Generation of a Transgenic Zebrafish (Danio rerio) Expressing the Transcriptional Repressor Inducible cAMP Early Repressor (ICER)

Theodora Tsatsos
ABSTRACT

Inducible cAMP early repressor (ICER) has been known to moderate cAMP antiproliferative actions. It has ovarian-specific properties and its gene product has been shown to act as a tumor suppressor. It has been previously shown that ICER has been targeted for expression in the ovaries via a transgenic mouse model to create an increased level of ovulation. In this paper, we cloned the following ovarian-specific promoters: CYP19A1, FSH, ZP3, and GDF9. Plasmid DNA was constructed with the aforementioned ovarian specific promoters using a three-fragment vector construction method and EGFP was used as a marker to follow whether plasmid DNA was successfully incorporated into the ovarian tissue. Upon vector construction, microinjection into zebrafish eggs was performed to produce the transgenic model. Upon transgenesis into the zebrafish model, Danio rerio, we ultimately want to test whether expression will cause an increase in ovulation. 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 livestock or fish.
Generation of a Transgenic Zebrafish (*Danio rerio*) Expressing the Transcriptional Repressor Inducible cAMP Early Repressor (ICER)

by

Theodora Tsatsos

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GENERATION OF A TRANSGENIC ZEBRAFISH (*DANIO RERIO*) EXPRESSING THE TRANSCRIPTIONAL REPRESSOR INDUCIBLE CAMP EARLY REPRESSOR (ICER)

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INTRODUCTION

Although leutinizing hormone (LH) and follicle stimulating hormone (FSH) are largely responsible for regulating the ovary, new research indicates that the ovary may be principally regulated by local growth factors, particularly growth and differentiation factor-9 (GDF-9), that are released from follicles as well as oocytes (Ge, 2005). The follicle, which is the regulatory network of the ovary consists of the oocyte, the inner granulosa, and the outer thecal cells. In zebrafish, the follicles contain one layer of granulosa cells that is situated next to the internal vitelline envelope. The thecal cells are located externally to the inner granulosa. The oocyte grows and develops through changes mediated by the follicle, a process that is known as folliculogenesis.

The follicles of the zebrafish ovary are very active and the follicles experience development in an asynchronous manner (Ge, 2005). Development of the oocyte can be divided into five separate stages which occur within approximately 10 days (Ge, 2005). Stage I includes the initial growth stage; Stage II is the pre-vitellogenic stage; Stage III is the vitellogenic stage; Stage IV is maturation; and Stage V is the mature egg stage (Ge, 2005). Zebrafish can produce many follicles at various stages year round, which is an aspect that makes this organism an exceptional one for study (Ge, 2005).

The developing oocyte relies largely on LH and FSH for maturation and later fertilization to occur. Some of the most significant phases in egg development include the establishment of primordial germ cells, their conversion into oogonia, and then into primary oocytes where meiosis occurs. Vitellogenesis follows, and is manifest by substantial oocyte growth and the accrual of nutritional resources necessary for future
embryo growth. Differentiation at this point is complete when the oocyte gains maternal RNA. Maturation is marked by meiotic divisions with ovulation occurring at the final phase of oocyte formation.

In some fish species, vitellogenesis is dictated by an increase in both FSH and LH expression levels, in addition to a decrease in the expression of GDF9 (Lubzens et al., 2010). Gonadotropin hormone receptors are thought to mediate the expression of GDF9, especially when stimulated with human chorionic gonadotropin, in zebrafish (Lubzens et al., 2010). The quality and number of oocytes produced in vitellogenesis is often determined based on energy resources available (Lubzens et al., 2010). Ovarian gonadotropin releasing hormone (GnRH) has been observed to mediate apoptosis in fish species and may have pro and anti apoptotic properties (Lubzens et al., 2010).

In all vertebrates, the egg is covered by a vitelline envelope called the zona pellucida (ZP) and is thereby regulated by various ZP proteins synthesized at the onset of oocyte growth. The synthesis of the ZP proteins occurs in the liver in zebrafish before being transported and incorporated into the ovarian zona radiata envelope layer (Lubzens et al., 2010). In zebrafish, estrogen regulates liver expression while regulation in the ovaries can be independent or under the influence of estrogen (Lubzens et al., 2010). Estrogen is believed to regulate the synthesis of ZP; however, further research in this area is required as the hormonal control for this process in fish has not yet been well established (Lubzens et al., 2010). The ZP protein groups display high conservation thought to be involved in hardening of the vitelline envelope upon fertilization (Lubzens et al., 2010).
Forming the egg is a multifaceted process, as the egg is a closed system that must be able to maintain itself and the embryo until hatching without the influence of external factors. All substances that contribute to egg development therefore, must be integrated into the developing oocyte.

