely White, the undergraduate student who assisted me throughout the course of the study, deserves special recognition for her contributions. Most notably, her careful skill in dissecting specific brain regions of interest from our zebrafish specimens proved invaluable to the study.

Lastly, I wish to dedicate this paper and its findings to the memory of my late friend Carina "Moony" Fink, who passed away July 18th, 2017. Their emotional support in the early stages of this study kept me motivated to continue on through numerous setbacks. I would not have been able to see this to the end without them, and I only hope my contributions to the field of neuroscience prove fruitful in the neuronal health and preservations of lives as precious as theirs.

Table of Contents

List of Tables

List of Figures

Introdction

Organismal longevity and the determining factors thereof have long been hot topics of inquiry and investigation within the life science community. All known forms of life are adapted over the course of millions of years to survive as long as their environments and physiology will allow for the purpose of passing their genetic material on to the next generation and propagating their respective species. After an organism reaches reproductive maturity, the mechanisms of metabolic and proliferative homeostasis responsible for sustaining said organism up to that point become gradually less efficient over time (Wang, Karpac, & Jasper, 2014). The debilitating effects of aging on an individual organism have been attributed to a number of genetic factors relating to each major system of organismal homeostasis. The primary focus of this study is an isoform of the "forkhead box" (Fox) transcription factor family known as "FoxO3", which has been linked to several homeostatic functions within the body, including longevity. The capacity for modern medicine to influence or manipulate these factors with the goal of prolonging an organism's predicted lifespan serves as the basis behind much longevity research (Cabreiro et al., 2011; Richardson, Galvan, Lin, & Oddo, 2015; Simonsen, Cumming, Brech, Isakson, Schubert, & Finley, 2008).

Stem cells and cellular regeneration processes are key mechanisms of FoxO3's effect on longevity. By definition, stem cells are non-differentiated pluripotent cells found within embryonic and adult organisms (Ulloa-Montoya, Verfaillie, & Hu, 2005). These cells have the capacity to self-renew themselves and adopt the genetic qualities of their surrounding cells, differentiating into cells of that type. Adult stem cells, while

sharing many qualities with their embryonic counterparts, differ primarily in their capacity for differentiation, being somewhat specialized to promote the genesis of specific cells within the body as needed whereas embryonic stem cells may differentiate into any cell type (pluripotency). Adult neural stem cells (NSCs) are one such example of these semi-specialized stem cells. In mammals, adult neural stem cells are found in the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus and in the subventricular zone (SVZ) of the cerebral cortex, and they gradually proliferate into nervous system-specific cell lineages including neurons, astrocytes, and oligodendrocytes, at a decreasing rate throughout the adult lifespan.

Although neuronal cell proliferation ceases in most regions of the brain upon reaching a certain age, the hippocampus and cortex continue to undergo neurogenesis (the production of new nervous system-specific cells) even after this point (Weissman, Anderson, & Gage, 2001). This has been attributed to the pool of NSCs in the hippocampus and cortex of the mammalian brain (Renault et al., 2009). Over time, this pool gradually depletes as the organism ages and neurogenesis becomes more and more necessary for systemic homeostasis. The degree of age/disease/trauma-induced neurodegeneration determines the degree of NSCs depleted at any given time, as FoxO regulates said depletion in response to neuronal cell death. In cases of brain injury such as stroke, total NSC count has been found to increase in number, which indicates that depletion of the NSC pool (and in effect the recovery of damaged nervous tissue) increases in response to major cell death in the central nervous system (CNS; Zhang et al., 2014).

Many transgenic animal models are used to investigate factors influencing longevity, including zebrafish (*Danio rerio*; Keller, & Murtha, 2004). While sharing fewer physiological traits with humans than other mammals, zebrafish have unique organismal qualities that could be applied to study questions about how FoxO influences longevity, including but not limited to their genetic tractability, transparency during embryonic development, and relative ease of rearing and testing in mass numbers. Zebrafish and humans share many common genes, including a variant of the FoxO gene known as FoxO3a, but homologies in physiological systems are generally more difficult to assess. Therefore, before proposing to study FoxO3a mechanisms in zebrafish to learn principles that may apply to human, it is important to first determine whether Fox3a even has the same relationship with age in zebrafish as seen in humans, namely, an increase in expression level with increased age.

FoxO3-Mediated Adult Neural Stem Cell Proliferation

Among the functions distinct to FoxO is homeostasis of adult NSCs through FoxO regulated gene expression. In an experimental study of the effects of FoxO3 manipulation in mice (Renault et al., 2009), brain sections of adult $FoxO3^{-/-}$ (knocked-out FoxO3 expression) and $FoxO3^{+/+}$ (wild-type) mice were stained with antibodies that recognized FoxO3 (but not FoxO1, FoxO4 or FoxO6 [significantly]) and Sox2 (a marker of NSCs/neural progenitors) and NeuN (a marker of neurons). FoxO3 was expressed in both the SGZ of the DG and in the SVZ of the cortex (Kaslin, Ganz, & Brand, 2008). Expression of FoxO3 was also detected in high levels from in vivo NSC niches and Sox2-positive cells in the SGZ and the SVZ (in a subset of NeuN negative cells and in

cells cointaining bromodeoxyuridine (BrdU), a genetic stain indicative of proliferation). Isolation of NSCs from DG and SVZ regions of postnatal and adult mouse brains and immunostaining on whole/dissociated NSCs revealed that FoxO3 is expressed in NSCs/progenitors derived from both postnatal and adult animals and is co-expressed with Nestin (a NSC/progenitor marker). Western blotting on protein extracts of neurospheres (systems of free-floating neural stem cell clusters) from NSCs of mice at various age milestones revealed expression of FoxO3 in NCSs and progenitors from every age group investigated, adult mice showing the highest degree of expression. This indicated that FoxO3 is expressed in adult NSCs and neural progenitors *in vivo* and *in vitro*.

Gene Expression in Self-Renewing NSCs

In a comparison of the phosphorylation status of FoxO3 in self-renewing versus differentiating adult NSCs via western blotting with phosphor-specific antibodies to Threonine 32 (T32), a larger percentage of FoxO3 was phosphorylated at T32 in differentiated progeny than in self-renewing NSCs (Renault et al., 2009). A luciferase assay using a FoxO reporter gene (to test the activity of endogenous FoxO3 in NSCs) revealed that the reporter gene was active in adult $FoxO3^{+/+}$ NSCs and slightly less active in $FoxO3^{-/-}$ NSCs (the remainder of which was likely due to partial compensation by other FoxO family members). This provided evidence of higher FoxO3 activity in self-renewing NSCs as opposed to differentiated progeny.

To determine whether the ablation of FoxO3 results in a decrease in NSC number *in vivo*, daily BrdU injections were given to adult $FoxO3^{+/+}$ mice and adult $FoxO3^{-/-}$

mice (viable and normal in outward appearance but prone to cancer and only live 1 – 1.5 years) for one week. When mice were sacrificed 1 month after injection, $FoxO3^{-/-}$ mice showed a significant reduction in the number of label-retaining NSCs compared to $FoxO3^{+/+}$ mice in both the SGZ and SVZ. When mice were sacrificed 1 day after injection, $FoxO3^{-/-}$ mice tended to have more BrdU-positive cells than $FoxO3^{+/+}$ mice in the SGZ and SVZ. Brains from adult $FoxO3^{-/-}$ mice were significantly heavier than brains of wild-type counterparts (both groups having similar weights at birth), indicating that inexpression of FoxO3 impairs natural regulation of NSC function, resulting in increased neuronal proliferation in the CNS.

