Generation and Partial Characterization of Testes Specific Promotor Sequences in Zebrafish (Danio rerio)

Rohini Rajendran

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Generation and Partial Characterization of Testes Specific Promoter Sequences in Zebrafish (*Danio rerio*)

The cAMP response element modulator (CREM) regulates cAMP-responsive genes and is an important component of the cAMP mediated signal transduction in spermatogenesis. CREM has a regulatory relationship with Inducible cAMP early repressor (ICER) which acts as a tumor suppressor and is a dominant negative transcriptional repressor with gonadal specific properties. ICER is also known to moderate cAMP antiproliferative actions and has been targeted in the past for expression in the ovaries of transgenic mouse models to generate higher levels of ovulation. Earlier studies on mice lacking the CREM gene have shown severe impairment in spermatogenesis.

Enhanced Green Fluorescent Protein (EGFP) is a versatile protein often used as a biosensor to determine the expression of proteins in cells. This is accomplished by introducing EGFP along with the desired protein in a vector construct into the organism. Fluorescence microscopy can detect the presence of EGFP and thus the protein if it is successfully introduced into the genome of the organism. Previously in Dr. Molina’s laboratory, CYP19A1 a gonadal specific promoter, was injected into zebrafish eggs. CYP19A1 was chosen due to its promising results using a three fragment vector construction method where EGFP was used as a marker to monitor whether it was effectively and specifically expressed in the testes. Once the vector construction was completed, the CYP19A1: EGFP promoter construct was injected into the zebrafish eggs generating transgenic zebrafish.
The main objective of this project was to show that CYP19A1 can function as a testes specific promoter that can be used to successfully express transgenes in the testes, with the potential to affect spermatogenesis in zebrafish. Western blots, PCR reactions, and immunohistochemical stains were performed to detect over-expression of EGFP in the testis of Danio rerio. The results of the study show that CYP19A1 was successfully expressed in the testes. The implications of the study indicate that the selected promoter region of CYP19A1 can be a testes specific promoter. This promoter can then be used in future experiments for the expression of relevant genes such as ICER, in the testes. For instance, the over expression of these genes in the testes of zebrafish can affect spermatogenesis and therefore have considerable implications for future research in fertility treatments and boosting livestock production.

Key words: ICER, cAMP, CYP19A1, EGFP, Transgenesis
GENERATION AND PARTIAL CHARACTERIZATION OF TESTES SPECIFIC PROMOTER SEQUENCES IN ZEBRAFISH (*Danio rerio*)

A THESIS

Submitted in partial fulfillment of the requirements

For the degree of Masters of Science

By

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Montclair State University

Montclair, NJ

2015
Acknowledgment

I would like to express my gratitude to my research advisor, Dr. Carlos Molina for accepting me into his lab and being a tremendous mentor. I learned a great deal from him over the months spent in his lab and it has become an integral and important period in my academic career. Without your guidance, I would not have been able to put together this thesis. Thank you for your support and encouraging me to grow as a research scientist.

Further, I would like also like to thank my committee members, Dr. Kirsten Monsen-Collar and Dr. John Siekierka for graciously contributing their invaluable time to serve on my thesis committee and guiding me through my defense. Thank you for your time and encouragement in this exciting research experience.

Last but not least, I would like to thank my family, my friends, and my lab partner for their unconditional support during the pursuit of my degree. The decision to pursue a degree while juggling a full time job has been challenging, thank you for your patience and understanding through this demanding, yet fulfilling chapter of my life.
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Introduction:

Cyclic adenosine monophosphate or cAMP is a ubiquitous second messenger that plays an important role in many biological processes, especially intracellular signal transduction in various organisms (Gellersen & Brosens, 2003). cAMP is synthesized from adenosine triphosphate (ATP) through adenylyl cyclase. cAMP-dependent protein kinase A (PKA) is a foremost receiver of cAMP downstream regulation. PKA is an enzyme that is present in the cytoplasm and is composed of two regulatory and two catalytic sub-units (Gellersen & Brosens, 2003).

When two molecules of cAMP bind to the regulatory subunits of PKA, they undergo a conformational change that activates the catalytic subunits, which can phosphorylate the target molecules in the cytoplasm (Gellersen & Brosens, 2003). Activated PKA can also diffuse through the nucleus altering the transcriptional activity of cAMP response element binding protein (CREB) and cAMP response element modulator (CREM) (Gellersen & Brosens, 2003).

CREB and CREM are members of the bZIP family of transcription factors, and is named for its highly conserved DNA-recognition basic region and leucine zipper (Walker et. al, 1998; Gellersen & Brosens, 2003). Both CREB and CREM are characterized by various alternative spliced forms, some of which lack the activation domains and thus function as transcription suppressors (Don & Stelzer, 2002). Inducible cAMP early repressor (ICER) is an example of one such suppressor isoform of CREM that can be activated by CREB and is involved in auto-regulating, as well as down regulating CREB expression (Don & Stelzer, 2002).
In the testes, both CREB and CREM are highly expressed. Their expression is controlled by G-protein coupled receptors in Sertoli cells due to the action of cAMP as well as interactions with FSH (follicle stimulating hormone) (Walker, et. al., 1998). CREM regulates cAMP-responsive genes and is an important component of the cAMP mediated signal transduction in spermatogenesis (Walker & Habener, 1996).