Overfishing has created a decline in resources, and the mass production of fish via eggs that have a high survival rate is becoming increasingly important. Previous studies focused on regulating the endocrine system in order to produce “high quality eggs” when in reality the development of the egg is a very dynamic and complicated process that is not yet well understood (Lubzens et al., 2010). Research is being aimed towards understanding what environmental factors may contribute to egg quality, including the role of vitamins (Lubzens et al., 2010).

Inducible cAMP early repressor (ICER) is encoded by a transcript that is produced from its gene family, cAMP-responsive element modulator (CREM) (Molina et al., 1993). ICER is a dominant negative transcriptional repressor that is stimulated by cAMP (Molina et al., 1993). It has been shown that FSH greatly improves the transcriptional stability of CREM via a different polyadenylation site (Molina et al., 1993; Foulkes et al., 1993). CREM is switched from antagonist to activator which builds to high levels beginning at the spermatocytic stage (Molina et al., 1993; Foulkes et al., 1993).

FSH, a gonadotropin released from the anterior pituitary, plays a pivotal role in commanding all features of gonadal growth and development in vertebrates. FSH is mediated by various neuroendocrine factors stemming from the hypothalamus and other
hormones from the gonads. Production of the hormone is stimulated from hypothalamic inputs such as GnRH. In zebrafish, the hypothalamus also plays a role in regulating the secretion of gonadotropin hormones (Lin & Ge, 2009).

The CYP19 gene is a highly conserved gene which encodes P450, a complex required for the synthesis of estrogen by granulosa cells (Tong & Chung, 2003; Morales et al., 2003). FSH stimulates P450 production in granulosa cells in response to a cAMP response element-like sequence (CRE) ovarian promoter (Morales et al., 2003). In fish, multiple CYP19 genes have been classified, and expression is present in the ovarian granulosa cells. Expression of this gene in zebrafish ovaries plays an important role in sex differentiation; however, the mechanism involved in the regulation of the expression of this gene is not yet well understood (Tong & Chung, 2003).

Growth differentiation factor 9 (GDF9) is a growth factor originating in oocytes and functions in mediating folliculogenesis. In zebrafish, GDF9 is produced in the oocyte and is chiefly expressed in the ovary (He et al., 2012). The highest expression is seen during early growth stages, and as the follicle develops expression typically decreases (Ge, 2005). This suggests that GDF9 plays a role in driving forward the process of folliculogenesis (Ge, 2005). GDF9 affects proliferation and differentiation of follicle cells by mediating their transcriptional activity (Ge, 2005). Ovarian GDF9 levels were shown to have very high expression in the primary growth follicle of zebrafish, which suggests that GDF9 may play an important role in the stages of early oogenesis (He et al., 2012).

The vitelline envelope, which surrounds zebrafish eggs consists of various ZP glycoproteins and functions not only as a barrier between egg and sperm, but also as a
shield for the developing embryo against the environment (Mold et al., 2009). Early on, ZP gene expression is specific to the oocytes and is restricted to the earliest stages of oocyte development (Mold et al., 2009). The activity of these ZP genes has been shown to be conserved in the promoter region. Regulation is tightly controlled in development in a very tissue-specific manner (Mold et al., 2009).

The zebrafish, *Danio rerio*, 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).

Transgenesis is a technique used to study gene expression and disease. Due to the swift embryonic development of zebrafish and the rapid development of organs by the first two days of life, zebrafish are an ideal candidate for study. The use of transposable elements replaced more antiquated techniques in zebrafish transgenesis since these
proved to have the best genomic incorporation (Suster et al., 2009). The Tol2 transposable element, derived from the medaka fish, has been the most successful element for the use of transgenesis and is extensively used for this purpose, although other Tol2 vectors have been identified via Gateway pathways (Suster et al., 2009). The Tol2 system has been more recently used in specific cell tissues for gene expression (Suster et al., 2009).

