Generation and Characterization Ovarian Specific Promoter Sequences of a Transgenic Zebrafish (Danio rerio)

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ABSTRACT

Inducible cAMP early repressor or, ICER, acts as a tumor suppressor with ovarian specific properties and moderates cAMP anti-proliferative actions. In this study, CYP19A1 was selected out of the four previously cloned ovarian specific promoters (CYP19A1, FSH, ZP3 and GDF9). The purpose of this experiment was to test whether a ~500 bp fragment of the CYP19A1 can function in the ovaries as a specific promoter that can be used to express transgenes in the ovaries. For this study, we generated transgenic Zebrafish that expressed Green Fluorescent Protein (GFP) with the CYP19A1 promoter and proceeded to characterize by four different methods which were western blot, direct fluorescent visualization of tissue, immunohistochemistry, and PCR. The results of the study show that CYP19A1 was successfully expressed in the ovaries which also indicates that the selected promoter region of CYP19A1 can be an ovary specific promoter. This promoter can then be used in future experiments for the expression of relevant genes such as ICER, in the ovaries. If successful, overexpression of these promoters in the ovaries of Danio rerio causes an increase in ovulation, this can have significant implications for future research for increasing the production of fish.

Key words: ICER, CYP19A1, EGFP, Transgenesis
Generation and Characterization Ovarian Specific Promoter sequences of a Transgenic Zebrafish (Danio rerio)

by

Riham Makhoul

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INTRODUCTION

As a ubiquitous second messenger cAMP or cyclic adenosine monophosphate has an important role which is played in the intracellular signal transduction in several different organisms. Through adenylyl cyclase, cAMP is synthesized from adenosine triphosphate. A receiver of cAMP downstream regulation will be the cAMP-dependent protein kinase A (PKA). PKA, an enzyme, is composed of four different sections, two are regulatory and the other two are catalytic subunits (Gellersen & Brosens, 2003).

In order to phosphorylate the target molecules in the cytoplasm the two molecules of cAMP bind to regulatory subunits of PKA which then undergoes a conformational change that will activate the catalytic subunits. Alteration of the transcriptional activity of cAMP response element binding protein (CREB) and cAMP response element modulator (CREM) can also activate PKA which can be diffused through the nucleus (Gellersen & Brosens, 2003).

Due to its leucine zipper and highly conserved DNA recognition region, CREB and CREM are members of the ZIP family of transcription factors (Walker et. al, 1998) (Gellersen & Brosens, 2003). According to Don and Stelzer in 2002, both CREB and CREM are known for their various alternative spliced forms and some may lack the activation domains which will result its function as a transcription suppressor. Don and Stelzer also stated that ICER is a suppressor isoform of CREM that is activated by CREB. ICER is an auto regulator and a down regulator of CREB expression (Don & Stelzer, 2002).

ICER as mentioned earlier is an isoform of CREM that encodes a repressor of cAMP responsive element and negatively auto regulates itself. Auto regulation is possible
due to the presence of P2, an intronic promoter, which is located near the 3' of the gene. Due to the close configuration of the region, it is possible to have an interaction between the binding sites. Over expression of ICER in cells can be seen by replacing the promoter region on ICER with different promoters.

A small protein that is encoded by the CREM gene is ICER, inducible cAMP early repressor. ICER functions as a gene suppressor that contains DNA-binding domain. ICER is expressed in testes and ovaries. Infertility in male mice have been seen from the knockout of the CREM gene (Nantel, et al., 1996). An example of how ICER may play a role in fertility is seen in transgenic mice models. The female mice have hyper-ovulation and the male mice have an enlargement of the testes (Muniz, et. al., 2012 in preparation). Zebrafish, Danio rerio, has been known to serve as a good experimental model that express ICER. Zebrafish secrete similar gonadotropins to humans such as the follicle stimulating hormone (FSH) and the luteinizing hormone (LH) (Goldstone, 2010).

The zebrafish emerged as an ideal candidate in vertebrate biology for studying various human diseases. Generation time for zebrafish is from three to four months, and since zebrafish reproduce year-round, they can be easily bred in a continuous fashion in the laboratory (Segner, 2009). Females spawn once per week and can produce up to or greater than 200 eggs (Segner, 2009). Zebrafish have external development, during which embryogenesis and development can be observed due to the translucency of the embryo (Segner, 2009). High fertility levels and short generation times also make zebrafish easy to manipulate for genetic study. Zebrafish are also particularly well suited as endocrine study models which is aided by an increase in the knowledge of the hypothalamic-hypophyseal-gonadal axis (Segner, 2009). Transgenic zebrafish strains have been shown
to be suitable in all life stages in studying endocrine disrupting compounds as phenotypic changes can be measured according to changes in gene expression (Segner, 2009).