CREB and CREM are cyclically expressed at different times during the sexual maturation of the testes and during the spermatogenic cycle (Walker & Habener, 1996). Mechanisms of alternative exon splicing and promoter usage cause the CREB and CREM to undergo programmed switches from activator to suppressor isoforms (Walker & Habener, 1996). CREs (cAMP regulatory elements) present in promoter region of CREB up regulates the activator CREB expression and CREs in CREM stimulates repressor isoform expression (Walker & Habener, 1996). Therefore, mediating the expression of the target gene involved in the development and production of germ cells can alter the regulation of spermatogenesis.

ICER, an isoform of CREM, encodes a repressor of cAMP responsive element and negatively autoregulates itself as well as the CREB/CREM system (Molina, et. al., 1993). ICER derives the ability to auto regulate due to the presence of an intronic promoter, P2, located close to the 3’ of the gene (Molina, et. al., 1993). Thus, the close configuration of the promoter region enables a cooperative interaction between binding sites as compared to widely spaced cAMP regulated promoters (Molina, et. al., 1993). Interestingly, replacing the promoter region on ICER with a different promoter can result in the over-expression of ICER in the cells.
Spermatogenesis is a developmental process in which diploid stem cells (spermatogonia) undergo a series of differentiations and result in the production of mature haploid sperm cells that contain a recombined genome (Schulza, et al., 2010). The differentiation of these germ cells occurs within the seminiferous tubules and are "nursed" by continuous and constant contact with somatic Sertoli cells present in the tubules which form the blood-testis barrier (Don & Stelzer, 2002) (Schulza, et al., 2010).

During spermiogenesis, the transcriptional factor CREM is present in higher levels in post meiotic cells (Foulkes, et. al., 1992). The activation of haploid germ cell specific genes that are involved in shaping the spermatozoan may be due to the expression of CREM (Foulkes, et. al., 1992.) Previous studies of the seminiferous tubules in infertile mutant male mice showed a CREM deficiency in post meiotic cells (Nantel, et al., 1996).

Nantel et. al, 1996 showed that CREM-deficient mice were low on late spermatids and completely lacked spermatozoa. Further their studies demonstrated that CREM-deficient mice had severe mutations in spermatogenesis due to a spermatogenic arrest. This impairment was also observed in many human cases of infertility (Nantel, et al., 1996). Henceforth, it can be hypothesized that an over expression ICER can have considerable implications in escalating CREM expression post meiotically. This can increase the number of spermatids and spermatozoa.

Danio rerio, commonly known as the Zebrafish has seen an emergence as a model for studies in vertebrate biology concerning human conditions and diseases. Zebrafish as a model for research studies has many advantages such as: ease of maintenance, cost effectiveness, and transparency of embryos which is ideal for
phenotypic screening (Segner, 2009). Another advantage is that Zebrafish can breed continuously in a laboratory setting because they breed year round and the females spawn about once a week producing around 200 eggs or more (Segner, 2009). Zebrafish are further ideal as a model due to its relatively short gestational period to adult life making it perfect for genetic studies. Zebrafish and humans have some analogous genes and significant conserved homology to disease-causing genes and proteins which make zebrafish ideal for endocrine studies (Segner, 2009). The versatility of zebrafish has led to scientists using them to produce transgenic strains that have been used in studies where observing different stages of life are essential while gene expression is being manipulated (Segner, 2009).

Rapid embryonic development of zebrafish and its organs makes it an ideal candidate for transgenesis. Transgenesis is a technique used in the study of gene expression that makes use of transposable elements to incorporate genomic expression. A plasmid DNA is inserted into a fertilized egg and allowed to integrate into the genome. This is then transmitted through the germ lineage. Commercially available kits, such as the tol2 kit derived from the medaka fish (*Oryzias latipes*), are used extensively in transgenic studies involving Zebrafish (Kawakami, 2004). In the past, transgenesis in Zebrafish has encountered three major problems “the labor of building complex expression constructs using conventional subcloning; low transgenesis efficiency, leading to mosaicism in transient transgenics and infrequent germline incorporation; and difficulty in identifying germline integrations unless using a fluorescent marker transgene” (Kwan, et al., 2007).
Using Gateway technology, the *tol2* system resolves these issues by permitting quick and easy construct building and site-specific recombination cloning (Kwan, et al., 2007). The kit further simplifies clone sharing, increases the probability of transgenesis, and eliminates fluorescent markers as a primary source of transgene identification (Kwan, et al., 2007). The relative ease with which zebrafish eggs can be microinjected with transposable elements has enabled the *tol2* system to become the established method of creating transgenic zebrafish (Kawakami, 2004). Tissue specific promoters can be attached to green fluorescent protein (GFP) to monitor the transgene expression.

