5-2018

The Expression of ICER Under the Control of the Ovarian CYP19A1 Promoter in Zebrafish Ovaries Danio rerio

James Reilly
Montclair State University

ABSTRACT

Infertility, according to the World Health Organization, is defined as the failure to conceive after twelve months of regular unprotected sexual intercourse. A recent study measuring the prevalence of female infertility estimated that 48.5 million women were affected globally (World Health Organization). To even begin to understand infertility is a colossal undertaking. Before we can hope to cure this disorder in humans we must first understand its pathogenesis in animal models. For this purpose, a study will be undertaken in zebrafish, *Danio rerio*, a popular model organism for reproductive studies (Segner, 2009). Based on a previous study in mouse models the ICER (inducible cAMP early repressor) protein has been identified as a potentiator of hyperovulation in mice (Muñiz). The ICER protein is an inducible early repressor of the cAMP second messenger system and a dominant negative autoregulator of its own expression (Molina, 1993). Based on the results of the Muñiz study, the present research will focus on the expression of ICER in zebrafish ovaries. First, it was proven via protein and RNA expression analysis that ICER is expressed in a stage-dependent manner in the zebrafish ovary during folliculogenesis. Next, based on this expression profiling, an ICER plasmid vector was designed with ICER under the control of the *cyp19a1* ovarian-specific promoter. *Cyp19a1* is a gonadotropin and estrogen inducible promoter predominantly expressed in the zebrafish ovary (Kazeto, 2001). This plasmid vector can be utilized in the future, in conjunction with the Tol2 transposase system in zebrafish, to study the effects of ICER overexpression in the ovary and hopefully elucidate some further understanding of the causes and pathology of infertility as well as possible treatment scenarios (Kawakami, 2007).

MONTCLAIR STATE UNIVERSITY

The Expression of ICER Under the Control of the Ovarian cyp19A1 Promoter in Zebrafish Ovaries Danio rerio

by

James Reilly

A Master's Thesis Submitted to the Facility of

Montclair State University

In Partial Fulfillment of the Requirements

For the Degree of

Master of Science

May 2018

College/School: College of Science and Mathematics

Department: Biology

Thesis Committee:

[redacted]

(Carlos Molina, Ph.D.)
Thesis Sponsor

[redacted]

(Chunguang Du, Ph.D.)
Committee Member

[redacted]

(John Gaynor, Ph.D.)
Committee Member

THE EXPRESSION OF ICER UNDER THE CONTROL OF THE OVARIAN CYP19A1 PROMOTER IN ZEBRAFISH OVARIES *DANIO RERIO*

A THESIS

Submitted in partial fulfillment of the requirements

For the degree of Master of Science

by

James Reilly

Montclair State University

Montclair, NJ

2018

ACKNOWLEDGEMENTS

First and foremost, I cannot adequately express my gratitude to my research mentor Dr. Carlos Molina. Not only for providing me the opportunity to work in his lab, but being with me every step of the way, from our first failed attempts to perform a subtracted hybridization on zebrafish ovarian mRNA, to our final successful production of an ICER pTol plasmid vector. I am beyond grateful for all your guidance, long hours in the lab, and endless encouragement and support. You've endowed me with the knowledge of what it truly means to be a research scientist: that while experiments may fail often and unpredictably, the true measure of a scientist is the ability to keep moving forward, to innovate and adapt constantly, and that the only true failure in science is to not try again. We're all the products of millions of years of mistakes that ended up being advantageous under the right circumstances, and that is knowledge I will carry with me forever. Thank you for the opportunities you provided me, and the lessons you taught.

My project would not have been completed without the support of my committee members: Dr. Chunguang Du and Dr. John Gaynor. Thank you so much for taking the time to sit through my dissertation, read over my manuscripts, teach me inside and outside of the classroom, and for accompanying me through this exciting and unforgettable experience of graduate school and defending a thesis.

Last, but certainly not least, I would like to thank my parents for always fostering an environment of encouragement and learning, because that is rare today. From constant trips by train to the Museum of Natural History, or walks to the public library, to summers spent preparing me for the next schoolyear so that I always went in with the knowledge necessary not only to succeed but to excel, you guys have been there with me

every step of the way. You supported me when I left science and the states and moved to Prague because I didn't know what I wanted to do with my life. You welcomed me back when I returned home to pursue my masters, after realizing biology was actually my passion. It is because of your passion and parenting that you have two children, one who can almost call himself a molecular biologist and another who will soon have degrees in both mathematics and education. You guys did good. Bianca, my sister, future teacher or mathematician, favorite child by her own estimation, I must thank you as well for being the thoughtful yang to my reckless yin in the house. Without your influence I wouldn't be half the man I am today. I spent seven years an only child before you were born, and then all of a sudden I had a sibling to share the world with. Really 3 siblings, Joseph and Thomas I have to thank you guys as well. The three of you taught me so much because the world is a different place when you have 3 pairs of eyes looking up to you. I'll amend that total one last time, round up and say 5 siblings because I can't not thank my two dogs Cody and Zoey. Having siblings is one thing, having a dog is another. While I was just a moon exerting some gravitational effects on my human siblings, they were each their own unique and varied worlds, but to a dog you're their whole world. And that is an honor I carry with me every day, because the world might be a better place if each of us acted like the person our dog thinks we are.

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

Infertility is a leading cause of distress among families in the modern world. The emotional toll cannot be quantified in numbers or enumerated by statistics. The sorrow is unique to each couple as they struggle to understand their inability to conceive. The causes are complex, the etiology a labyrinthine web. While science cannot begin to understand the poignant burden carried by these families, we can only hope to offer solace and solutions. Solace, that we are learning more about infertility with each passing day, and solutions, that modern medicine has within its grasp the ability to help any couple conceive.

Infertility, according to the World Health Organization, is defined as the failure to conceive after twelve months of regular unprotected sexual intercourse. A recent study measuring the prevalence of female infertility estimated that 48.5 million women were affected globally. Among women aged 20-44 1.9% were unable to conceive a first child, and among women who had had a first child 10.5% were unable to have a second child. These are termed primary and secondary infertility (Mascarenhas, 2012). Female age is widely known to be the single most important factor in conception, showing a gradual decline with age, and a sharp decline after 35 (Maheshwari, 2008). There is an increasing trend among women to delay childbearing often into the thirties and even forties. Given this trend, women should be made aware of the possibility of the rapid onset and loss of fertility in the mid to late thirties. A study from the NCHS finds the rate of women with impaired fecundity doubles from 16% at ages 25-34 to 33% at ages 35-44. The rate of spontaneous pregnancy loss mirrors this, with the rate nearly doubling from 17% at ages 25-34 to 31% at ages 35-44 (Mosher, 1988).

A recent review found the five most common diagnoses at presentation of infertility to be: ovulatory dysfunction (23.6% <35 yrs, 11.4% >35 yrs), endometriosis (4.5% <35 yrs, 3.1% >35 yrs), tubal factor (17.6% <35 yrs, 24.8% >35 yrs), unexplained (21.0% <35 yrs, 26.6% >35 yrs), and male factor (35.0% <35 yrs, 32.5% >35 yrs) (Maheshwari, 2008). Some data suggest that unexplained infertility is caused by diminished ovarian reserve. Premature ovarian failure is a disorder which causes ovarian loss of function before the age of 40. This disorder affects 1/10,000 women at age 20, 1/1,000 women at age 30 and 1/100 women at age 40 (Goswami, 2005).

While infertility continues to be a problem for many families worldwide, there are treatments available. According to the Society for Assisted Reproductive Technology (ART), 71,296 babies were born as the result of 242,168 ART cycles in the United States in 2016. In a year in which 3,945,875 babies were born in the United States this accounts for 1.8% of births (www.sart.org, www.cdc.gov).

The Endocrine System

Infertility at its core is a disruption of the normal functioning of the reproductive system. Reproduction is a fundamental component of life, and sexually reproducing organisms make a heavy investment into passing on their genetic material to the next generation. Due to its critical importance, the reproductive system is under tight regulatory control and the neuroendocrine system is the master regulator of this arena. The endocrine system is a chemical messenger system consisting of hormones, the glands which secrete those hormones, and the feedback loops of homeostasis that the hormones drive. The hypothalamus, in vertebrates, is the neural control center of the endocrine systems. All

animals with a nervous system have a neuroendocrine system and all vertebrates have a hypothalamus pituitary axis (Hartenstein, 2006).

The hypothalamic-pituitary-gonadal (HPG) axis consists of the hypothalamus and the pituitary gland and gonadal glands: the ovaries and testis. The hormones which make up the HPG axis are gonadotropin releasing hormone (GnRH) secreted by the hypothalamus, the gonadotropins (GTH): follicle stimulating hormone (FSH) and luteinizing hormone (LH) secreted by the anterior pituitary gland (APG), and estrogen and testosterone secreted by the gonads.

Focusing on the gonads, and more specifically the ovaries, can serve to highlight the complex homeostasis which the endocrine systems maintains. In the ovaries, FSH and LH stimulate the ovaries to produce estrogen and inhibin. Estrogen then forms a negative feedback loop by inhibiting production of GnRH in the hypothalamus. Inhibin, inhibits activin, a peripherally produced hormone that positively stimulates GnRH producing cells (Mukherjee et al., 1998; Wang et al., 2004). Meanwhile, a positive feedback loop between LH and estrogen helps prepare the ovary and uterus for ovulation and implantation. When the egg is released the empty follicle then begins to produce progesterone, which in turn inhibits the hypothalamus and APG thus stopping the estrogen LH positive feedback loop (Clelland and Yeng, 2009; Nagahama et al., 2008; Zhang et al., 2015). The idea of feedback loops and the delicate maintenance of homeostasis will play a central role in this paper.

At the molecular level, FSH and LH are glycoprotein polypeptide hormones. They share a common α subunit while maintaining distinct β subunits. FSH is primarily responsible for promoting early gonadal development and growth, stimulating follicular growth and estrogen production by binding its cognate FSH receptor (FSHR) (Kwok et al.,

2005; Mazon et al., 2011). The FSHR is expressed in both theca and granulosa cells. Meanwhile LH regulates the late stages of gametogenesis and final gamete maturation and release, stimulating androgen production by theca cells which acts as a substrate for estrogen production in granulosa cells. The LH receptor (LHR) is expressed on granulosa cells only. However, the FSHR has the ability to bind both FSH and LH (Kwok, 2005; So et al., 2005).

FSHR and LHR are typical G protein-coupled receptors (GPCRs) containing the canonical transmembrane region consisting of 7 transmembrane domains (Kwok, 2005; So, 2005). They detect extracellular signals and direct intracellular signal transduction ultimately activating a cellular response. Because they are gonadotropin receptors, and the most important regulators of gonadal development and function, FSHR and LHR expression is in turn under tight control by various endocrine and paracrine factors in the gonads (Clelland, 2009; Kwok, 2005). We will return to the regulation of FSH and LH shortly but first attention must be paid to the mechanism by which the gonadotropins exert their cellular affects while being unable to cross the plasma membrane themselves and directly affect the expression of the genes involved in ovulation.