To make use of transposable elements and to integrate genomic expression a technique called transgenesis is used. Into the fertilized egg, plasmid DNA is inserted which allows integration into the genome which is then spread into the germ lineage. Zebrafish is the ideal candidate for transgenesis because of the rapid embryonic development. Transgenesis was difficult in the past due to three issues. These three issues are low transgenesis efficiency, using conservative subcloning a compound expression construct can be made, and in transient transgenics mosaicism can be formed and rare germline incorporation; and there was a difficulty in identifying germline (Kwan, et al., 2007). Now, transgenesis is easier due to commercial available kits such as \textit{tol2}.

In this study the \textit{tol2} kit was used. The \textit{tol2} system resolves the three above issues by allowing fast and easy construct building and site specific recombination cloning. The kit has several main purposes to simplify the process. For instance, clone sharing is simple, transgenesis probability increases, and elimination of fluorescent markers as a primary source of transgene identification (Kwan, et al., 2007). The \textit{tol2} system has become the conventional method of creating transgenic zebrafish because the zebrafish eggs can be easily microinjected with transposable elements (Kawakami, 2004). To monitor the transgene expression green fluorescent protein known as GFP is attached to the tissue specific promoters.

Figure 1, shown below, is a diagram that shows the plasmid DNA being inserted into a zebrafish using the \textit{tol2} system. A vector construct and mRNA encoding transposase was inserted. The vector construct consists of \textit{tol2} elements at the ends,
CYP19A1 promoter, and EGFP.

Figure 2, shown below, shows the sequences of the CYP19A1: EGFP vector construct bracketed by the Tol2 genes. The purpose of mRNA is to translate the transposase protein and catalyzes the cutting out of the transposon construct from the plasmid donor. The excised DNA is then able to stably integrate into the genome resulting in transgenic zebrafish in 50-70% of injected fish (Kawakami, 2007). Once the vector construction was completed, the CYP19A1: EGFP promoter construct was injected into the zebrafish eggs generating transgenesis into the *Danio rerio* in Dr. Hatem E. Sabaawy’s Lab at Rutgers Medical School.
In the present study, Zebrafish was transfected with a plasmid containing a gonadal-specific promoter (aromatase gene: CYP19A1) and a reporter gene (enhanced...
green fluorescent protein, EGFP). Tissue samples from transgenic female fish were isolated and several techniques will be utilized to determine whether the expression of EGFP is tissue-specific when under the control of the aromatase promoter.

In the first experiment, genomic DNA extraction was done using TRIZOL reagent which was followed by PCR.

In the second experiment, a series of Western blots were performed on the tissues of interest taken from the transgenic zebrafish which will be incubated with either primary monoclonal or primary polyclonal antibodies, at varying concentrations, to determine the best antibody conditions for EGFP detection. Once optimal antibody conditions are determined, a second experiment will be performed.

In the third experiment, two western blots were performed with tissue samples derived from the same male and female transgenic zebrafish (samples include: male testis, intestines, heart, brain and female ovary and muscle), our main focus is the female transgenic zebrafish ovaries. One western blot was incubated with polyclonal anti-GFP antibody and the other with an antibody against the housekeeping gene, Beta actin. Due to the presence of the aromatase promoter, expression of EGFP is expected only in the testis and ovary tissues of transgenic zebrafish. The results that were obtained in the western blot experiments validated that CYP19A1 could be used to study ICER expression specifically in the ovaries.

Since the western blot was a success, a direct fluorescent visualization of the ovarian tissue experiment was done.

The final experiment was performed in order to observe the expression of GFP under the control of the CYP19A1 promoter in the transgenic Zebrafish tissue under the
microscope, immunohistochemistry will be performed using an anti-GFP polyclonal antibody.

MATERIALS AND METHODS

Mating Zebrafish

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 on September 23, 2013.

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 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. Once the embryos are hatched further testing will be conducted, these methods will be discussed below.