*Figure 1*: Insertion of Plasmid DNA into zebrafish using the *tol2* system
Figure 1 demonstrates the mechanism by which a plasmid DNA is inserted into a zebrafish using the \textit{tol2} system. A transposon donor which is a vector construct, containing inverted \textit{tol2} elements bracketing the ends, a promoter (CYP19A1 in this study), and EGFP, is co-inserted with mRNA encoding transposase into fertilized eggs. The mRNA translates the transposase protein and catalyzes the cutting out of the transposon construct from the plasmid donor (Kawakami, 2007). The excised DNA is then able to stably integrate into the genome resulting in transgenic zebrafish in 50-70\% of injected fish (Kawakami, 2007).

CYP19A1, a gonadal specific promoter was previously injected into zebrafish eggs and bred in Dr. Molina’s laboratory. CYP19A1, a gene involved in estrogen biosynthesis, encodes for aromatases that catalyze the conversion of androgens into estrogens (Hinfray, et al., 2013). The Leydig cells of testes of several different vertebrates, including fish, strongly express CYP19A1 proteins (Hinfray, et al., 2013). Due to its promising results, CYP19A1 was chosen in constructing a three fragment vector where Enhanced Green Fluorescent Protein (EGFP) was used as a marker to monitor whether EGFP was effectively and specifically expressed in the testes (Tsatsos, Master’s Thesis, 2013). Figure 2 depicts the sequence of the CYP19A1: EGFP vector construct bracketed by the \textit{tol2} genes.

Once the vector construction was completed, the CYP19A1: EGFP promoter construct was injected into the zebrafish eggs generating transgenesis into the \textit{Danio rerio}. The microinjection of eggs was performed at the laboratory of Dr. Hatem E. Sabaawy at the Rutgers Medical School (Sabaawy, 2014). The primary objective of this project was to show that CYP19A1 can function as a testes specific promoter that can
successfully express genes in the testes, and potentially affect spermatogenesis in Zebrafish. Although ICER can negatively auto-regulate its expression, the use of CYP19A1 as a promoter overrides the auto-regulation, thereby permitting over-expression of the gene.

**Fig. 2 A**: CYP19A reverse complement DNA sequences with minito2 sequences bracketing the ends. Red (3’ end), Yellow and Blue (5’ end of minito2) sequences show 100% identity with to2 R4/R2. Purple and green show 97% alignment with the complementary sequence for pENTR-EGFP. Yellow underline strikethrough shows late polyA signal.

**Fig. 2 B**: Upper half of the illustration is the to2 RNA with introns (dots), exons (lines), coding, and uncoding regions. The lower half shows the positions of the mini to2 vector, promoter region, EGFP, and the polyA tail. (Kawakami, Tol2: a versatile gene transfer vector in vertebrates, 2007)
Fertility treatments are making enormous advances giving older couples, and previously infertile couples the option of conceiving. However, more options are available for female infertility treatments, such as fertility drugs, artificial insemination, \textit{in vitro} fertilization, etc. On the other hand, there is paucity in the options available to treat male infertility. If successful, the over expression of these genes in the testes of zebrafish can potentially affect spermatogenesis and therefore have considerable implications for future research in fertility treatments.

\textbf{Material and Methods:}

\textbf{Zebrafish Mating Protocol:}

The injection of eggs with the CYP19A1: EGFP construct was done at the laboratory of Dr. Hatem E. Sabaawy at the Rutgers Medical School. 400 eggs were initially injected with different primers, including CYP19A1, ZP3, GDF9, or FSH. CYP19A1 was chosen previously as the most viable primer (Tsatsos, Master’s Thesis, 2013). The transparency of the embryos allowed for visual observation of EGFP under the microscope. The embryos that fluoresced were allowed to grow and result in the F1 generation. Males and females from the F1 generation were mated to produce the F2 generation containing some transgenic zebrafish.

An equivalent number of male and female Zebrafish were selected. Females are distinguished from the males by rounder and whiter bellies. Four pairs of Zebrafish were paired and placed in separate breeding tanks. One Zebrafish from the pair was transgenic and the other was a wild type. The number of transgenic males and females were the equal. A single layer of marbles was added to the bottom of the Zebrafish breeding tanks.
to collect the eggs. The water in the tanks was controlled at 25°C. The Zebrafish ovulates during the first half hour of daylight (or simulated daylight) and after being fertilized by the male, eggs could be found on the strainer. The eggs were then collected and placed in a separate tank and allowed to hatch.