The Cyclic-AMP (cAMP) Secondary Messenger System

A large number of endocrine and neuronal functions are mediated by the cyclic AMP (cAMP) dependent pathway (Sassone-Corsi et al., 1998). FSH and LH bind their transmembrane receptors which are coupled to stimulatory G proteins that activate adenylyl cyclase generating high levels of intracellular cAMP (Kwok, 2005). cAMP is thus critically important to gonadotropin signaling. Indeed, it has been shown that following LH stimulation, an increase in intracellular cAMP produces profound changes in expression of

those genes required for switching the pattern of steroidogenic hormone production, withdrawal from the cell cycle, and altering the structure of the follicle wall to allow ovulation (Park et al., 2003). Similarly, FSH induces estrogen biosynthesis through the cAMP-dependent pathway to regulate the expression of the *CYP19A1* gene which will be discussed in greater detail below (Parakh et al., 2006).

cAMP is the prototypical example of a second messenger: intracellular molecules which mediate the effects of first messengers. A typical second messenger system consists of five parts: first messenger, signal transducer, primary effector, second messenger, and secondary effector. The first messenger is typically a neurotransmitter or hormone, which being unable to cross the phospholipid bilayer, must rely on a transmembrane signal transducer to generate an intracellular response. The signal transducer takes the form of a transmembrane receptor that upon activation by the binding of its ligand undergoes a conformational change causing it to activate a nearby membrane bound protein known as the primary effector. The primary effector is an enzyme which in turn catalyzes the synthesis of a signal molecule that can diffuse into the cell. This signal molecule is known as a second messenger. The second messenger diffuses into the cell and activates a secondary effector protein, typically another enzyme, whose downstream effect will depend upon the specific secondary messenger system in question, but is usually the activation or repression of target genes (Willoughby and Cooper, 2007).

In the case of FSH and LH, they mediate their effects through the cAMP secondary messenger system (Mukherjee, 1998). In this system the first messenger (the hormones: FSH and LH) interacts with their particular signal transducer (the GPCRs: FSHR and LHR) to stimulate the primary effector (the enzyme: adenylyl cyclase). Adenylyl cyclase then

catalyzes the conversion of ATP into the second messenger cyclic-AMP. cAMP then works on the secondary effector enzyme, protein kinase A (PKA) to produce final downstream effects (Sassone-Corsi, 1998; Willoughby and Cooper, 2007). Specifically, the rise in intracellular cAMP concentration brought on by activation of adenylyl cyclase results in cAMP binding the regulatory subunits of PKA causing a dissociation between the regulatory and catalytic subunits allowing the active catalytic subunits to translocate into the nuclease and there phosphorylate substrate proteins (Sands et al., 2008; Sassone-Corsi, 1998). PKA translocates into the nucleus where it phosphorylates and thereby stimulates transcriptional activators capable of binding to cAMP response elements (CRE) which induce transcription from the promoters of cAMP responsive genes. A final step in the cAMP signaling cascade is the activation of the cAMP responsive transcription factors such as CREB and CREM, which in turn transactivate the transcriptional expression of cAMP responsive target genes (Molina et al., 1993). The critical role of cAMP-responsive transcription factors cannot be overstated and the next section of this paper will be devoted to an explanation of these transcription factors.

The cAMP Response Family: CREB CREM ICER

To fully explain how the cAMP system mediates gene expression we must understand the family of transcription factors through which it acts and the genes which encode these transcription factors. By analyzing the regulatory sequences of genes known to be affected by the cAMP pathway, promoter elements were identified where intracellular cAMP mediated a transcriptional response. This element became known as the cAMP response element (CRE). The consensus CRE site is an 8 bp palindromic sequence 5'-TGACGTCA-3' (Molina, 1993; Sands, 2008; Sassone-Corsi, 1988).

Following the discovery of the CRE, proteins were elucidated which showed high affinity for binding the CRE. These proteins became known as CRE-binding (CREB) proteins. All CREB proteins belong to the bZIP or basic-region leucine zipper transcription factor family. CREB proteins are coded for by the *CREB* gene. Structural and functional analysis of CREB proteins lead to the identification of the closely related cAMP response element modulator (CREM) protein, encoded by the *CREM* gene (Sassone-Corsi, 1998). *CREM* is a multiexonic gene that encodes a family of activators and repressors of cAMP-inducible transcription via alternative exon splicing (Molina, 1993). CREB, CREM, and ATF-1 make up the CREB subfamily of bZIP transcription factors. Like all bZIP transcription factors CREB family members contain the C-terminal basic domain, also known as the DNA-binding domain which mediates DNA binding by the transcription factors, and the leucine zipper domain that allows for dimerization. Because of their highly similar bZIP domains the CREB family can homo- and heterodimerize allowing the different activators and repressors to bind each other and mediate their effects. Another effect of their high bZIP domain homology is that all CREB family members recognize and bind the CRE. In addition to the DNA-binding domain, CREB family members also share a transactivation domains known as the phosphorylation (P-) box or kinase inducible domain (KID) (Meyer et al., 1993; Lonze et al., 2002). It is here where the cAMP-dependent PKA phosphorylates a serine residue within the P-box activating the proteins, which subsequently modulate CRE-mediated gene expression. Through a complex system of differential splicing, alternative promoter usage and autoregulation, cAMP signaling enhances the expression of CREB and CREM isoforms with differing transactivation and repression properties, that in turn due to their high homology of bZIP domains can homo-

and heterodimerize allowing for intricate interactions and myriad downstream effects (Molina, 1993; Sassone-Corsi, 1998). A diagram of the structures of the *CREB* and *CREM* genes, to better visualize these elements, taken from a 2000 paper by Bodor et al., is below.

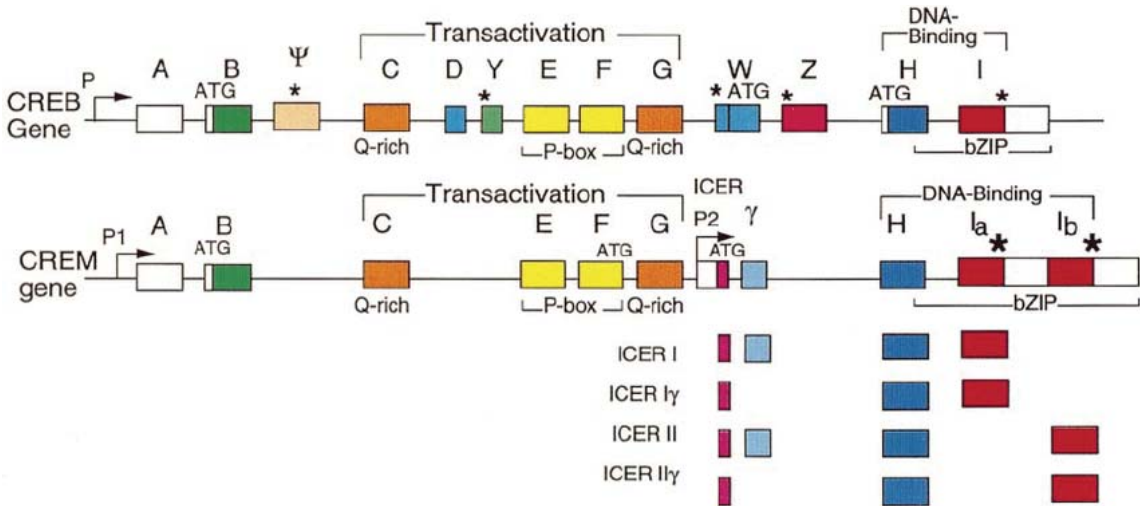

Figure 11 Fig. 1. Diagrammatic structures of the *CREB* and *CREM* genes showing repressor ICER isoforms generated by alternative start sites of transcription and translation (Bodor et al., 2000).

Perhaps the most interesting of these isoforms is the inducible cAMP early repressor (ICER). ICER is the product of the alternative utilization of an internal promoter, P2, of the *CREM* gene which produces a truncated protein consisting solely of the DNA binding domain of CREM. Because the ICER open reading frame only encodes the leucine zipper and basic regions of the *CREM* gene product, it has the unique ability to not only bind CRE but also to heterodimerize with other CREM proteins and with CREB. It is this ability which allows ICER to be a dominant negative repressor of the cAMP system. Also, unlike the other CREM isoforms ICER-mediated repression is based only on the stoichiometric concentration of ICER in the cell. This is because ICER lacks the P-box and thus cannot be phosphorylated by PKA. The ICER alternative promoter P2 of *CREM*

contains 2 pairs of cAMP autoregulatory response elements (CARE) which differ slightly from the consensus CRE. It is through these slightly altered CARE, that ICER is transcriptionally induced by CREB. Furthermore, ICER negatively autoregulates its own transcription in response to high ICER levels. Following ICER induction by CREB in response to cAMP, ICER competes with and represses CREB-mediated gene transcription, before finally repressing its own transcription through the CARE of the P2 of the *CREM* gene as shown in Fig. 12. Again, this repression is based solely on ICER concentrations and not the degree of phosphorylation. Because the cAMP second messenger system is an important signal transduction pathway in a wide variety of cells, it makes sense that the expression of cAMP responsive genes also exists under a tight system of control involving competing activation and repression (Molina, 1993; Stehle et al., 1993).

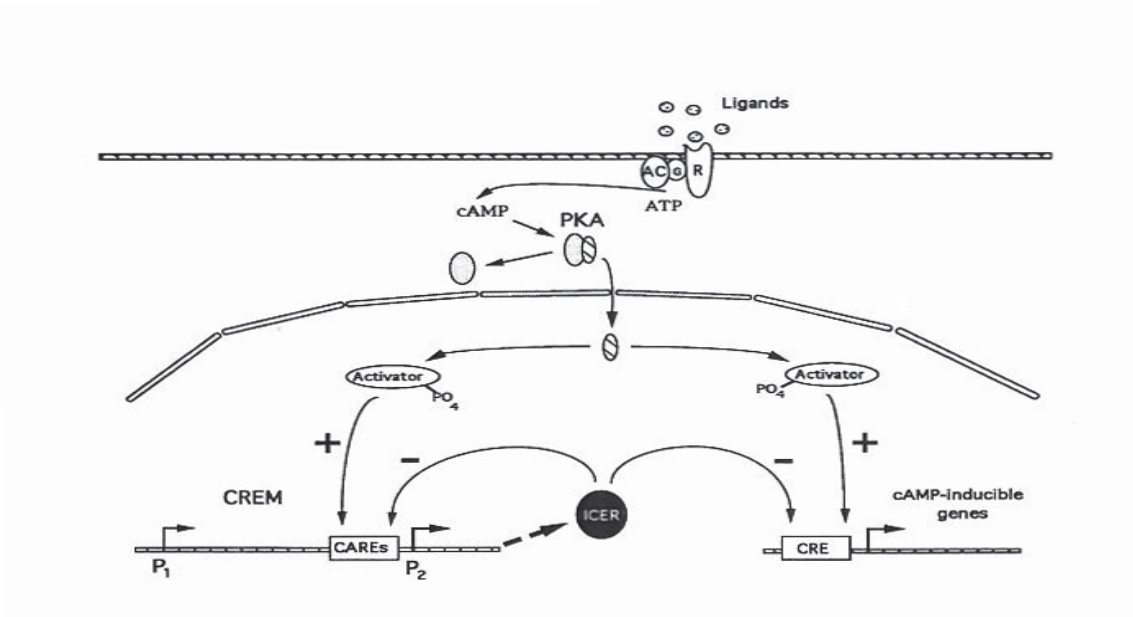

Figure 12 Figure 9. The Role of ICER in the Regulation of Gene Expression by cAMP (Molina, 1993).