DNA extraction and PCR
Genomic DNA extraction using TRIZOL reagent:

DNA was isolated from the tissues of the F2 generation of transgenic 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 procedure is as follows: Wash cells three times with ice-cold PBS. Add 1 ml (per 25cm² flask) passing cell lysate several times through pipette. Incubate at room temperature for 5 mins. Add 200μl chloroform to each tube, shake vigorously by hand for 15 sec and incubate at room temperature for 2-3 minutes. Centrifuge at 12,000 RPM for 15 minutes at 4°C. The TRIZol® Reagent from the Invitrogen DNA extraction kit (by Life Technologies, Grand Island, NY) was used for this purpose.

Collect phenol lower phase (red) and interphase after removing upper aqueous phase. Add 300μl of 100% ethanol and mix by inversion. Incubate at room temperature for 2-3 minutes. Centrifuge at 12000 RPM for 5 minutes at 4°C. Remove supernatant. Wash twice with 1ml 0.1M sodium citrate with 10% ethanol. At each wash incubate for 30 minutes with periodic mixing. Centrifuge at 12000 RPM for 5 minutes at 4°C. Following the two washes, suspend DNA pellet in 1.5 ml 75% ethanol, incubate 10-20 minutes at room temperature with periodic mixing. Centrifuge at 12000 RPM for 5 minutes at 4°C. Air-dry pellet. Dissolve DNA in 50-200ul ultrapure PCR grade water. Incubate samples at 55°C for 10 minutes to completely dissolve DNA. Centrifuge at 12000 RPM for 10 minutes at 4°C to pellet. Collect supernatant and store at -20°C.

PCR Protocol:

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. To each centrifuged tube add and mix 45μl of Platinum PCR SuperMix High Fidelity, Primer Solution, and Template DNA solution. Cap the tubes and load the tubes in the thermal cycler. To completely denature the template and activate the enzyme incubate the tubes at 94°C for two minutes. 35cycles of PCR amplification will be conducted as follows, denature 94°C for 30 seconds, anneal 55°C for 30 seconds and extend 68°C for 1 min per kb.

The primers used to amplify a 714bp fragment of GFP were

GACGTAACGGCCACAAGTT 3' the left primer and
TGCTCAGGTAAGTGTTGTCG 3' 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 wild type zebrafish was used as a negative control.

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 ovaries of Zebrafish. For this experiment, protein is extracted and subject to gel electrophoresis, the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) technique by BIORAD (BIORAD, 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 antibody. 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. Procedure is as follows below.

Part 1: Determination of optimal antibody (polyclonal vs. monoclonal) and the optimal concentration for EGFP detection by Western blot.

Rapid Total Protein Extraction from tissue sample:

Obtain the ovaries and muscle of female transgenic zebrafish to create tissue. Add 350mL of 1X Laemmli buffer to the ovaries and 400mL of 1X Laemmli buffer to the muscle. Homogenize the tissue using a plastic-Teflon homogenizer until samples were viscous. Heat 5 min at 95°C in heating block. Samples at room temperature were micro centrifuge for 10 minutes at 13,000 rpm. Transfer supernatant to a clean 500μl micro centrifuge tube. Store supernatant samples at -20°C for SDS/PAGE.

SDS/PAGE:

A Mini-PROTEAN precast gel was obtained commercially and used for SDS/PAGE Electrophoresis. Tape was removed from the bottom of prepared gels and gels were loaded into gasket. 1X Running Buffer was loaded into center of gasket until
wells were covered with buffer. After the buffer was loaded, the gasket was left standing until it was confirmed that no leaks were present in set up. Teflon comb was removed from gel and each well was rinsed with 1X SDS running buffer using a 1mL pipette. The gasket was loaded into electrophoresis chamber and was filled with recommended 1X SDS Buffer appropriate for 4 gels. A micropipette was used to load 10μL of female tissue samples (the ovary and muscle samples were obtained from total rapid protein extraction from female transgenic zebrafish), 5μL of control sample (negative control samples were obtained from HeLa cell extracts and the positive control samples were obtained from HeLa cell extracts transfected with the pCMV vector containing EGFP. Cells were confirmed to have successfully been transfected by microscopic observation of green fluorescence) and 5μL of Kaleidoscope Molecular Weight Ladder into appropriate wells. Once all wells were loaded, power supply was connected to the cell and run at 200 volts at constant voltage for 40 minutes. Once the tracking dye had reached the bottom of the gel, the power was disconnected. The electrode buffer was discarded and the upper chamber removed with attached gel sandwich. Plates were carefully pried apart and the gel was carefully removed.