**DNA Isolation and Extraction:**

DNA was isolated from the tissues of the F2 generation of transgenic zebrafish. Tissue samples from the brain, heart, intestines, and testis were obtained from dissected zebrafish. DNA isolation was performed by disrupting the tissue and cellular structures in order to create a lysate or a solution of the broken down cells. The tissue was washed three times with ice cold 0.1% PBS and placed in a culture dish. 1 ml of TRIzol® reagent was added to the dish and the cells were lysed by passing it several times through a pipette and the lysate was incubated at room temperature for 5 mins. 200 micro liters of chloroform were added to the tubes and vigorously hand shaken for 15 seconds after which it was incubated at room temperature for 2-3 mins. This lysate was then separated by centrifugation (at 12,000 rpm for 15 mins at 4°C) into cell debris, nucleic acids, and proteins. The TRIzol® Reagent from the Invitrogen DNA extraction kit (by Life Technologies, Grand Island, NY) was used for this purpose.

After centrifuging the lysate, the mixture separated into a phenol-chloroform phase (organic) that is red in color, an interphase, and a colorless aqueous phase. The lower phenol phase and interphase were collected and mixed with 300 μL of 100% ethanol. After being incubated at room temperature for 3 mins this mixture was further centrifuged (at 12000 rpm for 5 mins at 4°C). The supernatant was discarded and the
pellet was washed twice using 1ml 0.1M sodium citrate and 10% ethanol. It was then and incubated for 30 mins at room temperature and centrifuged again at 12000 rpm for 5 mins at 4°C.

Following the two washes, the DNA pellet obtained was suspended in 1.5ml 75% ethanol and incubated for 15 mins at room temperature with periodic stirring. This was further centrifuged (12000 rpm, 5 mins, 4°C). The pellet was then air dried and then dissolved in 150 µl of ultrapure PCR grade water. The sample was incubated at 55°C to allow the pellet to completely dissolve. To remove any impurities, the sample was centrifuged (12000 rpm, 10 mins, 4°C) and the supernatant was collected for PCR amplification.

**Polymerase Chain Reaction (PCR):**

The Invitrogen Platinum® PCR SuperMix High Fidelity kit was used to amplify DNA isolated from the zebrafish tissues. The purpose of amplifying the isolated DNA from the tissues was to detect the presence of EGFP. EGFP has 720 bp and can easily be visualized on the gel. The contents of this kit (45 µl of Platinum PCR SuperMix High Fidelity, primer solution, and template DNA) were added to the reaction tube containing the extracted DNA and agitated in order to create a mixture. The capped tubes were loaded onto the thermal cycler and incubated at 94°C for 30 seconds to completely denature the template and activate the enzyme. After that, 25-35 cycles of denaturing at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 68°C for 1 min were performed.
The primers used to amplify a 714bp fragment of GFP were

\[\text{GACGTAAACGGCCACAAGTT} \ 3' \ \text{the left primer and} \ \text{TGCTCAGGTAGTGGTTGTCG} \ 3' \ \text{the right primer. The primers were a part of the Invitrogen Platinum® PCR SuperMix High Fidelity kit. 50ng of genomic DNA was used for the DNA template. Genomic DNA from wt zebrafish was used as a negative control.}

**Gel Electrophoresis:**

The Qiagen QIAquick Gel Extraction Kit was used to purify the amplified DNA obtained by PCR. This was established using a 1% agarose gel electrophoresis stained with ethidium bromide to distinguish bands of DNA fragments. The gels were run at 100 volts for about an hour in a 1x TAE buffer solution. 4 μL of loading gel and around 20 μL of each tissue sample (Lane 1: DNA ladder, Lane 2: Genomic DNA of wt zebrafish, Lane 3: Male 1, Lane 4: Male 2) were added to each well.

**Protein Extraction and Western Blot:**

Western Blot is an analytical technique performed to detect the presence of specific proteins in a tissue, in this study- GFP in the testes of Zebrafish. For this protein is extracted and subject to gel electrophoresis, the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) technique by BIORAD (BIO-RAD, Hercules, CA) was used. The proteins in the tissue are denatured with an agent, in this case, β-mercaptoetanol and then subjected to gel electrophoresis in a Mini-PROTEAN precast gel was obtained commercially from Bio-Rad. The proteins are separated based on their ability to move through an electric current based on their molecular weight. The SDS
detergent is applied to maintain the proteins in polypeptide chains and prevent them from folding into secondary and tertiary structures.

In order to visualize the protein, the membrane is incubated with a primary antibody that recognizes and binds to the specific protein, EGFP which is not visible until it is incubated further with a secondary antibody directed against the primary AB. The membrane is washed before and after the incubation with the secondary antibody with 0.1% PBS in 0.1% Tween 20. The membranes are then scanned with the Odyssey Infrared Scanner so the protein could be detected. The quantity of protein obtained from each tissue sample is then measured by densitrometric analysis.

**Rapid Protein Extraction Protocol:**

A tissue sample of the Zebrafish was obtained and Laemmli buffer, at twice the volume of the tissue, was added to a micro-centrifuge tube. The tissues were thoroughly homogenized with a plastic-teflon homogenizer. The samples were then heated for 5 mins at 95°C on a heating block. Next, it was centrifuged at room temperature at 13000 rpm for 10 mins. The supernatant was transferred to a 500 μl micro centrifuge tube and then stored at -20°C and was ready for Western Blot.