The Inhibin-Activin-Follistatin System

Returning to the idea of fertility, ovulation is a very energy intensive investment in an organism, and as such it is under strict regulatory control. Thus far an overview of the endocrine system of regulation at the organismal level involving the feedback mechanisms of the hormones, FSH and LH, and the steroids, estrogen and testosterone, as well as the intracellular cAMP second messenger system and the roles of CREM, CREB and ICER has been undertaken. A further level of regulation is maintained through the inhibin-activin-follistatin system.

Activin and inhibin are growth factors belonging to the transforming growth factor beta (TGFβ) superfamily. Similar to FSH and LH the idea of shared and differing subunits come into play here. Activin is a homo- or heterodimer consisting of two β subunits: βA and βB. The dimerization system of the β subunits gives rise to three forms of activin: activin A (βAβA), activin B (βBβB), and activin AB (βAβB) (Lin et al., 2003; Wang et al., 2004). Inhibin is a heterodimer consisting of a β subunit and a specific α subunit. Activin acts by binding to a specific type II receptor which then recruits a type I receptor to allow for intracellular signaling. Inhibin does not have its own signaling receptor and instead competes with activin for its type II receptor (Poon et al., 2009). Follistatin, meanwhile, is a single-chain glycosylated protein that acts as a binding protein of activin and neutralizes its bioactivity (Popovics et al., 2011; Wun Li et al., 2013). Activin has a stimulatory effect on FSH release, while inhibin has an inhibitory effect on FSH. Neither has any effect on LH release in the pituitary. Follistatin is found to be expressed primarily in the ovarian follicles (Lin, 2003; Poon, 2009; Popovics, 2011; Wang, 2004; Wun Li, 2013).

Activin stimulates follicular growth and granulosa cell proliferation, and also stimulates the expression of FSHR and FSH-induced LHR in granulosa cells. Gonadotropins in turn stimulate activin βA and follistatin expression but suppress activin βB expression (Lin, 2003). The stimulation of activin βA and follistatin by human chorionic gonadotropin (hCG) is mediated by the cAMP-PKA pathway in cultured zebrafish follicle cells, whereas hCG inhibition of activin βB expression is signaled through a cAMP-dependent but PKA-independent pathway (Wang, 2004). The mechanisms of regulation of oogenesis and folliculogenesis are complex and involve significant crosstalk between different signaling pathways intracellularly, and different paracrine and endocrine systems on the organismal level. As mentioned earlier, FSH stimulates estrogen biosynthesis, and LH and estrogen form a positive feedback loop stimulating ovulation, while estrogen in turn inhibits GnRH production in the hypothalamus, all the while inhibin inhibits activin, which itself positively stimulates GnRH producing cells, and inhibin and activin themselves finally exert opposing effects on FSH completing the Gordian knot or serpent eating its tail regulatory framework of ovulation. Because no discussion of female reproduction is complete without mentioning estrogen we will turn briefly to the biosynthesis of estrogen next.

Cyp19A1 An Ovarian Specific Promoter

The goal of many experiments is to measure gene expression in a tissue specific manner. Most genes are under the control of promoters which respond to certain transcription factors and whose activity are mediated and regulated through external factors. Aromatase P450 is an enzyme responsible for the final step of estrogen biosynthesis from androgens. It catalyzes the aromatization of C19 steroids into C18

estrogens (Kazeto et al., 2001). The major site of estrogen biosynthesis is the ovary (Michael et al., 1997). Aromatase P450 gene expression in the ovary is regulated by FSH through the cAMP second messenger system. FSH causes an increase of intracellular cAMP, which through the cAMP pathway stimulates the expression of the *CYP19* gene encoding aromatase P450 (Kazeto, 2001; Michael, 1997).

Zebrafish have two *cyp19* genes: *cyp19a* which is predominantly expressed in follicular cells lining the oocytes within the ovary, and *cyp19b* which is expressed in the brain (Tanaka et al., 1995). Sequence analysis of the *cyp19a* gene identified many regulatory elements within its 2.5kb promoter region, including a TATA box, a steroidogenic factor-1 recognition site, estrogen receptor half site, androgen receptor half site as well as a CRE- like sequence (CLS) 5'-TGCACGTCA-3' which has been found to be critical for cAMP induction of aromatase in humans (Michael, 1997). Additional research has shown that *cyp19a* can be induced by estrogens, while it is inhibited by androgens (Tong and Chung, 2003). In zebrafish *cyp19a1* is an ovarian specific gene. For this reason, the *cyp19a1* promoter region can be utilized in a vector to drive the expression of genes in an ovary specific manner. Thus we chose to place transgenic ICER under the control of the zebrafish *cyp19a1* promoter in this study.

Zebrafish as a Model Organism

Zebrafish are rapidly becoming one of the most widely used model organisms in scientific research. The zebrafish is a tropical freshwater fish, that before becoming an animal model, was a popular aquarium fish. Zebrafish are inexpensive to maintain and relatively easy to care for. Their genome has been sequenced and they show a high degree of genetic identity to humans and higher vertebrates. Zebrafish have quickly become

popular organisms of choice in research into evolution, genetics, neurobiology, development, and reproduction.

Reproductive science is an important branch of medical research and infertility as mentioned previously is a persistent and devastating ailment globally. Zebrafish are ideal model organisms for reproductive studies because of their high homology with humans at the genomic and transcriptomic level (Howe et al., 2013; Zon and Peterson, 2005). They demonstrate a large degree of similarity within the neuroendocrine system of reproductive regulation (Segner, 2009; Ying Hoo et al., 2016). Additionally, zebrafish have a short generation time reaching sexual maturity within 3 months. Female zebrafish have bilateral ovaries which are structurally and functionally quite similar to those of humans. There are four stages of ovarian development in zebrafish: the primary oocyte stage, cortical-alveolar stage, vitellogenic stage, and finally the maturation stage. Females are morning breeders capable of spawning from 50 to well over 200 eggs in a spawning session. Zebrafish eggs are translucent and typically around 0.7mm in diameter. They undergo rapid embryonic development with precursors for major organs visible within 24 hours of fertilization, and most major organs reaching full development within 72 hours post fertilization (Segner, 2009; Ying Hoo, 2016). Their transparency and external development lend large advantages when compared with rodent models. For this reason, the use of fluorescent markers to study gene expression during embryonic development of zebrafish is very popular among researchers. The high fecundity, large brood size, small embryo size and rapid development of zebrafish lend themselves well to high throughput screening.

The close evolutionary relationship of zebrafish to higher vertebrates makes the zebrafish well suited to genomic investigation (Howe, 2013; Thisse et al., 2004). Just as in

mammals, the zebrafish reproductive system is under tight control by the three hormonal feedback control axes: the hypothalamic-pituitary-gonadal (HPG) axis, the hypothalamic-pituitary-adrenal (HPA) axis, and the hypothalamic-pituitary-thyroid (HPT) axis. In fish and other oviparous organisms, the HPG axis is sometimes referred to as the HPG-liver axis because the liver synthesizes the protein vitellogenin (VTG), an egg yolk precursor protein, that is the precursor of the lipoproteins and phosphoproteins of which the majority of the protein content of the yolk is comprised. VTG is synthesized under the control of estrogens produced by the ovaries (Segner, 2009; Ying Hoo, 2016). Zebrafish have asynchronous ovaries, that contain follicles at all stages of development. Females will spawn year round under laboratory conditions. The zebrafish follicle contains a large oocyte surrounded by the zona radiata or vitelline envelope. The follicular layer consists of an inner layer of granulosa cells separated from an outer layer of theca cells (Segner, 2009; Ying Hoo, 2016). Follicular development is separated into stages based on the size of the follicle: primary growth stage (PG), previtellogenic/ cortical alveolar stage (PV), early vitellogenic stage (EV), midvitellogenic stage (MV), late vitellogenic stage (LV), and full grown stage (FG) (Clelland and Peng, 2009; Koc et al., 2006; Selman et al., 1993). Because of high degree of functional similarity of hormonal regulation between the zebrafish reproductive system and humans they are a popular organism of choice in endocrine studies. Specifically, in chemical screenings to test whether compounds are endocrine-disruptive. Due to their ease of maintenance, large degree of evolutionary similarity to higher vertebrates, short maturation time, rapid development, high fecundity, and fully sequenced genome zebrafish are a phenomenal model organism well suited to scientific research. (Segner, 2009; Ying Hoo, 2016).

The Tol2 Trasngenesis System

Transgenesis involves the introduction of an exogenous gene into a living organism so that the organism will express the foreign gene and pass it on to its offspring. There are many methods to produce transgenic organisms and all carry unique advantages and disadvantages. Transposable elements (TE) or transposons are DNA sequence which can change their position within the genome. Generally divided into two classes: retrotransposons which function via reverse transcriptase and DNA transposons which encode the protein transposase, transposons were first discovered in maize (McClintock, 1950). Because of their ability to move within a genome, transposons can introduce or reverse mutations, alter the genotype and change the genome size. For this reason, many organisms have mechanisms to inhibit transposon activity. RNA interference is a common method of inhibiting transposon activity. Evolution also often deactivates transposons rendering them introns. Thus most vertebrate genomes contain large numbers of transposons which are naturally inactive. One exception is the Japanese rice fish (Oryzias latipes) also known as the medaka.

The medaka belongs to the class Actinopterygii of ray-finned fish similar to the zebrafish. In medaka fish the Tol2 transposable element was found to be autonomously active. Tol2 is an autonomous transposon about 4.7 kb in length that encodes a gene consisting of four exons which encodes for a fully functional 649 amino acid long transposase protein. Tol2 integrates via a cut and paste mechanism and does not modify the target site except for the introduction of an 8 bp duplication (Kawakami, 2007). Most fascinating of all Tol2 transposase can catalyze the transposition of a construct with the transposase coding region deleted and replaced by an exogenous DNA insert. A transposon

construct must simply contain two minimal cis regulatory sequences consisting of 200 bp and 150 bp of DNA from the left and right ends of Tol2, respectively, to be sufficient for transposition. These sequences contain 12 bp terminal inverted repeats and subterminal regions necessary for transposition (Kawakami, 2007). Any foreign DNA can be cloned between these sequences and inserted into an organism's genome when microinjected with an mRNA encoding the accompanying Tol2 transposase. This allows for a very flexible and easy to use transgenesis method. Once transposase activity has ended the excised DNA is now stably integrated into the genome (Kawakami, 2007; Kwan et al., 2007).

The Tol2 system has revolutionized the generation of transgenic organisms and is widely used in zebrafish research. Embryos can be microinjected with the Tol2 transposase mRNA and a Tol2 vector containing a gene of interest. About 50% to 70% of injected fish will produce transgenic offspring, at frequencies between 3% and 100% (Kawakami, 2007). The generation of stable transgenic lines is thus possible and within the ability of any researcher. The Tol2 system is particularly well suited for the study of genes with tissue specific expression. Simply place the gene of interest under the control of a tissue specific promoter and tissue specific expression can be easily quantified.

Goals of this Study

The cAMP system rears its head again and again as you investigate the intracellular mechanisms, by which the regulation of the HPG axis, are mediated. It might seem odd or even counterintuitive that one single factor can play such a crucial role in so many diverse yet intimately intertwined processes. It may even engender disbelief that the simple elevation of cAMP levels can allow such finely tuned responses. After all, once cAMP levels are elevated wouldn't they diffuse throughout the cell and render it refractory to any

further stimulation? While there is some evidence that ICER, through its interaction with cAMP in a concentration specific manner, plays a role in desensitizing and resensitizing cells to cAMP induction, the bigger picture is considerably more complex. But just as at the heart of every atom are protons, neutrons, and electrons, the cAMP system is composed of parts and ICER is a fundamental and unique component of the system. Thus we must first understand ICER to understand any of the higher degrees of control of cAMP-dependent pathways in the ovary.