Prior to the creation of the Tol2kit system, transgenesis was a cumbersome process (Kwan et al., 2007). Building the expression construct was laborsome, involved many steps, and was limited due to the availability of restriction enzymes (Kwan et al., 2007). Efficiency of transgenesis was shown to be low and led to issues with mosaicism and low incorporation of the construct (Kwan et al., 2007). Finally, without a fluorescent marker, it was difficult to identify construct integration (Kwan et al., 2007). Tol2kit transformed antiquated techniques by allowing site-specific recombination cloning, and constructs can be quickly and easily constructed (Kwan et al., 2007). Expression constructs are easily created, the Tol2 transposon offers a high probability of transgenesis, and transgenes can be easily identified via non-fluorescent marker methods (Kwan et al., 2007).

Since injecting zebrafish eggs has been proven to be an effortless and straightforward process, Tol2 has become the prevailing method in creation of transgenic zebrafish (Kikuta and Kawakami, 2009). Following this revelation, the method of expressing green fluorescent protein (GFP) in transgenic zebrafish was developed (Kikuta and Kawakami, 2009). Subsequently, tissue-specific promoters attached to the
GFP were constructed for expression in this organism (Kikuta and Kawakami, 2009). Problems with DNA integration, gene silencing, and DNA rearrangement along with low transmission frequency, that were encountered were dodged through the development of Tol2, and the cloning of DNA into these vectors was eventually facilitated by Gateway technology (Kikuta and Kawakami, 2009).

MATERIALS AND METHODS

Primer Design for Recombination Reactions

Primers for the recombination reaction were used to amplify DNA for CYP19A1, FSH, ZP3, and GDF9 and chosen via http://frodo.wi.mit.edu/. The expression clones for the BP reactions utilized the attB4 for the forward reaction and attB1r for the reverse reaction. The following primers were used for PCR: for CYP19A1, forward: 5’- GCCTACGTGAAAAAGCTCAGATGATG -3’; reverse: 5’- CCTCTGAACTGAGACGTACACTTT -3’. For FSH, forward: 5’- TGCAAATCAAATTACAAAATCAG-3’; reverse: 5’- ATGCAGCAAGTGTGTCTTCG- 3’. For ZP3, forward: 5’-CAAGTTTTTGTGAAGCCGGTTA-3’; reverse: 5’GCCCCTCCAGCTTAATTG-3’. For GDF9, forward: 5’- GCACTTGCACTGCACTTCTC-3’; reverse 5’-CGTCGCTCCCTCCCTTCTGAC-3’. The products were purified following the same protocol in order to perform the BP reaction. Gel electrophoresis was run to confirm identity of the promoters.
BP Recombination Reactions

Using the expression clones, BP recombination reactions were carried out using the MultiSite Gateway Three-Fragment Vector Construction Kit (Version H) for each of the promoters CYP19A1, FSH, ZP3, and GDF9. The entry clone was constructed using the aforementioned expression clones in combination with donor vector pDONRP4-P1R to create the L4/R1-flanked pENTR-'promoter.' PCR was done using the Invitrogen Platinum® Supermix High Fidelity protocol. Bacterial transformation was then successfully done in *E. coli* competent cells prior to a Minipreparation using the QIAprep Spin Miniprep Kit bench protocol. All reactions were carried out in accordance to the manufacturer’s protocol.

LR Recombination Reactions

Two-way LR recombination reactions were carried out using the MultiSite Gateway Three-Fragment Vector Construction Kit (Version H) to transfer the DNA fragments into the destination vector, pDEST R4-R3 Vector II. The reaction was performed with the L4/R1-flanked entry clone pENTR-'promoter’ and the L1/L2-flanked EGFP clone. The vector included a polyA tail to prevent degradation. For EGFP, donor vector pDONR221 was used for the L1/R2-flanked entry clone pENTR-EGFP. Upon reaction completion, bacterial transformation into *E. coli* was successfully done. All reactions were carried out in accordance to the manufacturer’s protocol.
Bacterial Transformation and Amplification

Bacterial transformation was done using One Shot® TOP10 Competent cells as per the MultiSite Gateway Three-Fragment Vector Construction Kit (Version H) and plated as per manufacturer’s instructions. Colonies were then added to LB medium in preparation to be harvested and purified using the Qiagen QIAprep® Miniprep Kit. Procedures were followed in accordance to manufacturer’s instructions.

Gel Electrophoresis

Integrity of DNA was established using a 1% agarose gel electrophoresis stained with ethidium bromide. Gels were run at 100 constant volts for at least 60 minutes in 1xTAE buffer. Gel extraction was done using Qiagen QIAquick® Gel Extraction Kit protocol. All procedures were done in accordance with manufacturer’s instructions.