Neurosphere Formation

In yet another experiment (Renault et al., 2009), NSCs from adult $FoxO3^{-/-}$ and $FoxO3^{+/+}$ mice were isolated and their ability to form primary (first-stage) neurospheres was tested. NSCs from adult $FoxO3^{-/-}$ mice formed primary neurospheres at a significantly lower frequency than NSCs from $FoxO3^{+/+}$ littermates. $FoxO3^{-/-}$ NSCs isolated from embryos/1-day-old mice formed primary neurospheres with the same frequency as $FoxO3^{+/+}$ NSCs. $FoxO3^{-/-}$ NSCs isolated from young adult/middle-aged mice formed neurospheres at a significantly lower frequency than $FoxO3^{+/+}$ NSCs, indicating that the absence of FoxO3 leads to depletion of NSCs in adult mice.

To determine whether FoxO3 is necessary for NSC self-renewal, primary neurospheres from $FoxO3^{-/-}$ and $FoxO3^{+/+}$ were dissociated and tested on their ability to form secondary neurospheres. $FoxO3^{-/-}$ NSCs from neonates formed secondary

neurospheres at the same frequency at $FoxO3^{+/+}$ NSCs. $FoxO3^{-/-}$ NSCs isolated from young/middle-aged adults generated fewer secondary neurospheres than $FoxO3^{+/+}$ NSCs. Concomitant deletion of FoxO1, FoxO3 and FoxO4 resulted in defects in neurosphere formation upon serial passage in culture. This proved the existence of functional redundancy among FoxO family members in cultured NSCs.

NSC Differentiation

Additional findings suggested that the ability of NSCs to generate different neural lineages is defective in the absence of FoxO3 (Renault et al., 2009). Progeny of $FoxO3^{-/-}$ and $FoxO3^{+/+}$ secondary neurospheres were examined after 7 days in differentiation conditions. Neurospheres generated from adult NSCs contained mainly astrocytes. Functionality was measured by scoring the number of differentiated secondary neurospheres formed at low cell density containing at least one immature neuron or one immature ogliodendrocyte. NSCs isolated from neonate $FoxO3^{-/-}$ and $FoxO3^{+/+}$ mice formed similar numbers of functional neurospheres while those isolated from young/middle-aged adult $FoxO3^{-/-}$ mice formed significantly fewer secondary neurospheres containing ogliodendrocytes than those isolated from $FoxO3^{+/+}$ mice. $FoxO3^{-/-}$ NSCs from idle-aged mice tended to form fewer neurospheres containing both neurons and astrocytes and more neurospheres containing only astrocytes than $FoxO3^{+/+}$ NSCs. Similar results were obtained when differentiated neurospheres were stained for all three cell fates (neurons, astrocytes and oligoodendrocytes) simultaneously.

NSC Depletion/Survival

Another experiment was designed to determine whether FoxO3 acts in the nervous system to regulate the NSC pool (Renault et al., 2009). $FoxO3^{lox/lox}$ mice were crossed with *Nestin-Cre* transgenic mice (which express the Cre recombinase in NSCs/progenitors from embryonic day 10.5). Cross mice displayed an ablation of FoxO3 protein in the brain but NOT in the majority of other tissues. Young/middle-aged adult cross mice had significantly heavier brains than control siblings. This difference in brain weight resulted from the impairment of FoxO3-mediated NSC depletion in cross mice. Extraction of BrdU-retaining cells from 3-month-old cross mice and control ($FoxO3^{lox/lox}$) siblings one month after a seven-day period of label injection revealed that young cross mice tended to have fewer label-retaining NSCs in the SVZ and SGZ than control siblings (but to NO statistical significance). The effects of FoxO3 loss in vivo were less pronounced in cross mice than control mice. NSCs isolated from cross mice had no FoxO3 protein expression for up to four western blot passages, and NSCs isolated from 9-month-old cross mice displayed significant defects in their ability to form primary neurospheres compared to NSCs from control littermates.

FoxO3 was also found to regulate quiescence and survival of NSCs in another experimental design (Renault et al., 2009). Cells dissociated from secondary neurospheres from young adult $FoxO3^{-/-}$ mice displayed a significant increase in BrdU incorporation in culture in comparision to cells from $FoxO3^{+/+}$ mice. Freshly isolated NSCs from adult $FoxO3^{-/-}$ mice displayed an increased number of cleaved caspase 3-

positive cells in comparison to $FoxO3^{+/+}$ NSCs. The level of apoptosis for cells dissociated from secondary $FoxO3^{-/-}$ neurospheres was lower than for cells dissociated from $FoxO3^{+/+}$ neurospheres. This supports the hypothesis that cell death is not a major consequence of FoxO3 loss. Additionally, that loss of quiescence may play a more prevalent role in NSC depletion *in vivo* in $FoxO3^{-/-}$ mice.

Moderation of Multiple Genes

To determine how and if FoxO3 coordinates the expression of a specific program of genes in NSCs, a genome-wide microarray analysis was performed on RNA isolated from two independent biological replicates (each in duplicate) from young adult $FoxO3^{-/-}$ and $FoxO3^{+/+}$ secondary neurospheres (Renault et al., 2009). Expression of a specific subset of genes was decreased in $FoxO3^{-/-}$ neurospheres in comparison to $FoxO3^{+/+}$ neurospheres (validated by reverse transcription followed by quantitative polymerase chain reaction). FoxO3-regulated genes were significantly enriched for genes that form a molecular signature for quiescence. FoxO3 was necessary for the expression of genes involved in cell quiescence and oxidative stress resistance, upregulation of genes involved in early neurogenesis, and inhibition of premature NSC differentiation. Microarray data comparisons with genes known to change with human aging indicated a correlation between FoxO3-regulated genes and genes regulated during aging in the brain.

Electrophoretic mobility shift assays (EMSA) conducted in NSCs revealed that FoxO3 could bind in vitro to FoxO binding sites present in regulatory regions of *Ddit4*,

Ndrg1, and *Otx2* (implied here to be direct targets of FoxO3; Renault et al., 2009). Chromatin immunoprecipitation (ChIP) conducted in NSCs revealed that FoxO3 is recruited to FoxO binding sites in the promotors of $p27^{KIP1}$ (implied here to be a cell cycle inhibitor involved in cell quiescence) and *Ddit4* (a known target of hypoxia-inducible factor 1) genes, indicating said genes to be direct targets of FoxO3 in NSCs. FoxO3 was NOT bound at the promotors of *Ndrg1*, and *Otx2* by ChIP, suggesting that these genes may NOT be direct targets of FoxO3 in NSCs. Lastly, after identifying FoxO3 directed targets in NSCs, $FoxO3^{-/-}$ and $FoxO3^{+/+}$ NSCs were compared on their ability to form neurospheres in low-oxygen (2%) versus atmospheric oxygen (20%) conditions. Adult NSCs showed increased ability to form neurospheres in low oxygen in comparison to atmospheric oxygen. $FoxO3^{-/-}$ NSCs did NOT display an increased ability to form neurospheres in low-oxygen in comparison to atmospheric oxygen. An alternative explanation for this result is that 2% oxygen (which mimics in vivo physiological oxygen concentrations in the mammalian brain) helps reveal differences between $FoxO3^{-/-}$ and $FoxO3^{+/+}$ NSCs.