Western blot:

Western blot casing was placed in a small container. Sponge pad was placed on top of black side of case and sponge pad was covered with transfer buffer. Filter paper was placed on top of sponge pad and was covered with transfer buffer. Gel membrane from SDS/PAGE was carefully placed on the filter paper, followed by nitrocellulose membrane (0.45 μm), filter paper and second sponge pad facing red side of western blot casing. The Western blot casing was tightly closed to ensure no air bubbles were present.
between layers within casing. The entire casing was placed in an electrophoresis chamber. The electrophoresis chamber was filled with transfer buffer. A frozen ice pack was placed inside the electrophoresis chamber to keep reaction cool during transfer. A magnetic stir bar was added to the chamber. The chamber was placed on top of a magnetic stirrer device and stirring rate was turned on medium speed. Electrophoresis chamber was connected to the power supply at 100V for 45 minutes. After 45 minutes, power supply was disconnected and nitrocellulose membrane was carefully removed from Western Blot encasing. Nitrocellulose membrane was placed in a container filled with blocking buffer to cover the membrane. Membranes were incubated overnight with agitation on a rocking platform at 42°C. After overnight incubation, the blocking buffer was discarded and primary antibody dilutions were prepared. Each of the 4 gel membranes were cut in half so that each gel contained each of the following lanes: positive control sample, negative control sample, and ovary sample. Four of the gels also contained the MW ladder. Each of the gels was placed in a separate container and was incubated with either Monoclonal or Polyclonal Primary Antibody at one of the eight antibody concentrations. The monoclonal antibody concentrations went as followed for the four gels: gel A 1μl AB: 500μl BB, gel B1 μl AB: 1000μl BB, gel C1 μl AB: 2000μl BB, and gel D1 μl AB: 3000μl BB. The polyclonal antibody concentrations went as followed for the four gels: gel A 1μl AB: 1000μl BB, gel B1 μl AB: 2000μl BB, gel C1 μl AB: 2500μl BB, and gel D 1 μl AB: 3000μl BB. The membranes were incubated at room temperature with agitation on a rocking platform for 6 hrs. After incubation was complete, primary antibodies were discarded. The membrane was washed four times with PBS in 0.1% Tween 20 for five minutes with gentle shaking and secondary antibodies
were diluted according to the calculations above. The membranes were incubated at room
temperature in the appropriate secondary antibody for 1 hour. After 1 hour, secondary
antibody was discarded and nitrocellulose membranes were washed with PBS in 0.1%
Tween 20. To wash, the membranes were covered with washing buffer and were
incubated for five minutes with agitation on a rocking platform. After five-minute
incubation, wash was discarded and membrane was covered with fresh washing buffer.
Membrane incubation with washing buffer was completed four times. A final wash with
PBS was performed as described for five minutes. The final wash buffer does not contain
0.1% Tween 20. Wash buffer was discarded and nitrocellulose membranes were removed
from containers and placed on absorbent paper to dry. Membranes treated with primary
monoclonal and primary polyclonal antibodies were scanned using the Odyssey Infrared
Scanner at IR Dye 680RD Goat anti-Mouse and IR Dye 800CW Goat anti-Rabbit,
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.

Part 2: Determine the expression of green fluorescent protein in transgenic zebrafish
tissue by Western blot.

Rapid Total Protein Extraction from tissue sample:

Tissue samples were obtained from one female transgenic zebrafish. Protein
samples were obtained from tissue using the rapid total protein extraction method
described above. A micropipette was used to load 10μL of female tissue samples (the ovary and muscle samples were obtained from total rapid protein extraction from female transgenic zebrafish), 5μL of control sample (negative control samples were obtained from HeLa cell extracts and the positive control samples were obtained from HeLa cell extracts transfected with the pCMV vector containing EGFP) and 5μL of Kaleidoscope Molecular Weight Ladder into the appropriate wells. The cells extracted were confirmed to have successfully been transfected by microscopic observation of green fluorescence.