**Western Blot Protocol:**

The SDS-PAGE gel was loaded and run in accordance with the manufacturer’s instructions (LI-COR®, Lincoln, NE). Four samples were selected and run on two separate gels: Control HeLa cell extract (negative control), HeLa-GFP cell extract (positive control), Protein extract from Testis (M7), and Protein extract from Ovary (F1). Proteins were transferred onto a nitrocellulose membrane by running the BIORAD
apparatus for 35 mins at 200 volts. After disconnecting the apparatus and carefully removing the nitrocellulose membrane, the membrane was placed in a blocking buffer by LICOR (contains Bovine Serum Albumin) and incubated overnight at 4°C.

The blocking buffer was then discarded and primary antibodies at various dilutions (to determine an ideal concentration at which the protein bands could ideally be observed) were added and incubated with either monoclonal (Anti-GFP mouse antibody from ABCAM®, Cambridge, MA) or polyclonal antibodies (Anti-GFP rabbit antibody from ABCAM®, Cambridge, MA). Each membrane was cut into two halves resulting in 8 membrane pieces. Each cut membrane included wells containing HeLa control, HeLa EGFP, Testis sample, Ovary sample. Four membranes were incubated at room temperature on a rocking platform for 6 hrs and treated with 5ml of Monoclonal (mouse) Antibody at dilutions - 1:500, 1:1000, 1:2000, 1:3000 and the other four membranes were also incubated at room temperature on a rocking platform for 6 hrs and treated with 5ml of Polyclonal (rabbit) Antibody at dilutions-1:1000, 1: 2000, 1:2500, 1:3000. After incubation period, the primary antibody solution was discarded and the membranes were washed with 4-5 ml of Rinsing Buffer four times for 5 minutes. Once optimal antibody conditions were determined, a second experiment was performed with the most ideal dilution conditions. Figure 3 depicts the ideal dilutions used in the second Western Blot.

<table>
<thead>
<tr>
<th>Gel 1</th>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Polyclonal Rabbit Anti-GFP</td>
<td>IR Dye® 800CW Goat anti-Rabbit</td>
</tr>
<tr>
<td></td>
<td>1μL Ab:3000μL BB</td>
<td>1μL Ab:5000μL BB</td>
</tr>
<tr>
<td>Gel 2</td>
<td>Monoclonal Anti-β-actin</td>
<td>IR Dye® 680RD Goat anti-Mouse</td>
</tr>
<tr>
<td></td>
<td>1μL Ab:5000μL BB</td>
<td>1μL Ab:5000μL BB</td>
</tr>
</tbody>
</table>

**Figure 3:** Polyclonal and Monoclonal Primary and Secondary Antibody Concentrations for Western Blots performed with ideal dilutions
The secondary antibodies used were IR Dye® 680RD Goat anti-Mouse Secondary Ab 1:5000 and IR Dye® 800CW Goat anti-Rabbit Secondary Ab 1:5000 from LI-COR®, Lincoln, NE. 5ml of anti-mouse secondary antibody (dilution 1:5000) was added to the four membranes treated with monoclonal antibody and incubated in dark for 1 hour. 5ml of anti-rabbit secondary antibody (dilution 1:5000) was added to the four membranes treated with polyclonal antibody and incubated in dark for 1 hour. After secondary antibody incubation, the membranes were washed with 0.1% Tween 20 for 4 times for 5 minutes. Membranes were incubated at room temperature in the appropriate secondary antibody for 1 hour. After 1 hour, the secondary antibody was discarded and nitrocellulose membranes were washed with 0.1% PBS in 0.1% Tween 20 by covering the membranes with the washing buffer and incubating it for five minutes with agitation on a rocking platform. After the five minute incubation, the wash was discarded and membrane was covered with fresh washing buffer. Membrane incubation with washing buffer was completed four times. A final wash with 0.1% PBS was performed as described for five minutes. The final wash buffer does not contain 0.1% Tween 20. The washing buffer was then discarded and nitrocellulose membranes were removed from containers and placed on absorbent paper to dry. The membranes treated with primary monoclonal and primary polyclonal antibodies were scanned using the Odyssey Infrared Scanner at 700 nm and 800 nm, respectively.

An equalization of loading GFP samples was done using the housekeeping gene, β-actin. In addition to the transgenic testis sample, homogenized intestine, brain, and heart from the male Zebrafish, along with ovary and muscle tissue from a female Zebrafish, were used to acquire more conclusive results of GFP expression in the testes.
(and ovarian) transgenic Zebrafish tissue. Western blots for the ovary and testis are expected to show a 27 kDa band showing the expression of GFP.