FoxO3a-Mediated Embryonic Neuronal Apoptosis

Approximately half of the neuronal cells produced through embryogenesis in vertebrate animals undergo programmed cell death (apoptosis). Activation of FoxO3a (a subclass of the FoxO gene) in neurons cultured in neurotrophic factor-deprived medium results in apoptotic reactions in select populations of neurons. A collaborative study was conducted by Sun Yat-sen and Peking University in China to determine the role(s) of

FoxO3a during embryogenesis in AB wild-type zebrafish by inducing morpholino-mediated knockdown of FoxO3a (Peng et al., 2007). The expression pattern of FoxO3a in zebrafish was identified and used as a reference point to analyze FoxO3a's physiological effects throughout embryonic development.

In situ hybridization was performed at select intervals during embryonic development in zebrafish. FoxO3a morpholino antisense oligonucleotides (FoxO3a-MO) was used to induce "loss-of-function" in FoxO3. Truncated green fluorescent protein fusion constructs were singly and simultaneously microinjected into the embryos. Acridine orange (AO) was used to label dying cells in zebrafish embryos. The effects of FoxO3a inhibition on the expression of select neural markers were examined at 9 hours post-fertilization. Quantitative polymerase chain reaction was then used to detect the expression of select genes and neural markers.

In situ hybridization revealed gradually increasing expression of FoxO3a in the nervous systems of zebrafish throughout embryonic development. Injection of FoxO3a-MO in developing embryos resulted in abnormal head morphology and sever brain shape abnormalities. A general increase in the number of AO-labeled cells was observed in embryos injected with FoxO3a-MO. FoxO3a knockdown via FoxO3a-MO injection resulted in a mild reduction in the expression of otx2 and pax2.1 genes, but no difference was found in the expression of hoxb2b. Transcripts of all neural markers of interest were significantly decreased in embryos injected with FoxO3a-MO. It was concluded that FoxO3a activity plays a significant role in the maintenance of normal neurodevelopment

during zebrafish embryonic development. Furthermore, FoxO3a loss-of-function does not affect neurogenesis induction but does lead to defects in neural development which might be mediated by suppressive expression of neural markers and increased apoptosis during late embryogenesis.

Up-Regulation of FoxO3a Expression with Progressive Organismal Aging

Building upon the foundation of Renault's 2009 study on the physiological effects of FoxO3a knockout, a new study by the Universities of Mosul and Zakho in Iraq set out to determine the degree of expression across different generations of albino mice (Mustafa, Salih, & Waheed, 2013). The mice were first categorized into five different age groups, including fetuses (E17), newborns (3 days old), young (1 month), adults (6 months) and aged (18 months), three mice per group. The mice were euthanized by means of cervical dislocation, their brains removed and transferred to pre-warmed CO_2-bubbled artificial cerebrospinal fluid before being cut into small pieces and gradually processed down to neurosphere clusters by means of fire polished Pasteur pipetting, centrifuging, and incubation.

Neurosphere samples from all five age groups were put through cell lysis and their RNA isolated via total RNA mini kit before conversion to cDNA via AccuPower® RocketScriptTM RT Premix lyophilized mastermix. Real-time polymerase chain reaction (RT-PCR) was then performed using FoxO3 and β-actin primers (the later serving as a reference gene) on the cDNA samples and the expression of FoxO3 was evaluated through the $2^{-\Delta\Delta CT}$ method, in which C_T indicates threshold cycle, ΔC_T (test) = C_T

(target, test) – C_T (ref, test), ΔC_T (calibrator) = C_T (target, calibrator) – C_T (ref, calibrator), $\Delta\Delta C_T$ = ΔC_T (test) – ΔC_T (calibrator), and $2^{-\Delta\Delta CT}$ = normalized expression ratio. The resulting data was then subjected to a one-way ANOVA test to determine statistical means ± standard error.

Analysis of RT-PCR data showed that FoxO3 was not only expressed in every age group studied, but the degree of FoxO3 expression increased significantly with progressive aging, embryonic and newborn mouse samples serving as the baseline. Young mice (1 month old) showed a 1.8-fold increase in FoxO3 expression compared to the baseline, adult mice (6 months old) showed a 2.4-fold increase, and aged mice (18 months old) showed a 5-fold increase. These findings strengthen the proposed implications of the Renault study (2009); firstly that FoxO3 is necessary for normal NSC functionality and homeostasis, and secondly that FoxO3 is responsible for normal up-regulation of genes responsible for developmental neurogenesis.

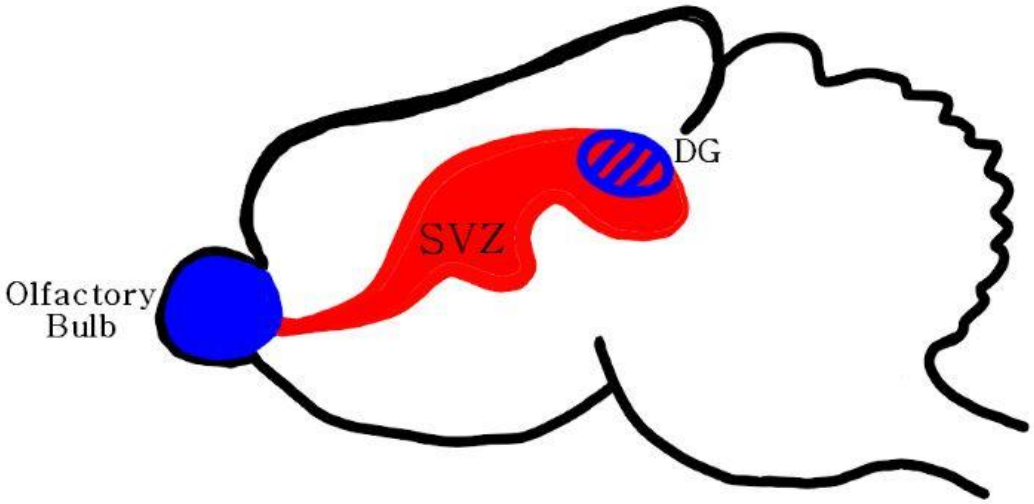

Figure 1. The regions highlighted in blue (the dentate gyrus of the hippocampus and the olfactory bulb) are sites of neurogenesis in the adult mammalian brain, while the region highlighted in red (the subventricuilar zone of the cerebral cortex) is responsible for neural cell proliferation or specialization into specific neural cell types.

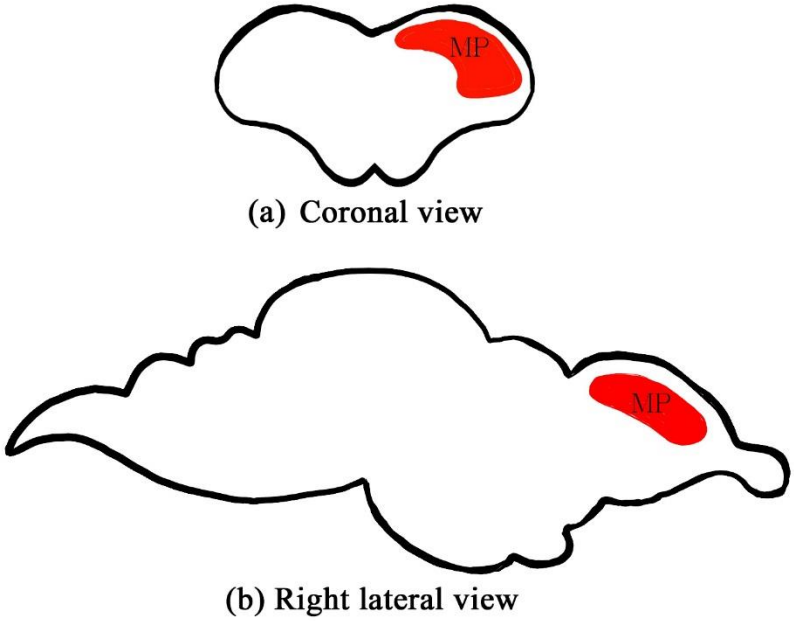

Figure 2. Front coronal (a) and lateral (b) views of the adult zebrafish brain highlighting the left medial pallium (red), a region structurally and developmentally homologous to the lateral areas of the mammalian dorsal telencephalon.