Sequence Analysis

DNA was sent to GENEWIZ (South Plainfield, NJ) for sequencing. Nucleotide BLAST search for zebra fish was used as confirmation that the promoter sequences were properly flanked (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

TOPO Cloning

TOPO cloning was performed using the Invitrogen user manual TOPO TA Cloning® for one-step inclusion of PCR product into a plasmid vector for storage. For the cloning reaction setup, 4 μl of PCR product was used and the reaction was carried out in accordance with the manufacturer’s instructions. Transformation was performed using
the One Shot® TOPO10 Competent Cells protocol. Two microliters of the TOPO Cloning reaction was mixed with One Shot® Competent \textit{E. coli} and subsequently incubated on ice for five minutes. Cells were heat-shocked for thirty seconds at 42°C without shaking. Upon being transferred to ice, 250 \(\mu\)l of S.O.C medium were added to the cells at room temperature and the tubes were placed on a shaker at 37°C for one hour at 200 rpm. Bacteria were then spread on plates and incubated at 37°C overnight. White or light blue colonies were then selected for analysis and added to LB medium and allowed to grow overnight while on a shaker at 37°C. Bacteria were harvested and purified using the Qiagen QIAprep® Miniprep Kit. A 1% agarose gel electrophoresis was performed to ensure that the DNA was of the expected size. The gel DNA fragment was excised and purified using the Qiagen QIAquick® Gel Extraction Kit for preparation for ligation.

Double Digest and Insertion into a pGL3 Luciferase Vector

A double digest was done upon TOPO using two restriction enzymes Knpl and XhoI to remove the DNA insert from the TOPO vector and insert it into pGL3 luciferase vector. This process was carried out using the Promega protocol. A 1% agarose gel electrophoresis was run to confirm the DNA identity, and sequencing was also done to confirm DNA matches. Ligation into pGL3 was then done using the TaKaRa protocol.

Microinjection of DNA Construct into Zebrafish Eggs

Microinjection was done at the University of Medicine and Dentistry of New Jersey on 16Apr2013. A 10 \(\mu\)L injection mixture of only CYP19a was prepared using
PCR-grade water. Since this procedure was done offsite, only CYP19a was chosen for microinjection. A total of 400 eggs were each injected with 2.5 nL.

RESULTS

Primer Design for Recombination Reactions

DNA sequences flanked with B4/B1 segments for CYP19A, FSH, ZP3, and GDF9 were used for the BP recombination reaction. Two gels were run in total for each of the above DNA sequences. Each of these gels in turn was rerun after PEG purification to remove unincorporated primers. A 1% agarose gel electrophoresis was run on the PCR fragments to confirm identity. Sequences were confirmed via a nucleotide blast search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the zebrafish genome database. Figure 1 shows the gel after PCR. Figure 2 features this DNA rerun through gel electrophoresis, after PEG purification. The second gel set was run upon PCR and is shown in Figure 3. Figure 4 shows these results upon PEG purification.
**Figure 1:** Lane 1 shows the DNA ladder. Lane 2 shows ZP3 (2,929 bp), lane 3 shows GDF9 (3,000 bp), and lane 4 shows CYP19a (1,823 bp). The black arrows to the left of the ladder indicate 3,000 bp, 2,000 bp, and 1,550 bp (from top to bottom).

**Figure 2:** The gel was rerun after PEG purification. Lane 1 shows the DNA ladder. Lane 2 shows GDF9 (3,000 bp), lanes 3 and 4 show CYP19a (1,823 bp). The black arrows to the left of the ladder mark 3,000 bp and 2,000 bp (from top to bottom).
BP Recombination Reactions

BP recombination reactions were run to obtain the L4/R1 flanked entry clones for each promoter using donor vector pDONRP4-P1R. Bacterial transformation into *E. coli* competent cells was done after the BP reactions, followed by a miniprep, and DNA sequencing was done to confirm that transformation was successful for each sample. For FSH (Figure 5), a BLAST search confirmed a 97% identity with a 3,101 base pair length. The BLAST search for ZP3 (Figure 6) produced a 97% identity match with a promoter length of 3,001 base pairs. For GDF9 (Figure 7), 98% identity was confirmed for a 3,000
base pair length. For CYP19A (Figure 8), a 98% identity was confirmed via BLAST for a promoter length of 1,823 base pairs.