Western blot:

Two Western blots were performed from the gel membranes obtained from SDS/PAGE described above. Western blots were performed as described but the gel transfer was completed for 1 hour instead of being incubated overnight. The primary and secondary antibody concentrations for SDS/PAGE were different. In the first gel, the primary antibody was Polyclonal Rabbit Anti-GFP 1μL Ab: 3000μL BB, product from Abcam, and the secondary antibody was IR Dye® 800CW Goat anti-Rabbit 1μL Ab: 5000μL BB, product from LICOR. In the second gel, the primary antibody was Monoclonal Anti-β-actin 1μL Ab: 5000μL BB, product from Abcam, and the secondary antibody was IR Dye® 680RD Goat anti-Mouse 1μL Ab: 5000μL BB product from LICOR. Incubation times and methods are just as described above in part one.

Direct fluorescence visualization of tissue

In order to visualize the nuclear DNA in either living cells or fixed cells a fluorescent stain is used. This fluorescent stain is known as 4', 6-diamidino-2-phenylindole, better known as DAPI. Gross cell morphology and the number of nuclei present can be determined by using DAPI staining. After the cells are stained the sample
is ready to be analyzed by a fluorescence microscope (Tarnowski, et al., 1991). The procedure used in this study is discussed below.

Ovarian tissue from EGFP-transgenic zebrafish was paraffinized and cut into 1 micron thick sections. Slides were washed two times with xylene 5 minutes each. Series of ethanol dilutions were done to rehydrate the tissue as follows: 100% ethanol two times for 3 minutes each and 95% ethanol, 70% ethanol and 50% ethanol (one time for 3 minutes each). The slide was washed two times with PBS for 5 minutes and stained with Vectashield (containing DAPI) and observed under fluorescent microscope using blue and green filters.

_Tissue extraction and IHC_

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 a key in detecting a signal from an antigen present in the tissue sample in question. One point five percent 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).
Part 1: Determination of GFP expression in testes and ovaries of Transgenic Zebrafish cells by Immunocytohistochemistry with reviewed polyclonal antibody concentration, deparaffinization and antigen-retrieval pH.

Deparaffinization Protocol for Zebrafish tissue samples:

Obtain 3 slides of ovarian tissues embedded in parafilm and mounted on a microscope slide. Heat the samples in a dry oven at 57°C for 30 minutes, positioned so as to allow drainage of melting paraffin. Perform the following washes: Xylene: 3 times for 5 min, Xylene 1:1 with 100% ethanol once for 2 min, 100% ethanol twice for 2 min, 95% ethanol once for 2 min, 75% ethanol once for 2 min, 50% ethanol once for 2 min, finally run cold tap water to rinse the slides for 1 min. Place the tissue samples in PBS for a quick rinse then add one drop of H2O2 for 10 minutes. Rinse with PBS 2 times.

Revised Antigen Retrieval for Zebrafish tissue samples:

Add the sufficient antigen retrieval buffer (0.01M Na Citrate) with three different pH: 6, 7 and 8 to cover the microscope slide in a small beaker. Place citrate beakers with sample slides in the microwaveable vessel containing boiling water (water bath). Boil samples for 5 minutes and from this point set microwave to medium-high power and continue to heat samples for additional 10 minutes. Remove the citrate beaker from water bath and cool to room temperature for 20 minutes. Use care with hot solution. Wash tissue samples with PBS 3 times. Draw circular hydrophobic barriers with a Liquid Blocker Pap Pen around the tissue samples for all 3 slides.

Staining Protocol for Zebrafish tissue samples:

Apply 1.5% Goat Serum Protein Blocking Buffer and incubate for 10 minutes at room temperature to block nonspecific background staining. Wash once in PBS. Apply
polyclonal anti-GFP primary antibody at 1:500, 1:2500 and 1:5000 concentration and
Pre-immunized Rabbit Serum negative control to each tissue circle respectively incubate
overnight at 4°C. Wash 4 times in PBS. Apply 1 drop of Biotinylated Rabbit Anti-
Polyvalent to each sample and incubate for 10 minutes at room temperature. Wash 4
times in PBS. Apply 1 drop of Streptavidin Peroxidase to each sample and incubate for
10 minutes at room temperature. Rinse 4 times in PBS. Add 30 μl (1 drop) DAB
Chromogen to 1.5 ml (50 drops) of DAB Substrate, mix by swirling and apply to tissue.
Incubate for 5 minutes. Rinse 4 times in PBS. Add a drop of crystal mount to each tissue
sample. Apply coverslip and view samples under microscope.