Statement of the Problem

A direct relationship has been found between FoxO3-dependent gene expression and the homeostasis of NSCs (Renault et al., 2009). Knocking out expression of the FoxO3 gene leads to more rapid depletion of the adult NSCs, which decrease rates of neurogenesis. Studies of naturally-occurring FoxO3 expression in mouse NSCs has revealed a general trend of increased expression over time, with adults showing higher expression levels than embryonic or newborn mice (Mustafa et al., 2013). Expression of FoxO3a in rat duodenum, an organ with high rates of cell proliferation, has also been shown to increase with age (Huang, Zhou, Huang, Zhou, Wei, & Shi, 2011). These studies suggest that FoxO3 is an important regulator of cell proliferation generally, including adult neurogenesis. However, the mechanisms through which Fox03a expression regulate proliferation and longevity are still not well understood. The current study was the first, to the author's best knowledge, to investigate the relationship between Fox03a expression and age in zebrafish. This is significant because if the same relationship between Fox03a expression and age is found in zebrafish as has been shown in rodents that would validate future investigation of Fox03a mechanisms in zebrafish, utilizing the unique technical advantages of this model system, to reveal generalizable principles of Fox3a actions. This in turn may promote advancements in age-specific treatments against neurodegenerative diseases.

Hypothesis

FoxO3a expression in the MP as measured by quantitative PCR will be greater in older zebrafish, with significant group differences predicted as follows: 2 year old > 1 year old.

Rationale for Hypothesis

In zebrafish, FoxO3a is gradually confined to the forebrain, midbrain, midbrain-hindbrain boundary and hindbrain throughout embryogenesis, during which it serves to regulate healthy developmental apoptosis in these regions (Peng et al., 2010). Furthermore, correlations between progressive organismal aging and the expression of metabolic/regulatory genes in the nervous system have been confirmed to exist in zebrafish through previous qRT-PCR studies (Menezes, Kist, Bogo, Bonan, & Da Silva, 2015).

Summary of Research Purpose

The aforementioned metabolic functions of FoxO3a have captured the interest of those within the scientific community conducting research into organismal longevity and disease treatment. As previously stated, zebrafish serve as a popular model for research into the mechanisms of neurodegenerative diseases such as Parkinson's due to the genetic and physiological parallels between their central nervous systems and those of humans (Babin, Goizet, & Raldúa, 2014). If FoxO3a has similar effects on neuronal stem cells and brain function in zebrafish and humans and shows similar relationships with age, that provides a basis of evidence for subsequent studies that use zebrafish to study additional aspects and possible therapeutic applications of FoxO3a mechanisms.

Method

Subjects and Design

This study aimed to determine FoxO3 expression levels in the forebrains of wild-type zebrafish at two different stages of adulthood. A two-part pseudo-experimental design was used to investigate this expression. The first study examined FoxO3a expression between the forebrains and hindbrains of five two-year-old zebrafish. The second study introduced "Age Group" as a non-manipulated independent variable analyzed at 2 levels: young adult (1 year old) and old adult (2 years old; Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995). The sample size was limited to 15 by the available resources for RNA extraction (explained further in Discussion section), so 8 young and 7 old fish were tested for forebrain Fox03 expression. For each fish from each group, FoxO3a expression in the forebrains was measured via q-PCR. A FoxO3a-specific RNA probe was used for q-PCR in accordance with published protocols (Peng et al., 2010). Various materials and pieces of analytical equipment were considered for the procedures necessary to isolate the gene and measure its expression, but ultimately the most cost-effective option was found in RNA extraction/cDNA synthesis kits offered by Bio-Rad.

Materials

As this study called for the analysis of a specific target gene, multiple steps were required to break down the necessary genetic material and isolate the gene of interest. To these ends, three specialized kits were selected from Bio-Rad Laboratories to prepare tissue samples for q-PCR. For isolation of RNA from dissected nervous tissue, the Aurum™ Total RNA Fatty and Fibrous Tissue Kit (containing PureZOL, DNase I, total

RNA wash low and high-stringency solutions and total RNA elution solution) was selected. To synthesize cDNA from the isolated RNA, an iScript™ cDNA Synthesis Kit (containing 5x iScript reaction mix, iScript reverse transcriptase and nuclease-free water) was purchased from the same distributor. Lastly, Bio-Rad's iTaq™ Universal Probes Supermix was purchased along with forward and reverse FoxO3a primers and a ROX-based flurogenic probe to prepare cDNA samples for q-PCR analysis.

Apparatus

A binocular dissecting microscope was necessary for the dissection and regional separation of zebrafish brains for use in this study. Preparation of genetic samples from dissected brain tissue required a tabletop Eppendorf centrifuge, fume hood, and a large-capacity refrigerated centrifuge. Assessment of RNA and cDNA concentrations for calculation of appropriate concentrations of samples to be used for q-PCR was determined by means of a Thermo Fisher NanoDrop™ Lite Spectrophotometer. Agarose gel electrophoresis for Western blotting of cDNA samples was prepared with a Bio-Rad Wide Mini-Sub® Cell GT Horizontal Electrophoresis System and PowerPac™ Basic Power Supply. Western blot analysis of agarose gel required the use of a LI-COR 4300 DNA Analysis System and its accompanying software. Finally, the q-PCR analysis required a Thermo Fisher Applied Biosystems 7300 Real-Time PCR System and its accompanying software.

Procedure

Animal care (Week 1, 2). Two fish broods representing the previously specified age groups (one year and two years old) were obtained from the laboratory of Dr. Ethan

Gahtan of Humboldt State University's Psychology Department and transported to the Biology Department's CORE Research Facility. The fish themselves were housed and reared in accordance with published protocols (Jorgensen, Morthorst, Andersen, Rasmussen, & Bjerregaard, 2008; Maack, & Segner, 2003).

Brain dissection (Week 1, 2). Fish were euthanized in an ice water bath before decapitation. Death was verified by an observed cease in opercular movement for a period no less than 10 minutes. Fish were then placed in Petri dishes of PBS to prevent drying and their telencephalons dissected and stored in microcentrifuge tubes until RNA extraction. Tissue samples were collected in equal volume between groups before being stored and frozen in a -80°C laboratory freezer with group-specific labels and categorized into "Young" and "Old" sample groups before RNA extraction.