**Immunohistochemistry:**

Immunohistochemistry is a procedure in which tissues are stained with a particular antibody in order to determine the location and distribution of target antigens in a tissue. Immunohistochemistry (IHC) is widely used in biological research and diagnosing diseases like cancer. While both chromogenic and fluorescent methods can be used to detect antigens in IHC, in this study we used the chromogenic method. In this method an insoluble colored precipitate is formed when a substrate such as DAB is added in the presence of the targeted antigen. Using the right antibody at the right concentration (to prevent non-specific binding) is key in detecting a signal from an antigen present in the tissue sample in question. One point five per-cent Goat Serum Protein Blocking Buffer was used to block any endogenous biotin enzymes before antibody staining. Abcam ab64261- Rabbit specific HRP/DAB (ABC) Detection Immunohistochemistry (IHC) kit (ABCAM®, Cambridge, MA) was used to stain the tissues in order to detect the presence of green fluorescent protein (GFP).

Tissue samples of the brain, intestines, and testis from Transgenic male # 6 [Figure 3] were embedded in cell blocks and then transferred onto slides using a microtome. The tissues were deparaffinized by baking them for 30 mins at 65°C and then rehydrated by serial washing in xylene, followed by washing them in 100%, 95%, 70%, and 50% ethanol for 3 mins each. Using a liquid repellent marker, each tissue section was
clearly defined. Hydrogen peroxide block was added to each of the sections and left to incubate for 10 mins, after which the sections were washed twice with 0.1 % PBS.

Heat induced epitope retrieval was performed for antigen retrieval in a microwave setting. Trisodium Citrate buffer was added to a microwaveable container in which the slides were placed. The samples were placed in the microwave and brought to boil for about 10 mins. They were then removed and allowed to cool to room temperature and washed three times with 0.1% PBS. IHC staining was performed in accordance with the manufacturer’s instructions (ABCAM®, Cambridge, MA). Figure 4 is a description of IHC samples and the respective antibody dilutions used. A protein block was used after which the tissues were incubated with the primary antibody at various concentrations.

The primary antibody used for this study was polyclonal anti-GFP. The primary antibody binds with the target antigen, which is GFP. A labeled secondary antibody, Biotinylated Rabbit Anti-Polyvalent, is added which binds to the primary antibody. The secondary antibody is further amplified by the addition of Streptavidin Peroxidase which increases the sensitivity of the antigen detection. A positive immuno-reaction that can be visualized by a dark brown stain is obtained when DAB Chromogen substrate is added to the tissues with the primary and secondary antibodies bound to the targeted antigen, GFP. It was further incubated with Biotinylated Goat Anti-Polyvalent, Streptavidin Peroxidase, DAB Chromogen in DAB Substrate, and counterstained with washes of 0.1% PBS between each incubation. The slides were then cover-slipped and ready for interpretation.
<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Description</th>
<th>Antibody Dilutions</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Control 1:500</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Exp (GFP) 1:500</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control 1:2500</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Exp (GFP) 1:2500</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Exp (GFP) 1:5000</td>
</tr>
</tbody>
</table>

**Figure 4:** Immunohistochemical sample descriptions and antibody dilutions

**Hematoxylin and Eosin:**

Hematoxylin and Eosin or H&E is a commonly used staining technique in animal histology to detect morphology of the tissues. Hematoxylin, a basic dye, stains the nuclei, ribosomes, endoplasmic reticulum, and other basophilic structures a purplish blue. Eosin, the acidic dye, stains eosinophilic structures like the cytoplasm a pinkish red. The deparaffinized tissue sections were stained according to manufacturer’s protocol in an automated staining machine (Leica Autostainer XL, by Leica Biosystems, Buffalo Grove, IL).

**Results:**

The Zebrafish mating protocol was successful. The males from the offspring were selected and DNA was extracted from subjects to verify if the transgenesis was successful. The extracted DNA was amplified by polymerase chain reaction.

**Polymerase Chain Reaction (PCR):**

To determine if CYP19A1 was incorporated into the zebrafish genome, DNA was isolated from tissue samples of zebrafish, amplified with PCR, and run through gel electrophoresis. Figure 5 shows bands at around 700 bp which is expected for transgenic
zebrafish. EGFP, which was incorporated into the vector construct with CYP19A1 as the primer, contains 720 bp and a PCR product with a band around 700 bp is a good indicator of a successful transgenesis. A band around 700 bp was observed in a gel run with ovarian tissue, but the results were not included.

Figure 5:
Lane 1 shows the DNA ladder.
Lane 2 is the genomic DNA from the wildtype zebrafish was used as a negative control.
Lane 3 and 4: samples of Males- M1 and M2 with bands around 700bp which indicate the presence of EGFP in the DNA isolated from zebrafish tissues.

Western Blot:
Next, protein was extracted from various tissue samples of male and female zebrafish and analyzed for the presence EGFP. Figure 6 depicts a western blot that analyzed the presence of EGFP in various transgenic zebrafish tissues. The tissue from the male testis (Lane T) tested positive for EGFP and the brain tissues (Lane B) also showed a weak expression of EGFP. This presence of EGFP in the testes as attested by the positive western blot indicates the successful integration of CYP19A1 in the
transgenic male zebrafish. The weak band in lane B containing the brain tissue may be
due to the isoform of the CYP19 gene, CYP19B1 inducing the expression of EGFP.