Part 2: Determination of GFP expression in different Transgenic Zebrafish tissues by
Immunocytohistochemistry with different polyclonal antibody concentrations diluted in
1.5% goat blocking serum.

Deparaffinization Protocol for Zebrafish tissue samples:

Obtain 3 slides of ovaries tissues embedded in parafilm and mounted on a
microscope slide. Heat the samples in a dry oven at 57°C for 30 minutes, positioned so as
to allow drainage of melting paraffin. Perform the following washes: Xylene: 3 times for
5 min, Xylene 1:1 with 100% ethanol once for 2 min, 100% ethanol twice for 2 min, 95%
ethanol once for 2 min, 75 % ethanol once for 2 min, 50 % ethanol once for 2 min, finally
run cold tap water to rinse the slides for 1 min. Place the tissue samples in PBS for a
quick rinse then add one drop of H2O2 for 10 minutes. Rinse with PBS 2 times.

Revised Antigen Retrieval for Zebrafish tissue samples:

Add the sufficient antigen retrieval buffer (0.01M Na Citrate pH: 6) to cover the
microscope slide in a small beaker. Place citrate beakers with sample slides in the
microwaveable vessel containing boiling water (water bath). Boil samples for 5 minutes and from this point set microwave to medium-high power and continue to heat samples for additional 10 minutes. Remove the citrate beaker from water bath and cool to room temperature for 20 minutes. Use care with hot solution. Wash tissue samples with PBS 3 times. Draw circular hydrophobic barriers with a Liquid Blocker Pap Pen around the tissue samples for all 3 slides.

Staining Protocol for Zebrafish tissue samples:

Apply 1.5% Goat Serum Protein Blocking Buffer and incubate for 10 minutes at room temperature to block nonspecific background staining. Wash once in PBS. Apply polyclonal anti-GFP primary antibody and negative control to the slides and incubate at room temperature for 1 hour. The wild type ovary slide has the antibody dilutions of the control being 1:500, the experimental being 1:500, another control being 1:2500 and another experimental being 1:2500. The first ovary tissue slide has the antibody dilutions of the control being 1:500, the experimental being 1:500, and another control being 1:2500. The second ovary tissue slide has the antibody dilutions of the experimental being 1:2500, control being 1:5000, the experimental being 1:5000. Wash the slides 4 times in PBS. Apply 1 drop of Biotinylated Rabbit Anti-Polyvalent to each sample and incubate for 10 minutes at room temperature. Wash 4 times in PBS. Apply 1 drop of Streptavidin Peroxidase to each sample and incubate for 10 minutes at room temperature. Rinse 4 times in PBS. Add 30 µl (1 drop) DAB Chromogen to 1.5 ml (50 drops) of DAB Substrate, mix by swirling and apply to tissue. Incubate for 5 minutes. Rinse 4 times in
PBS. Add a drop of crystal mount to each tissue sample. Dehydrate samples if required and apply coverslip and view samples under microscope.

RESULTS

The Zebrafish mating protocol was successful. The females 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 ran through gel electrophoresis. Figure 3 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.
Figure 3:
Lane 1 shows the DNA ladder.
Lane 2: sample of female with a band around 700bp which indicates the presence of EGFP in the DNA isolated from zebrafish tissues.
Lane 3 is the genomic DNA from the wildtype zebrafish was used as a negative control.

Western Blot:

Next, protein was extracted from various tissue samples of male and female zebrafish and analyzed for the presence EGFP. Figure 4, below, depicts a western blot that analyzed the presence of EGFP in various transgenic zebrafish tissues. The tissue from the female ovaries (Lane O) tested positive for EGFP and the brain tissues (Lane B) also showed a weak expression of EGFP. This presence of EGFP in the ovaries as attested by the positive western blot indicates the successful integration of CYP19A1 in the transgenic female 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 4 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 4B: 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 female transgenic fish as well as a male 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.
Beta-actin blot was scanned at 800nm and used as a loading control to ensure equal loading of sample tissues. The 42 kD β-actin band was observed in all the tissue samples (Figure 4). 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 5).

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).
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.

Direct fluorescent visualization of tissue

After the western blot portion of the experiment showed success, the direct fluorescent of the ovarian tissue was performed. Once the tissue was stained with DAPI, it was observed under a fluorescent microscope using blue and green filters as shown below in figure 6.