RNA extraction and cDNA synthesis (Week 3, 4, 5). Tissue samples were washed in PBS prior to use in accordance with published protocols (Lan, Tang, San Leong, & Love, 2009) after being transferred from storage to a workbench on ice. Each sample was treated with Bio-Rad's PureZOL™ RNA Isolation Reagent to deproteinate RNA and left to incubate at room temperature for 15 minutes before being homogenized. This process was repeated for each brain tissue sample in the age group. After thawing the samples on ice, RNA was isolated with DNase I, high and low-stringency wash solutions and other reagents from Bio-Rad's Aurum™ Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad, Hercules, CA, USA) and measured for RNA concentration by means of nanodrop, and cDNA for reverse transcription was synthesized with Bio-Rad's iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Expression of FoxO3a

was measured in units of cycle threshold (C_T) using Applied Biosystems 7300 Real-Time PCR System Software (Menezes et al., 2015). q-PCR was repeated 3 times suing different cDNA concentrations in order to determine the optimal cDNA concentration for this procedure (0.0011 ng/µl).

q-PCR (Week 6, 7, 8). Reaction samples for q-PCR were assayed through a q-PCR analysis system and the resulting data was graphed with fluorescence-indicated gene expression plotted along the y-axis and C_T values on the x-axis. C_T values represent the number of copies of a target gene in a given genetic sample and are calculated by the number of q-PCR cycles needed to detect their presence (Gibson, Heid, & Williams, 1996). The resulting output provides a numerical reference for the degree of expression of a target gene across multiple samples of varying conditions. In the case of this study, those conditions are age group and cDNA concentration.

Western blot (Week 8). Presence of FoxO3a in q-PCR samples was examined via Western blot analysis (see Figure 5). Expression comparison of the target gene to the complimentary ladder indicated a base pair length of under 100 bp. As FoxO3a contains 88 bp, this provided sufficient evidence of the target gene's identity.

Data Analysis

Mean FoxO3a C_T values of one- and two-year-old brains were subjected to an independent sample *t*-test. The resulting data was documented and organized by cDNA concentration and age group.

Interpretation of results. If this study's hypothesis were supported, expression of FoxO3a would be significantly greater in the forebrains of older zebrafish when

compared to those of younger fish, implying a functional/regulatory homology between the adult neural stem cells of zebrafish and mammals. Alternatively, if the data results in a failure to reject the null hypothesis of this study (that FoxO3a expression is not positively correlated with age), the functionality and regulation of FoxO3a in zebrafish would be assumed to differ significantly from mammalian FoxO3a.

Results

Study 1

Initial q-PCR runs involved a sample size of five older fish (~two years old). The brain was removed and separated into telencephalon and "whole-brain remainder" sections in order to ascertain a possible difference in FoxO3a expression between the two regions. The resulting expression curves of the first run were inconclusive, as the notably high concentration of cDNA synthesized from these samples made targeting the FoxO3a gene in particular difficult with the resources available.

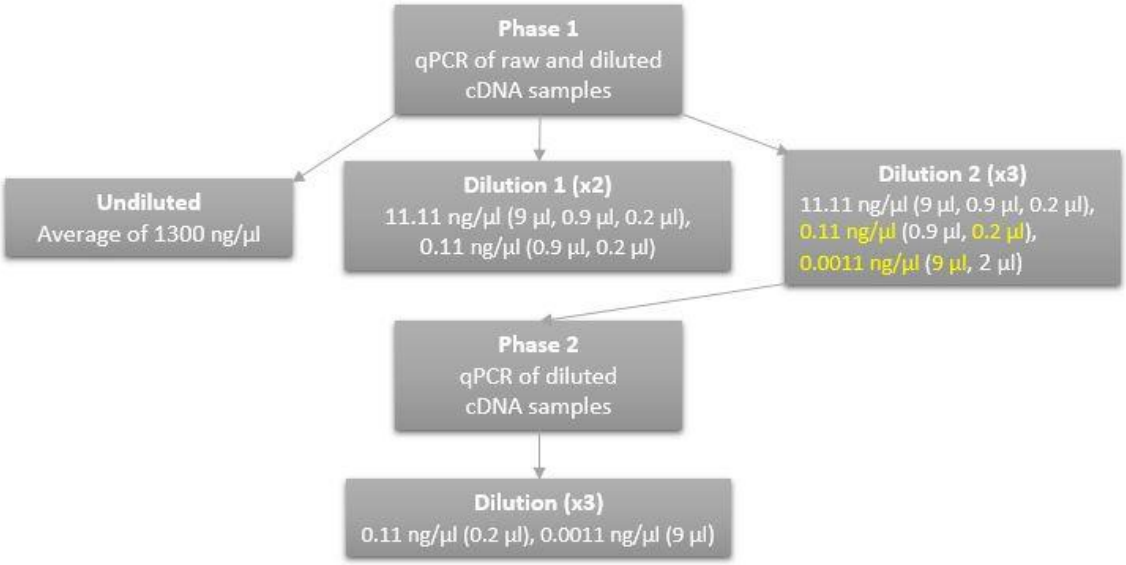

Figure 3. qPCR analyses in the first study involved various concentrations of tissue sample cDNA. Of the concentrations tested, two of them (0.11 ng/µl and 0.0011 ng/µl) were selected for implementation in the second study.

cDNA samples for the second q-PCR analysis underwent two rounds of dilution (three separate cDNA concentrations from the first analysis, two concentrations from the second) (see Figure 3). Samples from the third analysis underwent three rounds of dilution. From the results of this third analysis (see Figure 4), the two cDNA concentrations from which the most consistent expression curves (those displaying a definitive pattern of initial declination prior to increasing expression) were derived were selected as standards for the second round of experimentation (0.0011 ng/µl and 0.0005 ng/µl).

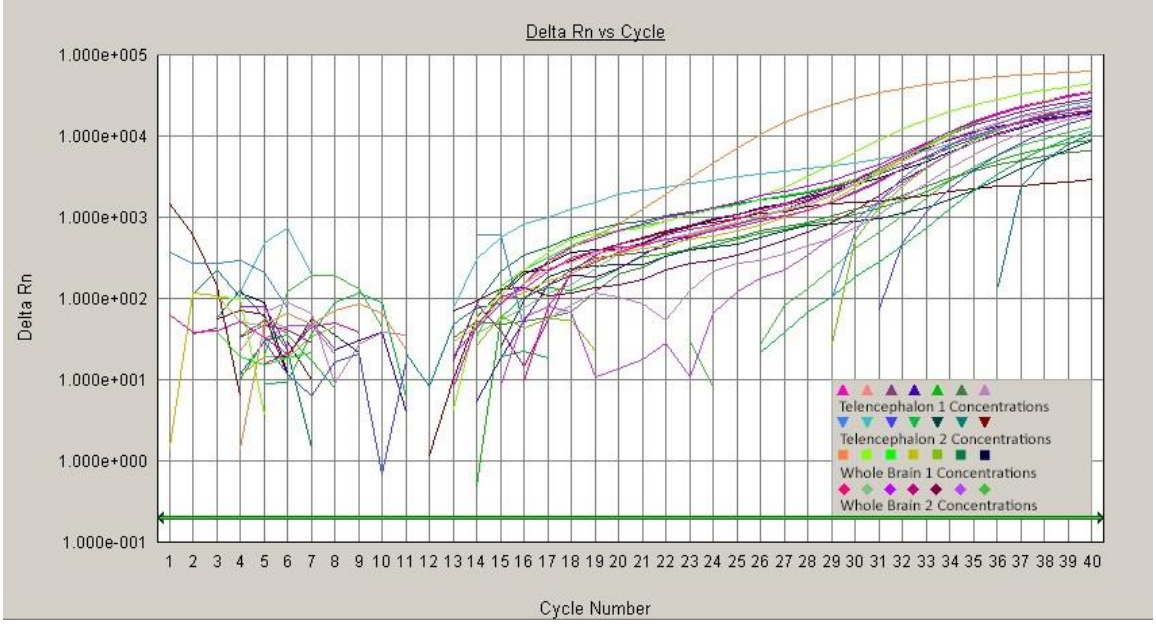

Figure 4. Two cDNA samples from the first study were selected from a sample size of ten (based on preliminary analyses of sample stability) and diluted into seven concentrations (5 ng/µl, 0.5 ng/µl, 0.11 ng/µl, 0.005 ng/µl, 0.0011 ng/µl, 0.0005 ng/µl and 0.00011 ng/µl), as indicated in the above graph's lower right-hand corner going left to right from highest to lowest. "Delta Rn" (on the Y-axis) refers to the ratio of fluorescent dye signals in the cDNA samples minus the baseline signal generated by the PCR machine, resulting in a quantitative value for gene expression. "Cycle Number" (on the X-axis) refers to the number of cycles of analysis necessary to detect expression of the target gene.