**Figure 6 A:** Infrared scanning of western blots of various transgenic fish tissue samples
with polyclonal antibodies. HeLa cells-control (HC), HeLa cells with EGFP (HG),
Intestines (I), Brain (B), Testis (T), Heart (H), Ovary (O), Molecular weight marker
(MW). **Figure 6B:** Loading control β-actin was equalized among all tissues.

**Western Blot of EGFP Polyclonal Antibody** depicting a membrane exposed to a
polyclonal antibody scanned at 800 nm using the Odyssey Infrared scanner. The dilution
of the primary polyclonal antibody used is 1µl Ab: 3,000µl BB and secondary Ab 1µl Ab:
5,000µl BB. The gel has samples taken from a male transgenic fish as well as a female
transgenic fish;

HC is loaded with HeLa cells;
HG is loaded with HeLa cells with EGFP
I- Protein from the intestines of transgenic male Zebrafish #7
B - Protein from the brain of transgenic male Zebrafish #7
T - Protein from the testis of transgenic male Zebrafish #7
H- protein from the heart of transgenic male Zebrafish #7
O: extracted protein from the ovaries of transgenic female fish #1

There is also a kaleidoscope molecular weight ladder loaded in the lane MW. Lanes HG, B, T and H show the expression of a protein about 27 kDa. The pink arrows indicate the expression of a protein about 27 kDa in lanes HG, B, T and H corresponding to EGFP.

**Figure 7: Densitometric analysis.** Relative density of EGFP intensity normalized to β-actin intensity

Beta-actin blot was scanned at 700nm and used as a loading control to ensure equal loading of sample tissues. The 42 kDa β–actin band was observed in all the tissue samples [Figure 6]. A low value of β-actin in the intestine could be due to degradation by proteolytic enzymes in the intestine. A quantitative analysis of the signal intensity of EGFP and β–actin bands was done through densitometry to confirm similar loading of all tissue samples. The relative density of EGFP intensity normalized to β-actin is depicted as a graph [Figure 7]
The transgenic zebrafish tissues were analyzed for EGFP expression by Western Blot using primary polyclonal antibodies at 1:3000 concentrations. EGFP expression was seen in the brain, testis and ovary at 27 kD. The intensity of the bands was similar in the testes and ovaries but much weaker in the brain. The bands present around 20 kD could potentially be degraded proteins.

The aromatase gene, CYP19 is present in two isoforms, CYP19A1 expressed mainly in the gonads and CYP19B1 present in the brain (Goldstone, et al 2010). The presence of a weak band in lane 4 containing the brain tissue may be due to the isoform of the CYP19 gene, CYP19B1 promoter inducing the expression of EGFP.

**Immunohistochemistry:**

Finally, the tissue samples were subjected to immunohistochemical staining wherein they were stained with the antibody, anti-GFP in order to determine the location and distribution of EGFP antigens in the tissue samples. Various concentrations (1 milliliter anti-GFP antibody: 500 ml 0.1% PBS buffer, 1: 2500, 1: 5000) of anti-GFP protein were used in immune staining the tissues of the male testis to determine the presence of EGFP in the tissues. A dark brown stain in the testis (and weakly in the brain tissues) confirmed the presence of EGFP in the zebrafish testes further indicating a successful transgenesis. The most ideal results were observed at 1:500 anti-GFP dilutions. Figure 8 B with the 1:500 anti-GFP dilution shows a stronger immunohistochemical signal when compared to Fig 8 D with the 1: 2500 anti-GFP dilution. The tissues in Fig 8 A, C, and E were negative controls that were subjected to a
non-immune serum instead of the primary antibody anti-GFP. Positive results were also obtained with ovarian tissues, but the results are not shown.

Hematoxylin and Eosin (H&E) stains were supplementarily performed to analyze the morphology of the testis and compare them to the immunohistochemical stains. The morphology (Figure 8) of the tissues that tested positive for EGFP indicates the presence of this protein in the Leydig and Sertoli cells of the testes. This substantiates the study by (Hinfray, et al., 2013) wherein isoforms of CYP19A1 were observed to be synthesized in the testes. Further, in corroboration with the western blot results, weak but positive results were observed in the brain tissue possibly due to the presence of CYP19A1b in the brain as was observed in a study on embryonic expression of brain aromatase cyp19a1b in zebrafish by Lassiter and Linney in 2007.
Figure 8 a-f: Immunohistochemical stains for verifying the presence of green fluorescence protein (GFP) in the male testis at various concentrations, male intestine, and brain tissue. The darker brown stain (arrows) indicates a positive reaction for GFP. Non immune serum was used as a negative control.
Figure 9: Left: Immunohistochemical stain of male transgenic Zebrafish #6 male (anti GFP 1:2500 pH 6), showing expression of GFP (blue arrow) in Sertoli cells and Leydig cells. Right: Schematic representation of testis tissue cell types, the green arrow represents Leydig cells and the orange arrow represents Sertoli cells. (Menke, et. al., 2011)

Discussion:

Zebrafish has served as a versatile model for transgenic studies because of its short gestational period, the large number of eggs produced, and the translucency of its eggs allowing for visual observation of embryonic development (Segner, 2009). The zebrafish eggs were microinjected with transposable elements with relative ease resulting in viable transgenic zebrafish that could be used for this project.