The goals of this research are two-fold. First to determine whether ICER is expressed in the zebrafish ovary by stage. If this turns out to be the case, then we will move on to our second goal: the generation of a transgenic zebrafish expressing ICER under the control of an ovarian promoter. Because ICER is such an important part of the cAMP second messenger system which in turn is a critical element of neuroendocrine regulation, we wish to ultimately examine the effects of the overexpression of ICER in zebrafish ovaries. Previous research, in mouse models, has demonstrated that female mice overexpressing ICER are hyperovulatory. A two-fold increase in ovulation rate was observed in the immature transgenic mice compared to the wild type in response to exogenous gonadotropin treatment. Additionally, mature cycling transgenic mice display a significantly enhanced ovulation rate compared to the wild-type (Muñiz). Many studies have also been undertaken examining the role of ICER in spermatogenesis but the literature on the role of ICER in folliculogenesis and oogenesis is significantly sparser. Given the utility of the zebrafish as a model organism for reproduction we believe an investigation of ICER in zebrafish ovulation is an important avenue of research. The implications of this research can have wide ranging effects from human fertility, where gonadotropins and

estrogen antagonists are frequently used to enhance ovarian activity prior to IVF treatments, to aquaculture, where the growing human population and demand for fish is placing significant strains on wild populations and farmed fish alike.

METHODS AND MATERIALS

Zebrafish Maintenance

Zebrafish were maintained in house at a water temperature between 24-30ºC, at a pH between 7.0 and 8.0, and a photoperiod of 10-hour light and 14-hour dark to encourage breeding.

SDS-PAGE

The Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis SDS/PAGE was performed according to the protocol set forth by the Mini-PROTEAN® Tetra Cell manual. Pre-made 4-20% polyacrylamide gels were utilized, and loaded with 20 µl of sample per well. Following loading the gels were placed in the electrophoresis gasket and tank and filled with buffer solution. Electrophoresis was performed for 35 minutes at a voltage of 200v.

Polymerase Chain Reaction (PCR) to Introduce NcoI Site

Polymerase Chain Reaction was performed using the Promege PCR Master Mix. Primers were designed and ordered from Eurofins Genomics. Left primer 1 had the sequence 5'-TGAGCTCGGATCCATGGACCCC-3'. Right primer 1 had the sequence 5'-AAGTGAAGGCCCATGAGGC-3'. Left primer 2 had the sequence 5'-TGAGCTCGGATCCATGGACC-3'. Right primer 2 had the sequence 5'-CCCATGAGGCCAGTTAATTAAGA-3'.

Following the Promega Protocol samples were prepared at the following volumes: 25µl of PCR master mix, 20µl of nuclease-free water, 2µl of template DNA, 1.5µl each of the left and right primers for total of 3µl for a total of 50µl. Amplification was then performed according to the protocol. An initial denaturation step of 2-minutes at 95ºC,

followed by 30 cycles with 95ºC denaturation for 30 seconds, annealing starting at 5ºC below the melting temp of the primers which was then increased by 1 ºC until the annealing temperature was reached, extension at 72ºC for 1 minute. Cycling was followed by a final extension of 5 minutes at 72ºC. Then the reaction was held at 4ºC.

Site-Directed Mutagenesis (SDM) to Introduce NcoI Site

Site-Directed Mutagenesis was performed using the Agilent Technologies QuikChange Lightning Site-Directed Mutagenesis kit. Primers were designed and ordered from Eurofin Genomics. The first primer t393g_A had the sequence 5'-CACGTCGTAGGGGTCCATGGATCCGAGCT-3'. The second primer t393g_B had the sequence 5'-AGCTCGGATCCATGGACCCCTACGACGTG-3'.

To set up the PCR reaction 2.5µl of 10X buffer, 17µl of nuclease-free water, 1.5µl of template DNA, 1µl each of 2 primers for total of 2µl, 1µl of dNTP mix and 1µl of polymerase was combined in each sample tube for a total of 20µl. PCR amplification was performed according to the QuikChange protocol as follows:

Cycling Parameters for the QuikChange Lightning Site-Directed Mutagenesis Method

Segment	Cycles	Temperature	Time
1	1	95°C	2 minutes
2	18	95°C	20 seconds
		60°C	10 seconds
		68°C	30 seconds/kb of plasmid length*
3	1	68°C	5 minutes

* For example, a 5-kb plasmid requires 2.5 minutes per cycle at 68°C.

This was followed by a DpnI restriction digest to digest parental dsDNA before proceeding to a miniprep and transformation of the PCR product into competent E. coli cells.

Restriction Digests

The following restriction enzymes were utilized in this study according to the manufacturer's specifications:

KpnI cuts 5'…GGTAC/C…3'

NcoI cuts 5'…C/CATGG…3'

For the NcoI/KpnI digestion of the SDM product the volumes used were as follows:

- 16µl DNA from SDM miniprep colonies
- 2µl 10X enzyme buffer
- 1µl NcoI
- 1µl Kpn1

The reaction was allowed to proceed for 10 minutes at 37ºC incubation.

For the NcoI/KpnI digestion of the PCR product the volumes used were as follows:

- 50µl DNA from PCR reaction
- 5µl 10X enzyme buffer
- 2.5µl NcoI
- 2.5µl Kpn1

The reaction was allowed to proceed for 15 minutes at 37ºC incubation.

For the NcoI/KpnI digestion of the pTol plasmid vector the volumes used were as follows:

- 16µl DNA pTol plasmid
- 2µl 10X enzyme buffer
- 1µl NcoI

- 1µl Kpn1

The reaction was allowed to proceed for 15 minutes at 37ºC incubation.

Successful restriction digests were confirmed utilizing gel electrophoresis.

Ligation

Ligation was performed using the Promega LigaFast Rapid DNA Ligation System according to the manufacturer's protocol.

For the ligation of the SDM product the volumes used were as follows:

- 0.8µl vector DNA
- 0.3µl insert DNA
- 5µl 2X Rapid Ligation Buffer
- 3µl T4 DNA Ligase
- 0.9µl Nuclease-Free Water
- For a total volume of 10µl.

For the ligation of the PCR product the volumes used were as follows:

- 2µl vector DNA
- 1µl insert DNA
- 5µl 2X Rapid Ligation Buffer
- 3µl T4 DNA Ligase
- 0µl Nuclease-Free Water
- For a total volume of 10µl.

The reactions were incubated at room temperature for 5 minutes.

RESULTS

ICER Protein Expression Peaks During Early Folliculogenesis

ICER protein expression is seen across all stages of folliculogenesis. However, there are significant changes in protein expression in a stage-dependent manner. As shown in Fig.1A ICER expression is heaviest at the PV stage of folliculogenesis. The strongest banding supports a peak in ICER expression at this stage. In the EV and MV stages ICER expression is still detectable at significant levels. There is a diminishing expression at the LV stage and minimal expression at PG and FG stages.

In Fig.1B we show ubiquitous ICER expression across tissue types, with predominant expression in the testis as expected based on the heavy previous research which has focused on the role of ICER in spermatogenesis. We also observed significantly measurable expression in the ovaries in support of further investigation into the function of ICER in ovulation. Fig.1C shows ICER ovarian expression across stages of folliculogenesis. In confirmation of the earlier data, ICER again shows strong expression at the PV, EV, and MV stages, however in this case we observed equal expression at PV and EV, with a reduction at MV. Furthermore, there was again diminished but perceptible expression at the PG, and LV stages. Notably there is no ICER expression at the FG stage. Fig.4D is data from a previous experiment showing the expected sizes of ICER as well as CREM isoforms (Molina et al. 1993). ICERγ, the focus of this study, is determined to be a protein somewhere in the range of 18-19.5 kDa. Our observations of ICER protein expression consistently supports this range.

ICER RNA Expression Shows Two Peaks at PV and LV Stages of Folliculogenesis

In order to quantify ICER RNA expression, semi-quantitative PCR analysis was undertaken. ICER RNA is expressed across all stages of folliculogenesis. In keeping with the protein expression data, the RNA expression is lowest at the PG and FG stages. However, in contrast to the protein expression data, RNA expression displays two peaks first at the PV stage and second at the LV stage. The LV stage actually appears to be the most significant stage of ICER RNA expression in the follicle. ICER mRNA expression shows an initial peak at the PV stage followed by a decline in the EV stage and a further decline in the MV stage. Then ICER mRNA expression surges at the LV stage before again declining at FG. Taken in context the cyclical stage-dependent expression pattern of ICER mRNA underscores the nature of its regulation and its negative autoregulation in response to cAMP levels. Having confirmed the expression of ICER in the ovaries is stage-dependent at both the RNA and protein levels, the construction of an ICER overexpressing plasmid vector under the control of an ovarian promoter is a next logical step in the further elucidation of the role of ICER in ovulation.

Introduction of an NcoI Site into ICER

Having confirmed that ICER is indeed expressed in the zebrafish ovary we turned our attention to the design of an ovarian specific plasmid vector for the overexpression of ICER in the zebrafish ovary. In order to insert our ICER construct in the plasmid vector it was first necessary to remove EGFP from the previously used and demonstrated construct. To do so we utilized two restriction sites NcoI and KpnI. This was a relatively straightforward digestion confirmed in Fig.5 lanes 5-8. Lanes 5-8 contain the pminTol2 plasmid vector (5863 bp) containing 1.8kb 5'UTR of *cyp19a1* as well as EGFP which runs

near the 750 band in keeping with its size of 768 bp. The ICER construct itself did not contain an NcoI site and in order to facilitate its insertion into pminTol2 we decided to introduce a novel NcoI site into ICER. We chose two methods for the introduction of this mutation into ICER. First we designed PCR primers with the NcoI site incorporated and utilized these primers to amplify ICER DNA containing our mutation. This was confirmed following a restriction digest with KpnI and NcoI and the results are shown in Fig.5.

Lanes 1 and 2 contain primer set 1 and 2, respectively, for zebrafish ICER wild type (wt). The ICER band appears just below 400 in keeping with its predicted size of 368 bp following digestion. Lanes 3 and 4 contain primer set 1 and 2, respectively, for zebrafish ICER knockout (ko) again with a band near the 400 mark. A second method of introducing the NcoI site mutation was undertaken to ensure the generation of mutants. In the second route we utilized site-directed mutagenesis to introduce the necessary T to G mutation (5'…CCATGT…3' into 5'…CCATGG…3'). SDM was performed and confirmed with SDS-PAGE. As shown in Fig. 6 all seven clones were positive for the mutation.

Insertion of Mutated ICER into the pmintol2 Plasmid

Finally, our amplified ICER products were digested with KpnI and NcoI and inserted into the pminTol2 cyp19a1prom plasmid via ligation. In Fig. 7 lanes 1, 5, 8-10 all show positive zfICERγ product running near the 400 marker. Lanes 2 and 7 show the EGFP of the original plasmid running near the 750 marker. Lane 3 contains no genetic material and lane 4 contains both ICER and EGFP the likely result of pipette error by the user resulting in two samples being placed in the well for lane 4. Material from lanes 1, 5, 8, 9, and 10 were removed and sequenced and the product was confirmed to be zfICERγ.