After determining the correct cDNA concentration for the PCR reactions, the second study was designed around examining age effects on Fox03a concentration. Fifteen zebrafish, divided into eight young fish and seven old fish, were processed as preciously described. Only the forebrains (telencephalon) were analyzed for FoxO3a expression, because previous research indicates this is the primary brain region where Fox03a expression undergoes age-dependent changes, and because supplies for RNA extraction limited me to 15 samples. Telencephalon tissue from each fish of each age group was used to produce cDNA, which was later diluted to the aforementioned concentrations of 0.0011 ng/µl and 0.0005 ng/µl (see Figure 5).

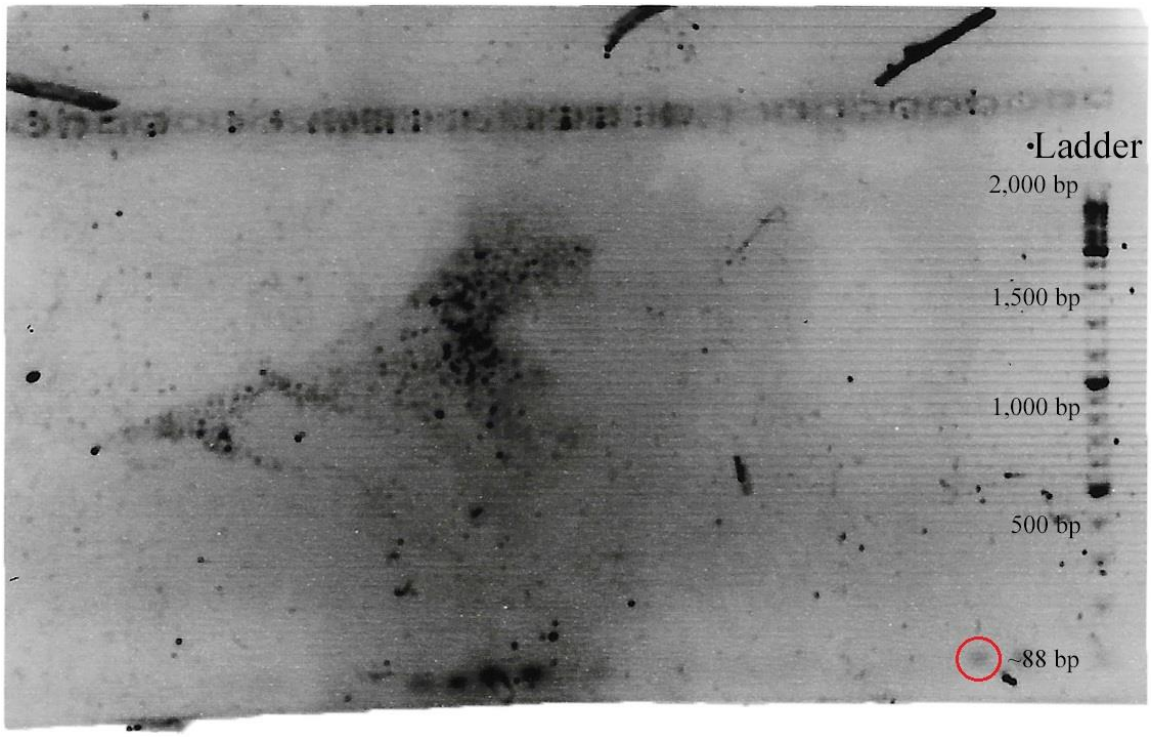

Figure 5. Western blot analysis of cDNA from trial run zebrafish brain tissue samples confirmed the expression of a gene roughly 88 bp in length, indicating the presence of target gene FoxO3a. The target band is circled in red.

Study 2

As with Study 1, the expression curves of these data showed a consistent range of C_T values (12-13 cycles of analysis prior to detection of gene expression). Lower concentrations of cDNA were tested in triplicate, volumes of each sample loaded into three different assay plate wells with the goal of averaging and validating the resulting data. These attempts were met with technical difficulties in the analytical software and followed by two consecutive attempts at troubleshooting by transferring cDNA samples into different tubes. These errors were attributed to the PCR machine itself, which was repaired two days later before a successful analysis could be conducted. This resulted in loss of sample quality due to multiple pipette transfers, with data showing no discernable pattern of expression resembling those of any previous analyses. Remaining reagents were then utilized to produce similar results to the concentration comparison run (C_T values of 12-13 cycles).

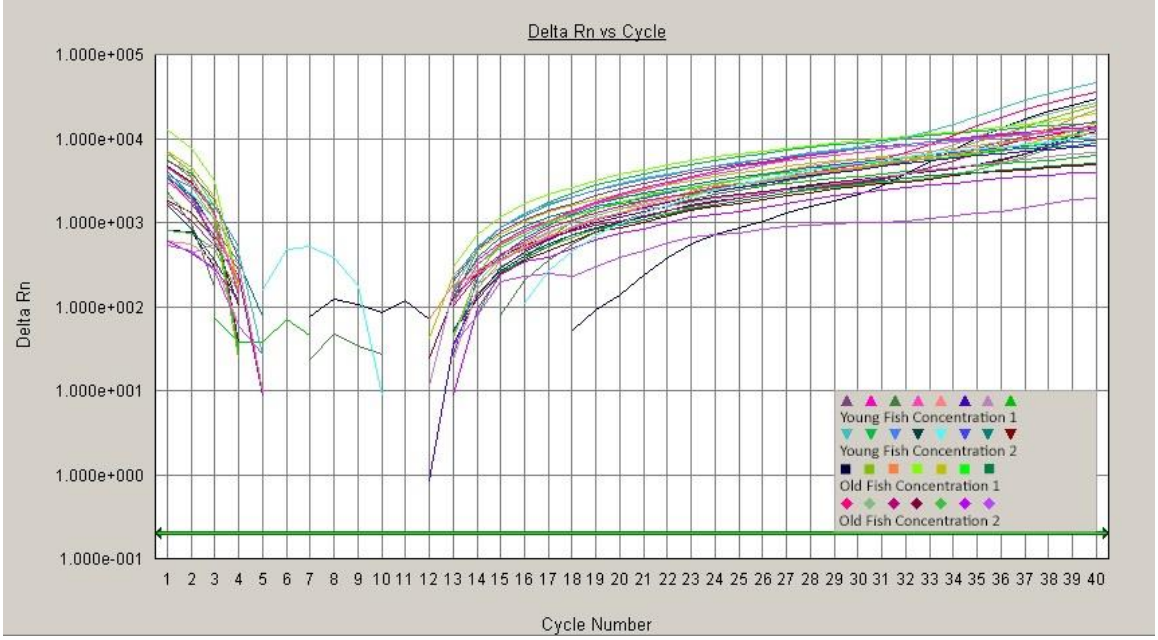

Figure 6. Comparative q-PCR of zebrafish telencephalon tissue at two different concentrations (0.0011 ng/µl and 0.0005 ng/µl) revealed no significant difference in expression of FoxO3a between samples of eight young and seven old fish.