Using the tol2 system, a three fragment vector construct with the promoter CYP19A1, the marker EGFP, and minitol2 genes was prepared and micro-injected into zebrafish eggs. CYP19A1 was picked as a promoter because it is a gene involved in estrogen biosynthesis from androgens that worked well in the three fragment vector.
construct. EGFP was used as a marker to determine if CYP19A1 successfully acted as a promoter in incorporating EGFP into the zebrafish genome.

The purpose of this study was to show that CYP19A1 can effectively function as a gonadal specific promoter. Successful integration of CYP19A1 would enable it to act as a testes specific promoter that can be used to successfully express transgenes such as ICER in the testes. CREM-deficient mice have previously been shown to experience arrested spermatogenesis leading to infertility (Nantel, et al., 1996). Over-expression of ICER, the isoform of CREM that negatively auto regulates CREM, could therefore potentially increase the number of spermatids and spermatozoa. ICER has been targeted in the past for expression in the ovaries of transgenic mouse models to generate higher levels of ovulation (Muniz, et. al., 2012 in preparation).

In order to determine if CYP19A1 performed successfully as a gonadal specific promoter and incorporated EGFP into the zebrafish genome, DNA was isolated from the male zebrafish tissues and analyzed. The tissue samples were amplified with PCR, subjected to western blot analysis, and immunohistochemically stained to test for the presence of EGFP. Each of the tests were positive which conclusively demonstrated the presence of EGFP in the tissues of both the testes and ovary (ovarian test results not shown), thereby evidencing the efficiency of CYP19A1 as a gonadal specific promoter. In brief, it can be summarized that the aromatase CYP19A1 gene is an effective tissue-specific promoter in zebrafish and can be used to study genes in the testes of transgenic zebrafish.

The successful microinjection of the CYP19A1: EGFP vector construct that resulted in transgenic zebrafish implies that CYP19A1 can be used as a gonadal specific
promoter. The tol2 system can now potentially be used to microinject zebrafish eggs with the isoform of cAMP response element binding protein (CREB) and cAMP response element modulator (CREM), ICER using CYP19A1 as the promoter in the vector construct.

While the results obtained were promising, the sample size used in this study (tissues obtained from brain, heart, intestines, and testes of 2 transgenic male zebrafish) was small. The reason for this was the limited quantity of the F2 generation available for research. Not all the fish from this generation were transgenic and some of the fish needed to be preserved to spawn offspring for the F3 generation. The small sample size limits the generalization that CYP19A1 can definitively be used as a gonadal specific promoter, but shows its potential as one. A statistical analysis could not be conducted due to the limited sample size. An increase in the number of specimens analyzed would produce more conclusive and definitive results.

Since the morphology of the F2 generation did not show any abnormalities, the remaining zebrafish will be allowed to reach adulthood and possibly breed the next generation. If viable F3 offspring are produced, they will be observed for EGFP expression and any mutations. Not all of the F3 generation will be expected to express EGFP in the testes or ovaries. However, a successfully spawned F3 generation could indicate that CYP19A1 can be used as a promoter with the tol2 system to integrate other genes like ICER into the zebrafish genome.

CREM-deficient mice in a study by Nantel, et. al (1996), showed a significant reduction in sperm production due to decreased late spermatid production, and all
together lack spermatozoa. Therefore, manipulating CREM expression, or that of its isogene- ICER and over expressing the gene could significantly improve the sperm production in the male reproductive organs. Although ICER negatively auto regulates its expression, as well as that of CREB/CREM system (Molina, et. al., 1993), hypothetically replacing the promoter region on ICER with another promoter such as CYP19A1 may lead to over-expression ICER in the cells.

Making use of the gateway technology, the *tol2* system could prove valuable in introducing ICER, with CYP19A1 as the promoter, into the *Danio rerio* genome to influence sperm production in male zebrafish. An amplification of ICER or CREM expression could have a substantial positive effect on fishing industry- where there is an increased demand for fish because of overfishing. CYP19A1 as a gonadal-specific promoter has the potential to increase ovulation thereby having significant implications for future research in fertility treatments and boosting livestock production. This could have a positive impact on fishing industries that are trying to keep up with the demand for food in a more health conscious society.

Over expression of ICER could also potentially lead to more significant research in male infertility treatments where there is a considerable scarcity in available options to males who, have a lower sperm count or are infertile, and wish to conceive. This avenue of fertility treatments could give hope to the previously infertile males and older couples that would like to have offspring of their own.
Works Cited