![Figure 5: FSH BLAST sequence after BP reaction shows 97% identity match.](image)
Figure 6: ZP3 BLAST sequence after BP reaction shows 97% identity match.
**Figure 7: GDF9**

**Sequence**

BP reaction shows 98% identity match.
LR Recombination Reactions

LR recombination was successful for each of the promoter sequences and DNA sequencing confirmed high identity matches for CYP19A (Figure 9), ZP3 (Figure 10), and GDF9 (Figure 11) only. BLAST was used to determine the identity matches between sequences. The sequencing confirms that EGFP is bound to the promoter sequence and that the reaction between the \textit{attL} and \textit{attR} sites was successful and thus generated the proper expression clone.

**Figure 8:** CYP19a BLAST sequence after BP reaction shows 98% identity match
**Figure 9A:** CYP19A forward DNA sequence after LR reaction.

**Figure 9B:** CYP19A reverse complement DNA sequences after LR reaction. Yellow and purple (5' end of miniTol2) sequences show 100% identity with Tol2 R4/R2. Green shows 98% alignment with the complementary sequence for pENTR-EGFP. Yellow underline strikethrough shows late polyA signal.
Figure 10A: ZP3 forward DNA sequence after LR reaction. Figure 10B: ZP3 reverse complement DNA sequence after LR reaction. Yellow and purple (5' end of miniTol2) sequences show 100% alignment with Tol2 R4/R2. Green shows 91% alignment with the complementary sequence for pENTR-EGFP. Yellow underline strikethrough shows late polyA signal.
**Figure 11A:** GDF9 forward DNA sequence after LR reaction. **Figure 11B:** GDF9 reverse complement DNA sequence after LR reaction. Yellow and purple (5' end of miniTol2) sequences show 100% identity with Tol2 R4/R2. Green shows 100% alignment with the complementary sequence for pENTR-EGFP. Yellow underline strikethrough shows late polyA signal.
PCR amplification was successfully done to amplify DNA and is indicated by the following 1% agarose gels in Figures 12 and 13.

**Figure 12:** Lane 1 shows the DNA ladder. Lanes 2 and 3 show the results of CYP19a (1,823 bp) after PCR. The black arrow to the left of the DNA ladder marks 2,000 bp.

**Figure 13:** Lane 1 shows the DNA ladder. Lanes 2 and 3 show the results of FSH (3,084 bp); lanes 4 and 5 show ZP3 (2,929 bp); lanes 6 and 7 show GDF9 (3,000 bp), after PCR. The black arrows to the left of the DNA ladder mark 4,000 bp and 3,000 bp (from top to bottom).

**TOPO Cloning**

TOPO cloning was used as a method for cloning our DNA into vectors for storage. This method is a stable way of maintaining DNA and is particularly useful in that it was designed to bond to the 3'-adenine overhang created by taq polymerase. The TOPO method takes advantage of this by using topoisomerase I to bind to the DNA at
specific sites. A 1% agarose DNA gel was run to confirm DNA size. Upon TOPO cloning, bacterial transformation was done.

Double Digest and Insertion into a pGL3 Luciferase Vector

A double digest using restriction enzymes Knpl and XhoI was run on the TOPO PCR product to positively confirm DNA integrity. Gels can be seen in Figure 14. DNA sequencing was then performed. A 98% identity match was confirmed for GDF9 and a 98% identity was confirmed for CYP19a using BLAST.

Figure 14: A. GDF9. Lane 1 shows the DNA ladder. Lanes 2-5 represent GDF9 (3,000 bp). The black arrow to the left of the DNA ladder marks the 3,000 bp mark. B. CYP19a. Lane 1 shows the DNA ladder; the remaining samples are CYP19a (1,823 bp). The black arrow to the left of the DNA ladder marks the 2,000 bp mark.

Ligation of the PCR product into pGL3 luciferase vector was done for the purpose of evaluating transcriptional activity in the cells that had been transfected with the DNA
construct. Bacterial transformation was subsequently done after ligation into the pGL3 vector. The luciferase gene is controlled by the promoters of interest (CYP19a and GDF9). A 1% agarose gel electrophoresis of recombinant pGL3 DNA was then done and can be seen in Figure 15A and 15B. The thick band at approximately 5,000 bp indicates undigested DNA, while the promoter product can be seen at the proper band size indicated by the black arrows. Figure 16 shows a gel of the pGL3 basic constructs after ultrapurification.