![A: Tissue with DAPI under fluorescent](image1)

![B: Tissue expressing GFP under fluorescent](image2)

Immunohistochemistry of transgenic zebrafish

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 female ovaries to determine the presence of EGFP in the tissues. A dark brown stain in the ovaries (and weakly in the brain tissues) confirmed the presence of EGFP in the zebrafish ovaries further indicating a successful transgenesis. The most ideal results were observed at 1:500 anti-GFP
dilutions. Figure 7 B with the 1:500 anti-GFP dilution shows a stronger immunohistochemical signal when compared to Fig 7 D with the 1:2500 anti-GFP dilution. The tissues in Fig 7 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 ovaries and compare them to the immunohistochemical stains. The morphology (Figure 7) of the tissues that tested positive for EGFP indicates the presence of this protein in the ovaries. 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.
Ovaries tissue expressing GFP under the fluorescent microscope

Figure 8: left: Schematic of ovary tissue cell types, the blue arrow represents granulosa cells. right: Transgenic #1 female ovaries anti GFP 1:2500 pH 6, the blue arrow shows expression of GFP in granulosa cells (Ge, W. 2005). See below.

DISCUSSION

Zebrafish serve as ideal model for transgenic studies. Zebrafish have short gestational period; the embryonic development of the zebrafish is translucent, and a larger number of eggs are produced (Segner, 2009). 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.

The purpose of this experiment was to show that CYP19A1 can function in the ovaries as a specific promoter that can be used to express transgenes in the ovaries. To determine if CYP19A1 was successful as a specific promoter and EGFP has been incorporated into the genome, several tests have been conducted. DNA was isolated from the zebrafish tissue and analyzed, via PCR, western blot analysis, direct fluorescent visualization and immunohistochemistry. Detailed conclusions of all four tests are below. Each of the tests had promising results which conclusively demonstrated the presence of EGFP in the tissues, thereby evidencing the efficiency of CYP19A1 as a specific promoter.

The most effective antibody concentrations for monoclonal and polyclonal were analyzed by western blot for the ovary, positive (HeLa cells confirmed to express GFP) and negative (HeLa cells) control samples. Four separate gels were run using four different antibody concentrations on identical samples. The monoclonal antibody concentrations were 1:500, 1:1000, 1:2000 and 1:3000 and primary polyclonal antibody concentrations: 1:1000, 1:2000, 1:2500 and 1:3000. Using the Odyssey Infrared Scanner at a wavelength of 700 nm for the monoclonal antibody and of 800 nm for the polyclonal antibody the results were obtained.

The gel with the least amount of background and the most precise, sharp bands at the expected molecular weight is the optimal antibody concentration. The optimal concentration for both the primary monoclonal and primary polyclonal antibody was at the 1:3000 concentrations. In the positive control and the ovaries were expected to have
bands of 27kDa. Bands of 100kDa were observed in all the lanes. The absence of bands of 27kDa could have been possibly due to two reasons. The first reason would be that the protein was not expressed under the control of the promoter. The second reason could be that the expression of EGFP was low which was not detected even at the highest concentration of the antibody.

The polyclonal antibody yielded a higher degree of nonspecific protein binding compared to the gel treated with monoclonal antibody. The bands of 27 kDa corresponding to EGFP were seen in two lanes positive control and ovaries. The band was absent which was expected since that lane was the negative control.

The polyclonal antibody is more efficient in detecting EGFP compared to the monoclonal antibody. This was proven by the presence of the band at the expected molecular weight in the gel. Due to the above fact, experiment two will consist of analyzing the transgenic zebrafish tissue by western blot using the primary polyclonal antibody of 1:3000 which showed the best quality bands in experiment one. To normalize the results the tissues with anti-beta-actin monoclonal antibody were performed by western blot at 1:5000 concentrations. In all the transgenic zebrafish tissue (male intestines, male brain, ovaries, and female muscle) the beta-actin concentration was analyzed using the monoclonal antibodies at 1:5000 concentrations via western blot to normalize the data. There were discrete bands of 42kDa corresponding to beta-actin in all lanes but in the male intestines. There is no need to normalize the data because the 42kDa band was present in all the beta-actin transgenic tissues expect for the intestinal tissue. The absence of the band in the intestine can possibly be due to the proteolytic enzymes that had degraded the beta-actin protein.
Using the primary polyclonal antibodies at 1:3000 concentrations via western blot the EGFP expression in all the transgenic zebrafish was analyzed. Expression of EGFP of 27kDa was seen in the lanes. The thickness of the band was larger in the ovaries compared to the other tissues. The weakest intensity of the band was shown in the brain tissue of the zebrafish. CYP19, an aromatase gene, is present in two isoforms. The first isoform is CYP19A1, which is expressed in the gonads. The second isoform is CYP19B1, which is expressed in the brain (Goldstone, et al 2010). CYP19B1 may have induced the expression of EGFP, which could have caused the presence of a weak band in the brain tissue. From the data analyze above, we can conclude that the CYP19A1 gene can be used to study ICER in the ovaries of a transgenic zebrafish because it is an effective tissue-specific promoter.