C_T values from the second study were averaged and subjected to a 1-tailed independent samples *t*-testing. As the purpose of this study was to measure the expression of FoxO3a between two independent age groups of zebrafish and to determine the nature of any significant differences between groups, a 1-tailed independent samples *t*-test was selected. C_T values for both "high-" and "low-concentration" cDNA samples provided inadequately-high *p*-values for rejection of the null hypothesis, which is to say neither of these *p*-value was less than 0.05 (see Table 1). This indicates no significant difference between data sets. These results suggest that expression of FoxO3a in the telencephalons of zebrafish do not significantly fluctuate with progressive organismal aging.

Table 1: Data Analysis of qPCR Results

Age Group	*N*	*M*	*SD*	Cohen's *d*	*t* Stat	*df*	*p*	*t* Crit.
High Conc. Young CT	8	12.6245	0.7522	0.3488	-0.2041	8	0.4217	1.8596
High Conc. Old CT	7	12.7865	1.9783					
Low Conc. Young CT	8	12.8122	1.0155	0.6151	1.2226	8	0.1281	1.8596
Low Conc. Old CT	7	12.3499	0.3136					

Note. "High Conc." = 0.0011 ng/µl, "Low Conc." = 0.0005 ng/µl

Discussion

Summary of Main Findings

The data collected from this study indicate a failure to reject the null hypothesis that there is no significant change in expression of FoxO3a across age groups. Cycle thresholds for each cDNA sample averaged at 12-13 across all samples and between both selected cDNA concentrations of 0.0011 ng/µl and 0.0005 ng/µl (see Table 1). The magnitude of these relationships is consistent with simple chance or noise. Furthermore, *t*-testing of data averages revealed no significant differences between C_T values. The absence of a significant correlation between expression and the aforementioned variables of brain region and age group contradicts previous findings from related studies showing greater expression of FoxO3a in older animals in comparison to their younger counterparts (Maiese, 2015; Mustafa, Salih, & Waheed, 2013).

Relevance of Study to Understanding FoxO3a's Relationship with Aging

The static expression of FoxO3a in zebrafish across age groups in this study suggest possible differences in the gene's functionality between zebrafish and mammals. FoxO genes are significantly correlated with longevity across animal models, as they transcribe various functions pertaining to tissue homeostasis. However, subfamily variants of the gene differ in such aspects as expression pathway and functionality (Martins, Lithgow, & Link, 2016). In particular, FoxO3 and its sub-variant FoxO3a are unique to vertebrate animals, as are the FoxO1, FoxO4 and FoxO6 subfamilies. Invertebrate animals only have one FoxO gene, with select species-specific variants serving the same roles as FoxO3a in vertebrates (e.g. dFoxO in *Drosophila*

melanogaster). It therefore stands to reason that the expression or functionality of a gene shared among vertebrate animals may differ between species. The findings of this study beg for a more thorough examination of the extent to which the zebrafish model may be applied to studies of neurophysiological development and homeostasis in humans.

Limitations of This Study

While the relative consistency of FoxO3a expression across age groups implies difference in the gene's functionality between zebrafish and mammalian brains, various aspects of this study stress the need for further investigation. The first of these key issues is the small sample size. With limited reagents available for q-PCR preparation, 25 reactions were available between the trial runs and final iteration runs, that is to say the analyses carried out upon establishing ideal cDNA dilutions. Previous studies investigating gene expression in developing fish have made use of at least 20 fish per age group (Maack, & Segner, 2003). While the data trends of this study indicate consistency in the expression of FoxO3a across age groups, increasing the sample size of both groups would provide greater significance to these data.

Technical difficulties proved to be another factor in this study's shortcomings, specifically hardware/software malfunctions. With only one q-PCR machine available, the study was compromised when this machine malfunctioned and attempts at troubleshooting via multiple sample transfers led to the decreased sample quality. Additional factors contributing to difference between studies include but may not be limited to sample quality, user error in sample handling/preparation, inaccuracy of the nanodrop system in determining sample concentration, or the mere one-year age

difference between “Young” and “Old” fish. Given this study’s limitations, the potential of FoxO3a as a therapeutic target to improve brain and cognitive aging outcomes must be evaluated on the basis of current published studies. The decision to pursue research on FoxO3a for this purpose must also consider its potential relative to other approaches to improve brain and cognitive longevity.

FoxO3a in Longevity Interventions

At present, there exist few studies investigating the application of FoxO3a manipulation as a therapeutic agent in promoting longevity of nervous tissue. However, various studies have looked into the relationship between FoxO3a expression and various diseases. Deletion of FoxO3a in mammals has been found to inhibit apoptosis and promote tumorigenesis, while overexpression of the gene suppresses tumorigenesis both *in vitro* and *in vivo* (Yang, & Hung, 2009). FoxO3a-dependent gene expression pathways have also shown to play vital roles in gene therapy designed to improve cardiac function (Kumarswamy et al., 2012). As FoxO3a serves multiple homeostatic roles that are highly dependent on the gene’s localization and epigenetic factors, research into alternative treatment types based on other cross-species “longevity genes” may provide more answers pertaining to the limitations of FoxO-based therapies.

FoxO3a Alternatives

While FoxO3a shows significant promise as a source of insight into the contributing factors and possible measures for counteracting age-related disease progression, translational research pertaining to FoxO3a and other genes promoting longevity in humans can be negatively affected by matters of metabolic and

environmental differences between human and animal models (de Magalhaes, 2014). Specifically, the lack of genetic diversity in animal model strains complicates translational relevance to humans given our notable genetic diversity and epigenetic factors distinct to different human populations. Research and clinical trials pertaining to the inhibition and expression of genes known to affect human models of age-related disease show significant promise as an alternative to the use of animal models (Dai, Sinclair, Ellis, & Steegborn, 2018).

Future Directions

While many structural parallels can be drawn between zebrafish and mammalian brains, the evident contrast in age-dependence of FoxO3a expression, a vital housekeeping gene, implies a notable difference in functionality. To these ends, additional research into the structural and functional similarities in neurophysiology between zebrafish and mammalian models are required to confirm the extent of this difference. An extended variant of this design utilizing triplicate runs of larger sample sizes with two or more age groups across multiple zebrafish broods would provide a more accurate basis for making such determinations.

With the findings of this proposed study as a foundation, another study investigating the differences in FoxO3a expression across age groups and sexes would provide further insight into the extent of the gene's functionality across species. Studies of longevity in *Drosophila melanogaster* have revealed multiple age- and sex-specific genetic differences which serve as direct determinants of survivorship and mortality across multiple strains of flies (Nuzhdin, Pasyukova, Dilda, Zeng, & Mackay, 1997).

Additional studies of animal models commonly used in neuro-developmental research have revealed considerable variation in sex-specific gene expression (Eliot et al., 2016). Further investigation into these common trait loci is necessary to determine the extent to which current animal models may be applied to translational longevity research.