ICER-protein expression

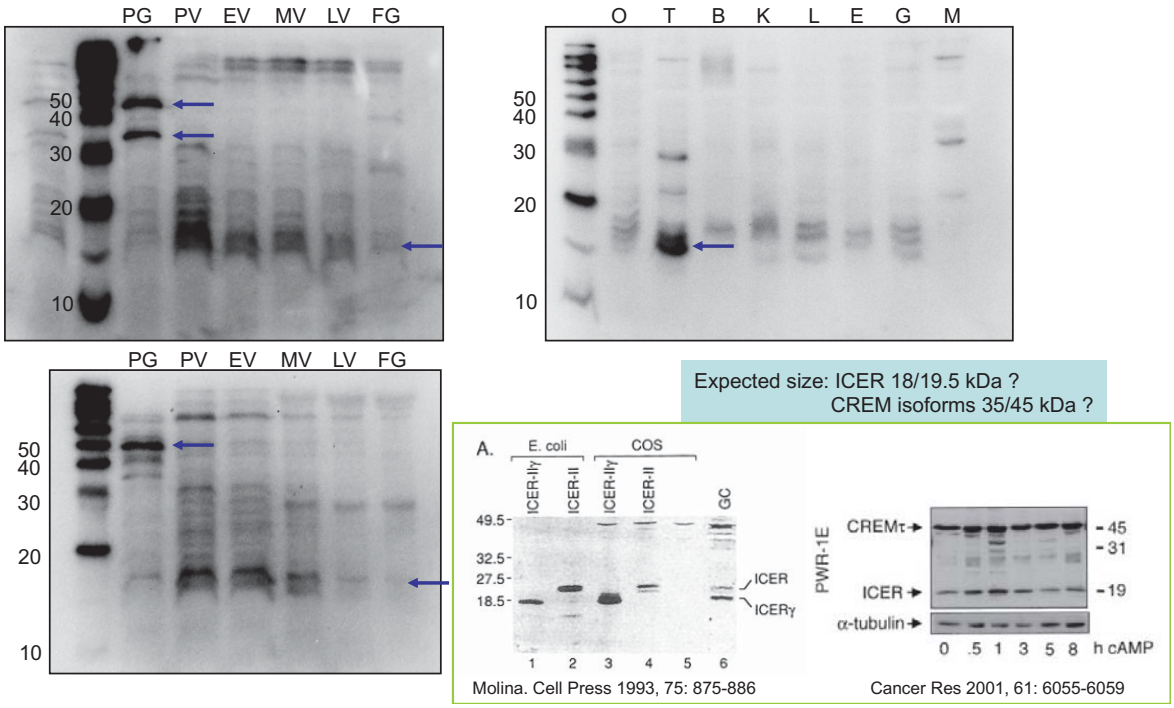

Figure 1 ICER Protein Expression

A. ICER and CREM expression by stage. CREM expression peaks at the PG stage. ICER expression on the other hand peaks at PV before showing a significant decline through the EV, MV, and LV stages with faint signal at the FG stage. B. ICER expression by tissue. ICER is ubiquitously expressed across tissue types with significant expression in the testis. ICER expression is also detectable in the ovaries. C. ICER and CREM expression by stage. Again CREM expression peaks at the PG stage. ICER expression on the other hand peaks at PV before showing a significant decline through the EV, MV, and LV stages with faint signal at the FG stage. D. Data from Molina, 1993 demonstrating expected ICER and CREM sizes. Depending on the specific ICER isoform the expected ICER product size is 18 kDa or 19.5 kDa. CREM isoforms will generate an expected product of 35 kDa or 45 kDa.

icer-RNA expression

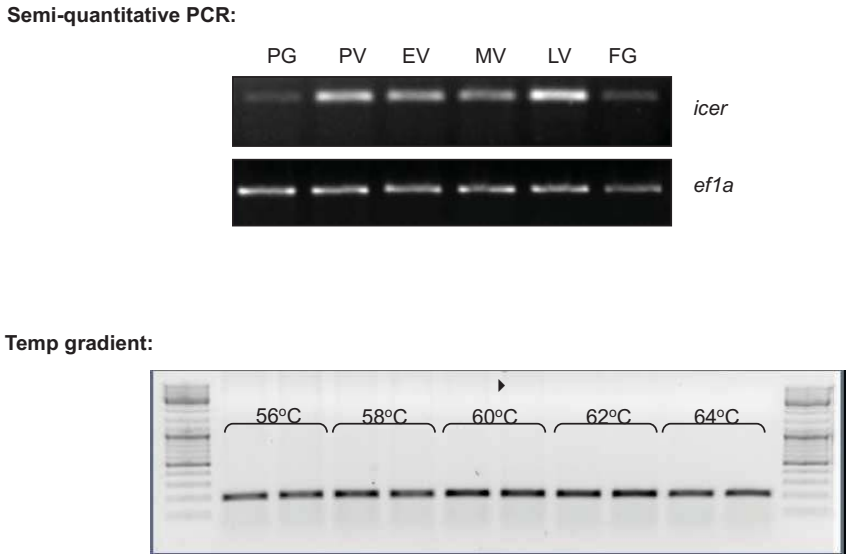

Figure 2 ICER RNA Expression
ICER RNA expression based on semi-quantitative PCR. Results show faint but detectable ICER RNA expression at the PG stage. ICER RNA expression increases in the PV stage before showing a slight decline in signal intensity at the EV and MV stages. A second peak in intensity is seen at the LV stage before another decline and a faint but detectable signal at the FG stage.

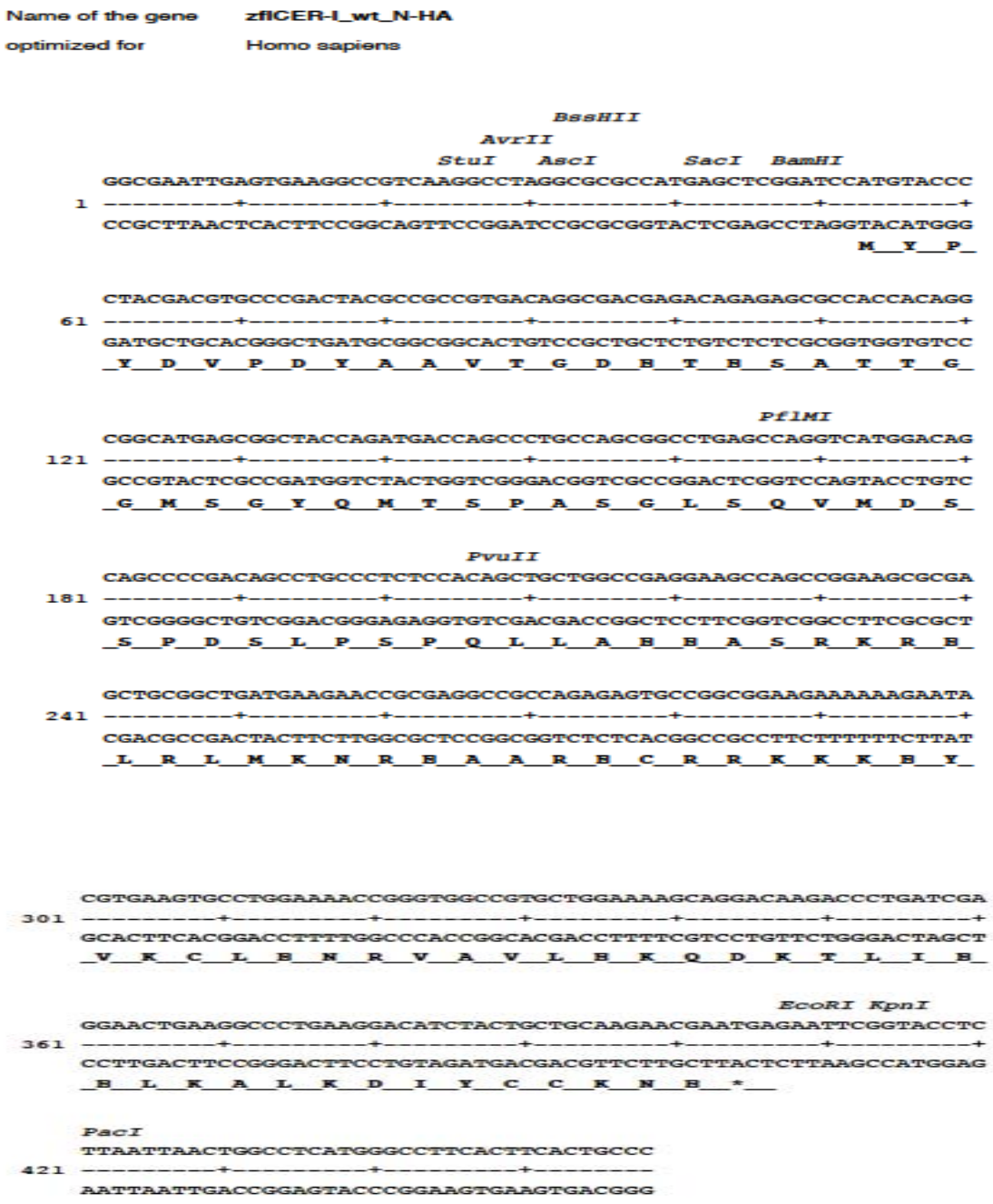

Figure 3 ICER sequence prior to insertion of novel NcoI site.