The female ovaries (tg1) samples were tested at three different pH of Na Citrate of pH 6, 7, and 8 at the following dilutions of 1:500, 1:2500, 1:5000 and the control. At pH 7, the ovaries and negative control had a strong background, which indicates that the sample is not an ideal negative control for the experiment. Dilutions 1:500, 1:2500, 1:5000, 1:10000 of the ovary tissue at pH 7 shows a decreasing background as the antibody increases, this may be due to the non-specific binding. Dilution of 1:500 shows a stronger background whereas dilution of 1:10000, the background is lessened as therefore a stronger cell signal can be seen. The concentrations of 1:500, 1:2500, 1:5000, 1:10000 of the female ovary tissues showed similar results at pH 6 compared to pH 7. Unfortunately, both sets of transgenic tissue samples at the various Na Citrate pHs and dilutions show strong background which interferes with GFP expression. The experiment concluded that there were no significant changes between the different pH levels of the
0.01M Na Citrate for the ovaries samples. Due to no significant changes further experiments were performed using 0.01M Na Citrate at pH 6.

After the western blot portion of the experiment showed success, the direct fluorescent of the ovarian tissue was performed. Once the tissue was stained with DAPI, it was observed under a fluorescent microscope using blue and green filters. The ovary tissue from transgenic zebrafish under DAPI stain showed marked fluorescence in the nucleus of live cells. The same tissue under fluorescent microscope showed cellular expression of GFP in a similar pattern as seen with DAPI. Due to the successful results of the experiment, immunohistochemistry would be performed.

To optimize the best signal of GFP expression under fluorescent microscope several different trial and errors were performed. The transgenic zebrafish tissue was tested via immunohistochemistry using different polyclonal antibody concentrations and different deparaffinization and antigen-retrieval techniques. The best results obtained were at the 1:5000 antibody concentrations with a 30-minute microwave boiling deparaffinization procedure. The different pH for antigen retrieval did not cause a change in the results proving that pH did not contribute to optimization of the GFP signal. The ovary tissues did show GFP under the fluorescent microscope.

As indicated by the blue arrows in figure 8 the ovary tissue shows GFP signaling. The expression of GFP appears to correlate with the follicle cells also known as granulose cells. Several studies have shown that the granulosa cells that surround developing oocytes in the ovary of zebrafish express high levels of Cyp19A1 (Chiang, Evelyn Feng-Lin, et al.2001, Dranow, Daniel B., et. al., 2013,) implying that the signals observed in the ovary tissue examined was from granulosa cells. As expected, the
Aromatase promoter, Cyp19A1 induced expression of GFP in the ovaries and specifically in the estrogen-producing cells.

From all the gathered information above, the purpose of this experiment was to show that CYP19A1 can function in the ovaries as a specific promoter that can be used to express transgenes in the ovaries was approved. The results of the study show that CYP19A1 was successfully expressed in the ovaries which also indicated that the promoter region of CYP19A1 can be an ovary specific promoter. In the future, there should be a larger sample size to confirm the promising results.

In conclusion, the construct did successfully produce an ovarian-specific transgenic zebrafish which can have important implications if hyperovulation is an observed phenotype. Aside from zebrafish being an ideal organism to manipulate, it also has direct implications for the fishing industry. Due to the growing demand for fish as a result of overfishing, a significant challenge in this industry is the production of large numbers of eggs with high survival, and current research is focused on manipulating the endocrine system to optimize spawning (Lubzens, 2009). If hyperovulation can be induced by targeting ovarian-specific promoters, it can have a substantial impact on the food industry, particularly when taking into consideration the growing world population and its effect on the depletion of natural resources.
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