Figure 15: A. shows the results upon the double digestion and insertion into pGL3 vector for GDF9 (3,000 bp). The black arrow indicates 3,000 bp B. shows these results for CYP19a (1,823 bp). The black arrow indicates 2,000 bp.
Microinjection

Of the 400 eggs injected, 100 viable embryos resulted. Of the 100 viable embryos, eighty five zebrafish survived, and are growing. Additionally, thirty five ‘monsters,’ in which zebrafish develop abnormally, resulted. Once they reach reproductive maturity, they will be mated and checked for incorporation of the construct.

DISCUSSION

Zebrafish are a good model for ovulation studies due to their ability to frequently produce large numbers of eggs and short generation times. Translucency of the embryo also allows direct observation of embryogenesis and development (Segner, 2009). They are particularly ideal candidates in studying the endocrine system since phenotypic changes in response to gene expression can be easily measured, and also because there is a great deal of knowledge regarding their hypothalamic-hypophyseal-gonadal axis (Segner, 2009).
The purpose of this study was to clone several ovarian-specific promoters in zebrafish using Tol2 to ultimately create a transgenic zebrafish. It was previously shown, in an ovarian-specific transgenic mouse model, that ovulation was increased upon ICER expression (Muniz et al. N.d). ICER increased the ovulation rate two-fold in the mouse model upon treatment with external gonadotropins (Muniz et al. N.d).

Several ovarian-specific genes are regulated by cAMP pathways in response to gonadotropin hormone signaling (Muniz et al. N.d). The cAMP reaction is regulated by various transcription factors, one of which is CREM, and its various isoforms like ICER (Muniz et al. N.d). These genes encode various transcriptional activators or repressors of genes that activate the cAMP signaling response (Muniz et al. N.d). ICER has been linked with the transcriptional repression of FSH stimulating genes during the process of folliculogenesis, and is distinctive in that because it is a negative transcriptional repressor of cAMP transcription, it can be induced in response to hormone stimuli (Muniz et al. N.d).

In this study, as confirmed by gel electrophoresis and sequencing, DNA promoters were successfully cloned through PCR. DNA promoter sequences of interest were successfully amplified, with gel electrophoresis indicating bands at proper markers and DNA sequencing confirming alignment from a range of 97% to 98% identity for each ovarian-specific promoter. This successful amplification allowed for a straightforward preparation of the promoter entry clones and EGFP for the LR reaction.

This study aimed to clone several ovarian-specific promoters in zebrafish through utilization of the Tol2 system. Of these promoters, three constructs proved to be
successful enough for injection: CYP19a, GDF9, and ZP3. Reverse complement DNA sequences, as seen in Figures 9-11 showed high identity matches for the promoter sequence, for EGFP, and for Tol2, indicating successful generation of the intended expression clone. The polyA tail showed was shortened in the construct, and only a fraction of the 221 bp element was represented. Because the polyA tail is important for translation and overall stability of the molecule, it can affect gene expression, and if it is too short, mRNA can be degraded.

Although all of the constructs were successful, only CYP19a was chosen for microinjection because this technique was done at a facility where observation and training was the intent. Microinjection resulted in eighty five viable fish post-fertilization. When these zebrafish (F0 generation) grow to adulthood, they will be bred and the resulting generation (F1 generation) will be observed for expression of EGFP in the ovaries. Because the promoter sequence is bound to EGFP, fluorescence may indicate successful implementation of the DNA construct into the ovaries. Although injected with the CYP19a construct, it is not expected that all eighty five zebrafish will carry and express CYP19a in the ovary. The Tol2 system does offer a better likelihood of transgenesis when compared to more conventional methods; however, the system is not immune to other issues that may arise following DNA incorporation (Kwan et al., 2007). Misintegration of the DNA may occur, as well as loss of endogenous gene function, improper gene expression, harmful mutations, or upon mating, abnormal development of offspring (Reenen et al., 2001).
ICER and CREM are both expressed in the ovaries and can increase ovulation in the transgenic mouse model (Muniz et al. N.d). It is thought that increased levels of LH were seen in this model possible as a side effect of ICER expression, and that ICER mediates ovulation by inhibiting the degeneration and resorption of the ovarian follicle (Muniz et al.). ICER has been previously linked to repressing CYP19a which responds to FSH levels; however, in mouse studies where CYP19a was mutated, females were observed to be sterile due to interruptions in folliculogenesis (Muniz et al. N.d).

Upon growth and successful mating of the zebrafish, if integration of the construct can successfully produce ovarian-specific transgenic zebrafish, it 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.
REFERENCES


Supplemental Figure #1 shows a representation of the BP (top figure) and LR (bottom figure) reactions.
Supplemental Figure #2 shows a representation pGL3-Basic Vector