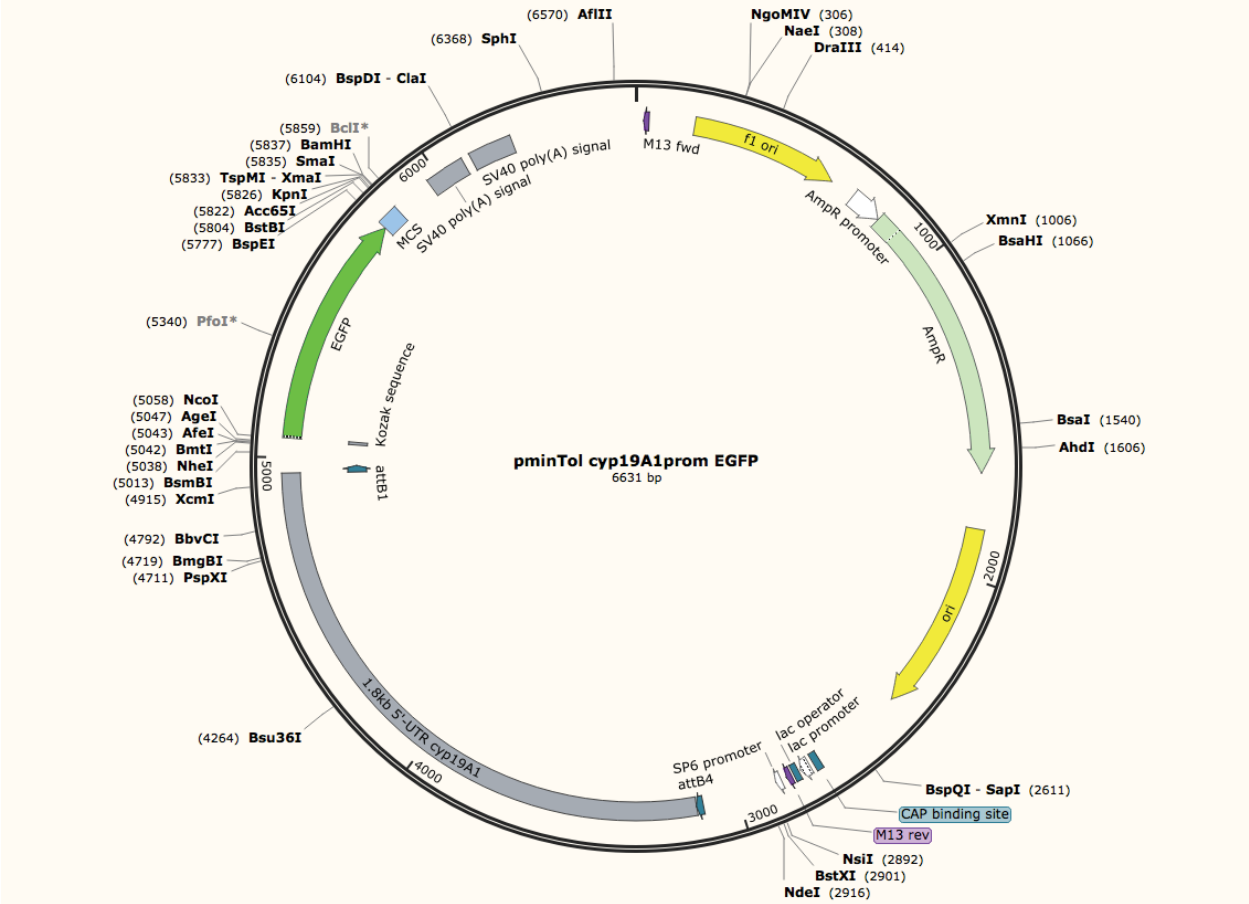

Figure 4 Original pminTol2 cyp19A1prom plasmid containing EGFP

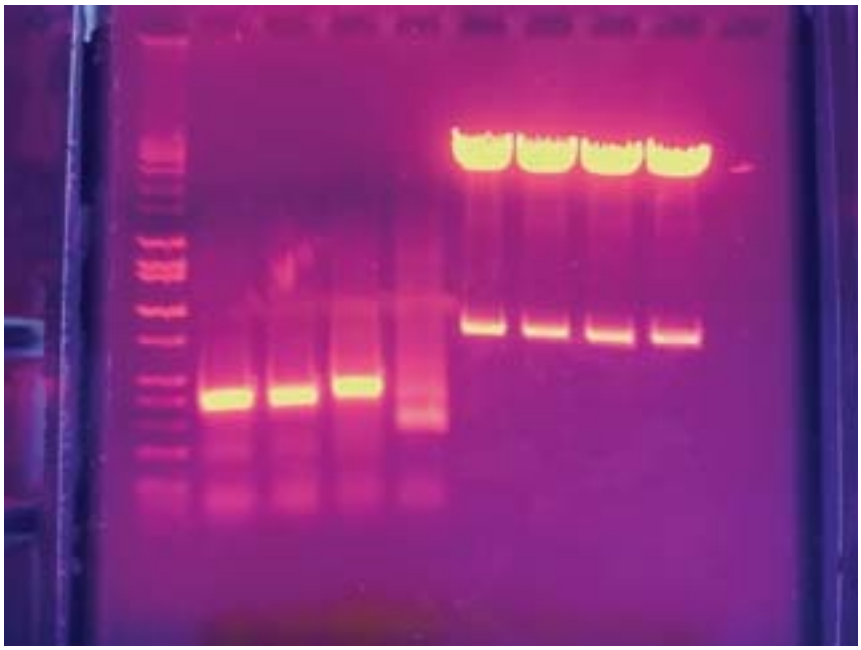

Figure 5 Digestion with NcoI and KpnI
Lanes 1 and 2 contain primer set 1 and 2, respectively, for zebrafish ICER wild type (wt). The ICER band appears just below 400 in keeping with its predicted size of 368 bp following digestion. Lanes 3 and 4 contain primer set 1 and 2, respectively, for zebrafish ICER knockout (ko). Again, the ICER band appears just below 400 in keeping with its predicted size of 368 bp following digestion. Lanes 5-8 contain the pminTol2 plasmid vector (5863 bp) containing 1.8kb 5'UTR of cyp19a1 as well as EGFP which runs near the 750 band in keeping with its size of 768 bp.

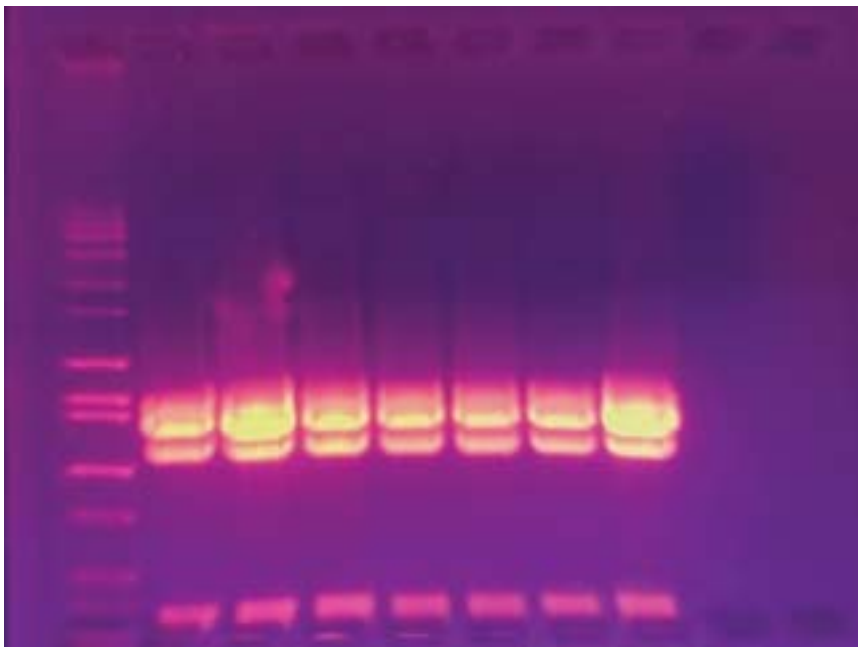

Figure 6 Wild Type Site-Directed Mutagenesis
SDM of wt zfICERγ to introduce the NcoI restriction site followed by digestion with NcoI and KpnI. The detection of two bands shows successful restriction digest confirming that all seven clones in lanes 1-7 are positive for the introduced mutation.

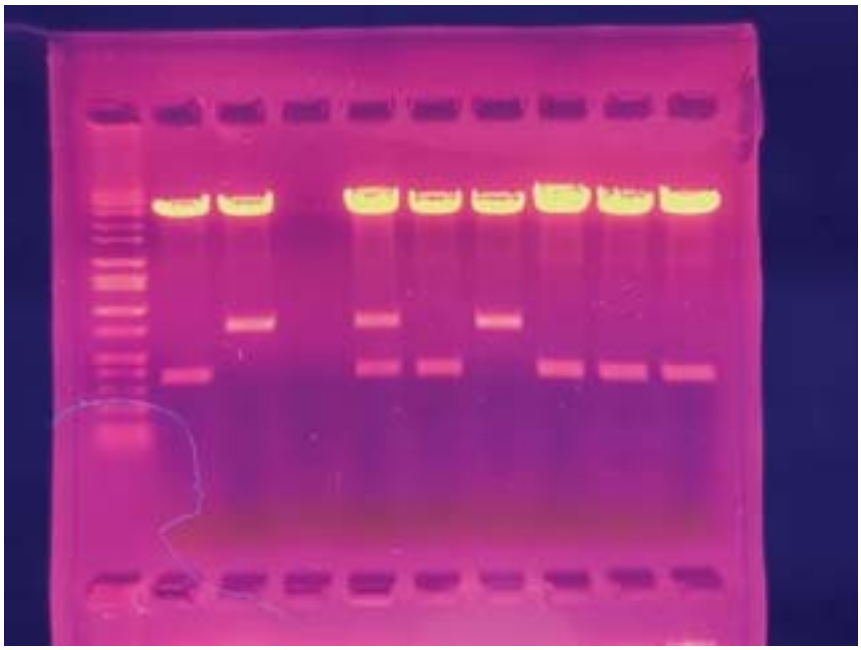

Figure 7 NcoI KpnI digestion after ligation wt zfICER pminTol2 (1.8kb 5'UTR cyp19A1).
Lanes 1, 5, 8-10 all show positive zfICERγ product running near the 400 marker. Lanes 2 and 7 show the EGFP of the original plasmid running near the 750 marker. Lane 3 contains no genetic material and lane 4 contains both ICER and EGFP the likely result of pipette error by the user resulting in two samples being placed in the well for lane 4.
Expected size: ICER-368 bp, EGFP-768 bp, pminTol2-5863 bp
Material from lanes 1, 5, 8, 9, and 10 were removed and sequenced to confirm the product was in fact ICER.

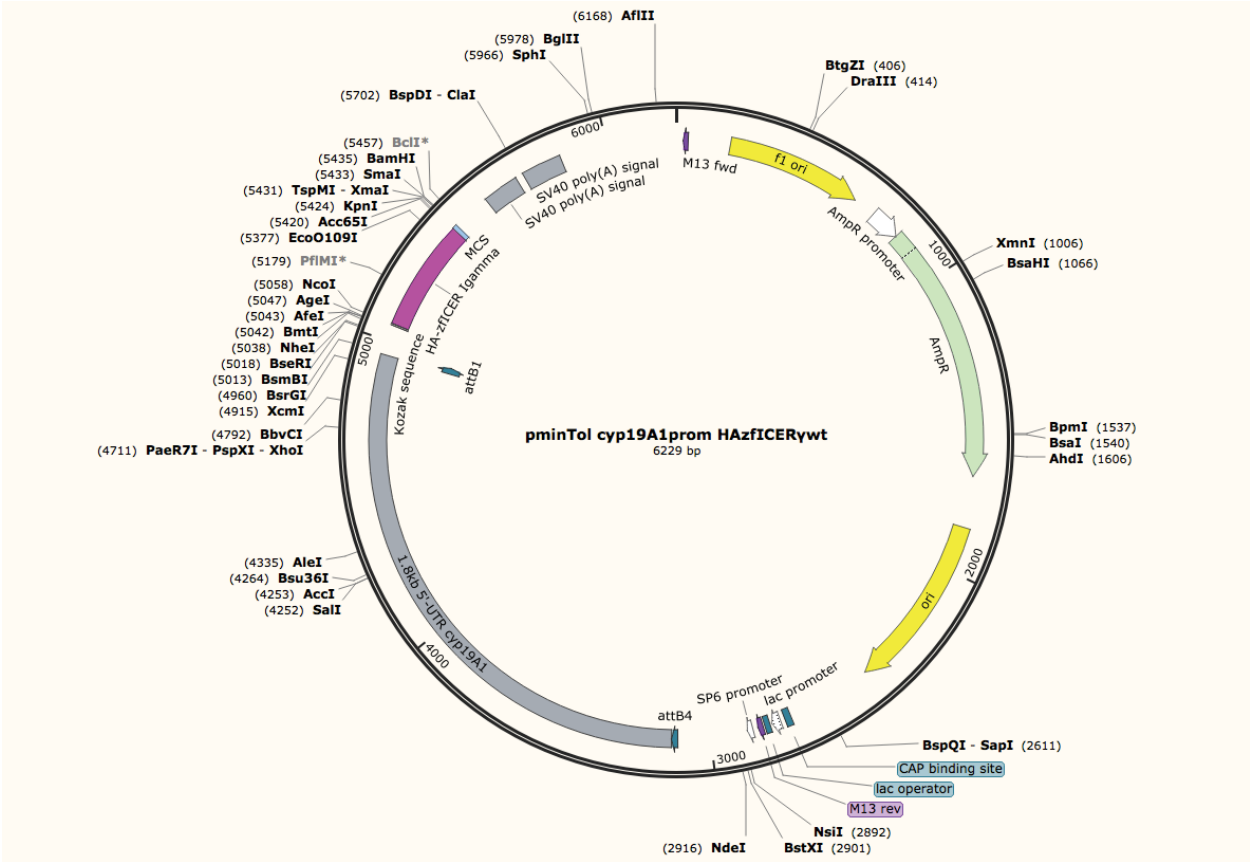

Figure 8 pminTol2 cyp19A1prom HAzfICERγ wt

The final plasmid construct containing zfICERγ downstream of the cyp19a1 5'UTR.