References

Babin, P. J., Goizet, C., & Raldúa, D. (2014). Zebrafish models of human motor neuron diseases: advantages and limitations. *Progress in Neurobiology, 118*, 36-58. doi: 10.1016/j.pneurobio.2014.03.001

Cabreiro, F., Ackerman, D., Doonan, R., Araiz, C., Back, P., Papp, D., ... Gems, D. (2011). Increased life span from overexpression of superoxide dismutase in Caenorhabditis elegans is not caused by decreased oxidative damage. *Free Radical Biology and Medicine, 51*, 1575-1582. doi: 10.1016/j.freeradbiomed.2011.07.020

Dai, H., Sinclair, D. A., Ellis, J. L., & Steegborn, C. (In Press). Sirtuin activators and inhibitors: Promises, achievements, and challenges. *Pharmacology & Therapeutics*, Available Online 22 March 2018. doi: 10.1016/j.pharmthera.2018.03.004

de Magalhaes, J. P. (2014). Why genes extending lifespan in model organisms have not been consistently associated with human longevity and what it means to translation research. *Cell Cycle, 13*, 2671-2673, doi: 10.4161/15384101.2014.950151

Gibson, U. E., Heid, C. A., & Williams, P. M. (1996). A novel method for real time quantitative RT-PCR. *Genome Research, 6*, 995-1001. doi: 10.1101/gr.6.10.995

Huang, P., Zhou, Z. Q., Huang, R. H., Zhou, B., Wei, Q. W., & Shi, F. X. (2011). Age-dependent expression of forkhead box O proteins in the duodenum of rats. *Journal of Zhejiang University Science B, 9*, 730-735. doi: 10.1631/jzus.B1000298

Jørgensen, A., Morthorst, J. E., Andersen, O., Rasmussen, L. J., & Bjerregaard, P. (2008). Expression profiles for six zebrafish genes during gonadal sex differentiation. *Reproductive Biology and Endocrinology*, *6*, 25-37. doi: 10.1186/1477-7827-6-25

Kaslin, J., Ganz, J., & Brand, M. (2008). Proliferation, neurogenesis and regeneration in the non-mammalian vertebrate brain. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *363*, 101-122. doi: 10.1098/rstb.2006.2015

Keller, E. T., & Murtha, J. M. (2004). The use of mature zebrafish (Danio rerio) as a model for human aging and disease. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 138*, 335-341, doi: 10.1016/j.cca.2004.04.001

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., & Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Developmental Dynamics*, *203*(3), 253-310. doi: 10.1002/aja.1002030302

Kumarswamy, R., Lyon, A. R., Volkmann, I., Mills, A. M., Bretthauer, J., Pahuja, A., ... & Harding, S. E. (2012). SERCA2a gene therapy restores microRNA-1 expression in heart failure via an Akt/FoxO3A-dependent pathway. *European Heart Journal*, *33*, 1067-1075. doi: 10.1093/eurheartj/ehs043

Lan, C. C., Tang, R., San Leong, I. U., & Love, D. R. (2009). Quantitative real-time RT-PCR (qRT-PCR) of zebrafish transcripts: optimization of RNA extraction, quality control considerations, and data analysis. *Cold Spring Harbor Protocols, 4*, 1-12, doi: 10.1101/pdb.prot5314

Maack, G., & Segner, H. (2003). Morphological development of the gonads in zebrafish. *Journal of Fish Biology*, *62*, 895-906, doi: 10.1046/j.1095-8649.2003.00074.x

Maiese, K. (2015). FoxO Proteins in the Nervous System. *Analytical Cellular Pathology*, *116*, 191-203. doi: 10.1155/2015/569392

Martins, R., Lithgow, G. J., & Link, W. (2016). Long live FOXO: unraveling the role of FOXO proteins in aging and longevity. *Aging cell*, *15*(2), 196-207. doi: 10.1111/acel.12427

Menezes, F. P., Kist, L. W., Bogo, M. R., Bonan, C. D., & Da Silva, R. S. (2015). Evaluation of age-dependent response to NMDA receptor antagonism in zebrafish. *Zebrafish*, *12*, 137-143. doi: 10.1089/zeb.2014.1018

Mustafa N. Gh.; Salih S. Gh. and Waheed I. N. (2013). FoxO3 gene expression in neural stem cells isolated from mice brain. *Journal of Cell and Molecular Biology*. *11*: 59-65. doi: 10.1016/j.stem.2009.09.014

Nuzhdin, S. V., Pasyukova, E. G., Dilda, C. L., Zeng, Z. B., & Mackay, T. F. (1997). Sex-specific quantitative trait loci affecting longevity in Drosophila melanogaster. *Proceedings of the National Academy of Sciences*, *94*, 9734-9739. doi: 10.1073/pnas.94.18.9734

Peng, K., Li, Y., Long, L., Li, D., Jia, Q., Wang, Y., ... & Peng, Y. (2010). Knockdown of FoxO3a induces increased neuronal apoptosis during embryonic development in zebrafish. *Neuroscience Letters*, *484*, 98-103. doi: 10.1016/j.neulet.2010.07.068

Renault, V. M., Rafalski, V. A., Morgan, A. A., Salih, D. A., Brett, J. O., Webb, A. E., ... & Palmer, T. D. (2009). FoxO3 regulates neural stem cell homeostasis. *Cell Stem Cell*, *5*, 527-539. doi: 10.1016/j.stem.2009.09.014

Richardson, A., Galvan, V., Lin, A. L., & Oddo, S. (2015). How longevity research can lead to therapies for Alzheimer's disease: The rapamycin story. *Experimental Gerontology*, *68*, 51-58. doi: 10.1016/j.exger.2014.12.002

Simonsen, A., Cumming, R. C., Brech, A., Isakson, P., Schubert, D. R., & Finley, K. D. (2008). Promoting basal levels of autophagy in the nervous system enhances longevity and oxidant resistance in adult Drosophila. *Autophagy*, *4*, 176-184. doi: 10.4161/auto.5269

Ulloa-Montoya, F., Verfaillie, C. M., & Hu, W. S. (2005). Culture systems for pluripotent stem cells. *Journal of Bioscience and Bioengineering*, *100*, 12-27. doi: 10.1263/jbb.100.12

Wang, L., Karpac, J., & Jasper, H. (2014). Promoting longevity by maintaining metabolic and proliferative homeostasis. *Journal of Experimental Biology, 217*(1), 109-118, doi: 10.1242/jeb.089920

Weissman, I. L., Anderson, D. J., & Gage, F. (2001). Stem and progenitor cells: origins, phenotypes, lineage commitments, and transdifferentiations. *Annual Review of Cell and Developmental Biology, 17*, 387-403. doi: 10.1146/annurev.cellbio.17.1.387

Yang, J. Y., & Hung, M. C. (2009). A New Fork for Clinical Application: Targeting Forkhead Transcription Factors in Cancer. *Clinical Cancer Research, 15*, 752-757.
doi: 10.1158/1078-0432.CCR-08-0124

Zhang, R. L., Chopp, M., Roberts, C., Liu, X., Wei, M., Nejad-Davarani, S. P., ... & Zhang, Z. G. (2014). Stroke increases neural stem cells and angiogenesis in the neurogenic niche of the adult mouse. *PloS One, 9*, e113972-e113972. doi: 10.1371/journal.pone.0113972