DISCUSSION

The generation of stable transgenic zebrafish expressing ICER under the control of the ovarian *cyp19a1* promoter is an important first step in elucidating the pivotal and important role that ICER plays in reproduction. Here we demonstrate the construction of a Tol2 plasmid vector containing the ICER gene downstream of the 1.8 kb 5'UTR of *cyp19a1*, an ovarian specific aromatase gene. First it was necessary to prove that ICER was indeed expressed in the ovaries in vivo. ICER was shown to be ubiquitously expressed across multiple tissue types with the highest expression in the testis followed by the ovaries. Protein and RNA expression were then measured in the ovaries specifically. ICER protein expression peaked at the PV stage and declined through the EV, MV, and LV stages with a sharp decline at the FG stage. ICER RNA expression showed two peaks at the PV and LV stages as measured by semi-quantitative PCR. Having confirmed that ICER is expressed in the ovary and that its expression is stage dependent we moved on to the generation of an ICER Tol2 vector for insertion into the zebra fish genome.

Two methods were used to generate an ICER construct containing flanking NcoI and KpnI restriction sites. In order to introduce the NcoI restriction site it was necessary to change 5'…CCATGT…3' into 5'…CCATGG…3' within the ICER construct. We utilized PCR to amplify a mutated ICER DNA sequence by designing primers intended to insert a G in place of a T thereby introducing a new NcoI restriction site into our construct. This proved successful and was confirmed via restriction digestion with KpnI and NcoI followed by gel electrophoresis. Additionally, site-directed mutagenesis was utilized in a similar manner introducing the T to G mutation and confirmed by restriction digest and gel electrophoresis. Finally, a pTol2 vector was created by first removing EGFP from a

previously designed vector which expressed EGFP under the control of the *cyp19a1* promoter region. Our new ICER construct was then inserted into the pminTol2 *cyp19a1* promoter vector by digestion with NcoI and KpnI followed by ligation to create a new vector containing ICER. Again gel electrophoresis confirmed the existence of an ICER sized product within the pTol2 vector.

To confirm that this product was indeed ICER, material was excised from lanes 1, 5, 8, 9, and 10 and sent to be sequenced. Sequencing data did then confirm that this product was indeed ICER proving the successful insertion of ICERγ into the pminTol2 cyp19a1prom vector. This vector can be utilized in future studies, with the Tol2 system, to generate a line of transgenic zebra fish that can be induced with gonadotropin stimulation to overexpress ICER within the ovaries.

Table 1
Expression patterns of various growth factors, receptors and steroidogenic enzymes during zebrafish follicle development.

Gene (other names)	Stage I	Stage II	Stage III-1	StageIII-2	Stage IV	References
inhba1(activin βA1)	+	++	++++	++++	+++	Wang and Ge (2004), DiMuccio et al. (2005)
inhba2(activin βA2)	+	+	+++	+++	+++	DiMuccio et al. (2005)[a]
inhbb(activin β=)	+	+	++	++	ND	Wang and Ge (2004)[b]
acvr2a(ActRIIA)	+	+	+	++	+	DiMuccio et al. (2005)[a]
acvr2b(ActRIIB)	+	+	++	+	+	DiMuccio et al. (2005)[a]
amh	+	++	–	UD	UD	Rodriguez-Mari et al. (2005)
bmp15	+	+	+	+	+	Clelland et al. (2006)
cyp11a1(P450scc)	+	–	−−−	−−−	−−−−−	Ings and Van Der Kraak (2006)[c]
cyp17a1(P450c17)	+	–	−−	−−	−−−−−	Ings and Van Der Kraak (2006)[c]
cyp19a1(P450AromA)	+	+++++	++	++	UD	Ings and Van Der Kraak (2006)[c]
egf	ND	+	–	−−	ND	Wang and Ge (2004)[b]
egfr	ND	+	++	++	ND	Wang and Ge (2004)[b]
fts(follistatin)	+	++	+++	−−	ND	Wang and Ge (2004)[b]
fshr	+	++	+++	+++	ND	Kwok et al. (2005)[b]
gdf9	+	–	–	−−	ND	Liu and Ge (2007)[b]
hsd17b1(17β-HSD1)	+	++	−−−	−−−	−−−	Ings and Van Der Kraak (2006)[c]
hsd17b3(17β-HSD3)	+	–	+++++++	+++++++	+++++	Ings and Van Der Kraak (2006)[c]
hsd20b(20β-HSD)	+	+	+	+	+	Zhou et al. (2007a,b)
hsd3b(3β-HSD)	+	+	–	–	−−−−−−	Ings and Van Der Kraak (2006)[c]
lhcgr	+	+	++	+++	+++++	Kwok et al. (2005)
pacap2	ND	+	++	+++	+++	Wang et al. (2003)
star	+	−−	+	+	−−−−−−−	Ings and Van Der Kraak (2006)[c]
tgfb1	+	+	–	−−	−−−−	Kohli et al. (2003)[a]
tgfbr1(ALK5, TβRI)	++	+	+	+	+	Kohli et al. (2003)[a]
vpac2r	ND	+	+	+	+	Wang et al. (2003)
mPRα	+	+	+	+++	+++	Kazeto et al. (2005), Tan et al. (2009a,b)
mPRβ	+	+	+	+++	+++	Kazeto et al. (2005), Tan et al. (2009a,b)

The level at stage I is set as one + and the increase in the number of + symbol indicates an increase in expression levels while the – symbol indicates decrease in the expression level. ND: not determined. UD: undetectable. All studies measured mRNA levels with the exception of mPRs where protein levels were determined.

[a] These studies did not separate stage I and II follicles and they were assigned the same expression level.

[b] These studies separate stage III follicles into three groups: early vitellogenic (EV), mid-vitellogenic (MV), and folly grown (FG) follicles. Data from the EV and FG follicles were grouped into stages III-1 and III-2, respectively.

[c] These studies did not separate stages III-1 and III-2 follicles and therefore, the same value was assigned to both stages III-1 and III-2.

Table 1 Expression patterns of various growth factors, receptors, and steroidogenic enzymes during zebrafish follicle development (Clelland, 2009).

As can be seen in the table above from a 2009 review of the expression patterns of various growth factors, receptors, and steroidogenic enzymes during folliculogenesis, there are an extensive number of paracrine/autocrine factors produced within the ovary that interact with the gonadotropins to modulate follicle development and oocyte maturation (Clelland, 2009). Some of these have been mentioned previously while others are beyond the scope of this research. Furthermore, this serves to highlight how important ovulation is to an organism, and due to its importance and the investment on the part of the organism to maintain the system, just how tightly controlled all the events which lead to the final production of a mature oocyte are. The cAMP second messenger system is critically involved in the endocrine system and cAMP itself plays an essential role in follicular development, atresia, and luteinization in the ovary (Wang et al., 2007). The critical role of cAMP in mediating FSH and LH signaling, as well as its involvement in the inhibin-activin-follistatin system of endocrine regulation, underscore the importance of understanding the manner in which the cAMP system itself if regulated to further clarify the intricate feedback loops and regulatory mechanisms of the ovarian cycle. Temporally restricted gene expression is vital to many diverse biological processes. And it is abundantly clear that the precise timing and synchronization of the pattern of gene expression and ovulation, is integral to the maintenance of fertility and that the result of any disruption of this highly coordinated system is infertility. By understanding the mechanisms of the regulation of the system which itself underlies the regulation of the endocrine system we can begin to shed new light on the complex mysteries of fertility, and the pathogenic factors causing infertility.

There is a final part of the cAMP second messenger system which hasn't been discussed yet. Earlier it was mentioned that it almost defies belief that cAMP concentrations can have such myriad effects within a cell once cAMP concentrations begin to ubiquitously rise intracellularly. It was long believed that cAMP signal transduction depended on random collisions between signaling components within the cell (Martin and Cooper, 2006). However, starting in the late 1980's our understanding of the cAMP system drastically changed. As mentioned previously a fundamental step of the cAMP system is the synthesis of cAMP itself by the activity of adenylyl cyclase (AC). Long viewed as a single entity that was itself regulated by hormones in either a stimulatory or inhibitory manner through the actions of stimulatory or inhibitory G proteins of the GPCR, it has since been discovered that there actually exist 10 AC isoforms, 9 membrane-bound and 1 soluble (Willoughby and Cooper, 2007). This discovery turned the established paradigm of the cAMP system on its head. It rapidly emerged that these ACs are constrained to distinct regions within the plasma membrane, opening the door to the idea that cAMP signaling is compartmentalized within the cell (Houslay, 2009).

Now no man is an island, and if my scientific education has taught me nothing else, neither is an enzyme. As Newton's third law attests for every action an equal and opposite reaction, there naturally exists a family of enzymes which are the yin to adenylyl cyclase's yang. These are the cyclic nucleotide phosphodiesterases (PDE) which degrade the phosphodiester bond in the second messengers cAMP and cGMP, returning them to their acyclic forms 5'-AMP and 5'-GMP (Wang, 2007). Sequestered PDE isoforms act in concert with AC, albeit in an opposing manner, to sculpt gradients of cAMP, generating signal compartmentalization and revealing the presence of discrete signaling complexes

within cells which are termed cAMP microdomains (Houslay, 2009). The discovery, acceptance, and proof of theory of cAMP microdomains was pivotal in understanding the inexplicably diverse effects cAMP could exert within the environment of a single cell.

The organization and regulation of cAMP microdomains are incredibly intricate and detailed and their discussion could fill another thesis entirely. Rather than diving deep into the complexities of the cAMP microdomains I would rather discuss their implications within the paradigm of ICER and the ovary, which is after all what this research is about. The many PDE family members and their isoforms have numerous different localizations and functions. Of utmost significance to the research at hand are the PDE3 and PDE4 families. Oocytes predominantly express the PDE3 family, and inhibition of PDE3A blocks oocyte maturation, while PDE3A-deficient mice ovulate a normal number of oocytes but are infertile because their oocytes fail to undergo spontaneous maturation (Vezzosi and Bertherat, 2011; Wang, 2007). Furthermore, research into PDE3 inhibitors in primates showed a dose-dependent ability of PDE3 inhibitors to block the resumption of meiosis in macaque oocytes, suggesting the possibility of PDE3 inhibitors to function as contraceptives (Jensen et al., 2002).

PDE4D is expressed in granulosa cells and is critically important to the ovarian follicle. Inhibition of PDE4 in follicle culture causes oocyte maturation in the absence of gonadotropin stimulation, while subcutaneous injection of PDE4 inhibitors induced ovulation in rats (Vezzosi and Bertherat, 2011). Additionally, PDE4 inhibition in FSH-primed rats induced ovulation in the absence of LH or hCG, indicating the possible function of PDE4 inhibitors as a substitute for LH or hCG in ovulation, and when coadministered with a subeffective dose of hCG, PDE4 inhibitors enhanced the ovulatory response

(McKenna et al., 2005). However, mice which are deficient in PDE4D exhibit delayed growth, decreased viability, and reduced female fertility. PDE4D null females show impaired ovulation and a diminished sensitivity of the granulosa cells to gonadotropins (Jin et al., 1999). The ablation of the PDE4D gene caused the formation of luteinized unruptured follicles with entrapped oocytes in mouse models again leading to infertility (Park et al., 2003). Basically the inactivation of the cAMP/PDE4D regulatory feedback loop disrupts the correct coordination of the pattern of gene expression and ovulation in response to gonadotropin-induced cAMP elevation. Rather than being transiently elevated, the consistent elevation of cAMP leads to desensitization of the gonadotropin receptors to further signaling, inducing premature luteinization without oocyte release causing infertility. The role of ICER, the only cAMP inducible repressor of cAMP-dependent gene expression in the ovary, in relation to PDE3 and PDE4 remains to be studied. In fact, the relative dearth of studies of ICER in the ovary is the basis for this research, which can hopefully pave the way to further investigation of ICER in ovulation. However, in the absence of specific evidence of ICER interactions with PDEs in the ovaries, data from the cardiovascular system might provide some clues as to the effects of ICER in relation to PDEs.

Yan, Miller, and Abe in a 2007 paper on the regulation of PDE3 and ICER in the heart describe a PDE3A-ICER positive feedback loop, in which ICER represses PDE3A gene transcription. In brief, CREB activation in response to cAMP induction, initiates ICER expression. ICER then represses PDE3A expression, by interacting with CRE within the PDE3A promoter. PDE3A downregulation leads to increased local cAMP concentration, which activates PKA. PKA activation leads to more ICER protein elevation

completing the loop (Yan et al., 2007). Sustained ICER induction promotes cardiac myocyte apoptosis by suppressing CREB-mediated transcription and survival protein Bcl-2 expression (Ding et al., 2005; Yan, 2007). Angiotensin II (Ang II) and β–adrenergic receptor agonist isoproterenol (ISO) utilize cAMP signaling to regulate contraction, growth, and cell death in cardiac myocytes. Chronic exposure to Ang II and ISO promote cardiac dysfunction through the PDE3A-ICER positive feedback loop (Ding, 2005; Yan, 2007).

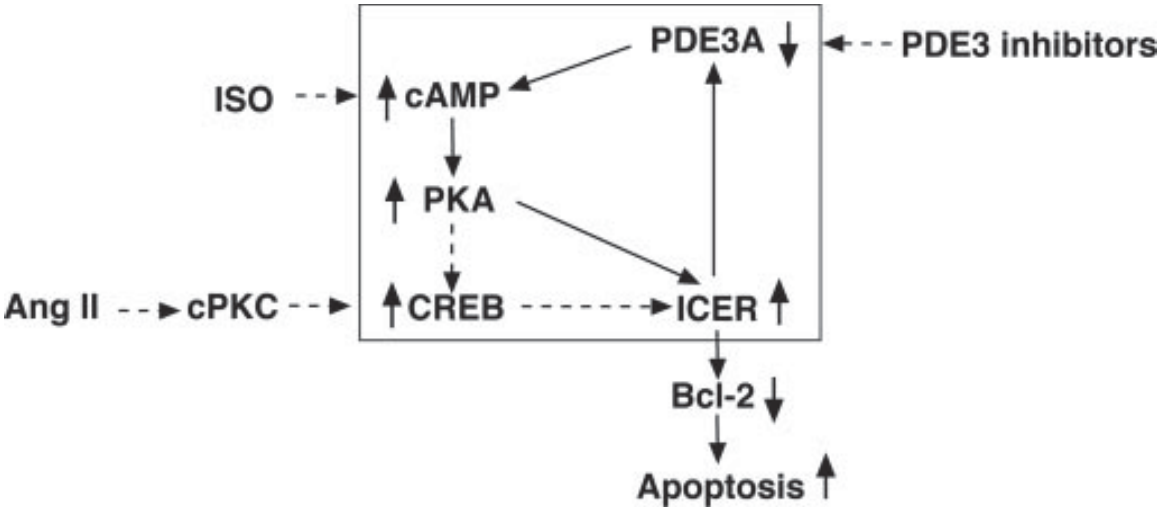

Fig. 10 Scheme of the PDE3A-ICER feedback loop (Ding et al., 2005)

PDE3 inhibition in the reproductive system blocks the resumption of meiosis in macaques and blocks oocyte maturation in mice causing infertility by an unknown mechanism, while PDE3 inhibition via ICER induction in the cardiovascular system promotes cardiac myocyte apoptosis, causing heart failure (Jensen, 2002; Vezzosi and Bertherat, 2011; Wang, 2007; Ding, 2005; Yan, 2007). Based simply on this, the effects of ICER overexpression in the ovary clearly deserve further study. Different tissues react differently to ICER disequilibrium. Chronic reduction of ICER correlates with an increase in CREB activity and expression of ATF3 in adipose tissues leading to systemic insulin

resistance in obese mice (Favre et al., 2011). ICER protein levels were found to be significantly reduced in prostate cancer cells and undetectable and uninducible by cAMP in a prostate tumor cell line (Yehia et al., 2001). Clearly ICER antagonism of CREB, as well as PDE, represents a dynamic system of temporally and spatially restricted gene expression important in diverse tissues, systems and processes. Further investigation of the role of ICER in fertility, and its relationship with PDEs in the ovary, can hopefully lead to new discoveries and deeper understanding of the ovulatory process. Although still limited, the data on ICER induction in mice supports the notion that ICER plays a major role in ovulation. Therefore, further research into its role in zebrafish ovulation made possible by the creation of an inducible ovarian specific ICER overexpression plasmid described herein, may in the future lead to novel therapies in the field of reproductive medicine.

REFERENCES

1. Aytekin, Y., & Yüce, R. (2008). Ovary maturatıon stages and histological investigation of ovary of the Zebrafish (Danio rerio). *Brazilian Archives of Biology and Technology*, *51*(3), 513-522.
2. Bodor, J., Bodorova, J., & Gress, R. E. (2000). Suppression of T cell function: a potential role for transcriptional repressor ICER. *Journal of leukocyte biology*, *67*(6), 774-779.
3. Cheshenko, K., Brion, F., Le Page, Y., Hinfray, N., Pakdel, F., Kah, O., ... & Eggen, R. I. (2007). Expression of zebra fish aromatase cyp19a and cyp19b genes in response to the ligands of estrogen receptor and aryl hydrocarbon receptor. *Toxicological sciences*, *96*(2), 255-267.
4. Clelland, E., & Peng, C. (2009). Endocrine/paracrine control of zebrafish ovarian development. *Molecular and cellular endocrinology*, *312*(1-2), 42-52.
5. Cooper, D. M. (2003). Regulation and organization of adenylyl cyclases and cAMP. *Biochemical Journal*, *375*(Pt 3), 517.
6. Ding, B., Abe, J. I., Wei, H., Xu, H., Che, W., Aizawa, T., ... & Berk, B. C. (2005). A positive feedback loop of phosphodiesterase 3 (PDE3) and inducible cAMP early repressor (ICER) leads to cardiomyocyte apoptosis. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(41), 14771-14776.
7. Favre, D., Le Gouill, E., Fahmi, D., Verdumo, C., Chinetti-Gbaguidi, G., Staels, B., … Abderrahmani, A. (2011). Impaired Expression of the Inducible cAMP Early Repressor Accounts for Sustained Adipose CREB Activity in Obesity. *Diabetes*, *60*(12), 3169–3174. http://doi.org/10.2337/db10-1743
8. Foulkes, N. S., Schlotter, F., Pévet, P., & Sassone-Corsi, P. (1993). Pituitary hormone FSH directs the CREM functional switch during spermatogenesis. *Nature*, *362*(6417), 264.
9. Goswami, D., & Conway, G. S. (2005). Premature ovarian failure. *Human reproduction update*, *11*(4), 391-410.
10. Hartenstein, V. (2006). The neuroendocrine system of invertebrates: a developmental and evolutionary perspective. *Journal of Endocrinology*, *190*(3), 555-570.
11. Hoo, J. Y., Kumari, Y., Shaikh, M. F., Hue, S. M., & Goh, B. H. (2016). Zebrafish: a versatile animal model for fertility research. *BioMed research international*, *2016*.
12. Houslay, M. D. (2010). Underpinning compartmentalised cAMP signalling through targeted cAMP breakdown. *Trends in biochemical sciences*, *35*(2), 91-100.
13. Howe, K., Clark, M. D., Torroja, C. F., Torrance, J., Berthelot, C., Muffato, M., ... & McLaren, S. (2013). The zebrafish reference genome sequence and its relationship to the human genome. *Nature*, *496*(7446), 498.
14. Hull, M. G., Glazener, C. M., Kelly, N. J., Conway, D. I., Foster, P. A., Hinton, R. A., ... & Desai, K. M. (1985). Population study of causes, treatment, and outcome of infertility. *Br Med J (Clin Res Ed)*, *291*(6510), 1693-1697.

15. Hunzicker-Dunn, M., & Maizels, E. T. (2006). FSH signaling pathways in immature granulosa cells that regulate target gene expression: branching out from protein kinase A. *Cellular signalling*, *18*(9), 1351-1359.
16. Jensen, J. T., Schwinof, K. M., Zelinski-Wooten, M. B., Conti, M., DePaolo, L. V., & Stouffer, R. L. (2002). Phosphodiesterase 3 inhibitors selectively block the spontaneous resumption of meiosis by macaque oocytes in vitro. *Human Reproduction*, *17*(8), 2079-2084.
17. Jin, S. L. C., Richard, F. J., Kuo, W. P., D'Ercole, A. J., & Conti, M. (1999). Impaired growth and fertility of cAMP-specific phosphodiesterase PDE4D-deficient mice. *Proceedings of the National Academy of Sciences*, *96*(21), 11998-12003.
18. Kawakami, K. (2007). Tol2: a versatile gene transfer vector in vertebrates. *Genome biology*, *8*(1), S7.
19. Kazeto, Y., Ijiri, S., Place, A. R., Zohar, Y., & Trant, J. M. (2001). The 5′-flanking regions of CYP19A1 and CYP19A2 in zebrafish. *Biochemical and biophysical research communications*, *288*(3), 503-508.
20. Kwan, K. M., Fujimoto, E., Grabher, C., Mangum, B. D., Hardy, M. E., Campbell, D. S., ... & Chien, C. B. (2007). The Tol2kit: a multisite gateway-based construction kit for Tol2 transposon transgenesis constructs. *Developmental dynamics*, *236*(11), 3088-3099.
21. Kwok, H. F., So, W. K., Wang, Y., & Ge, W. (2005). Zebrafish gonadotropins and their receptors: I. Cloning and characterization of zebrafish follicle-stimulating hormone and luteinizing hormone receptors—evidence for their distinct functions in follicle development. *Biology of reproduction*, *72*(6), 1370-1381.
22. Li, C. W., & Ge, W. (2013). Regulation of the activin-inhibin-follistatin system by bone morphogenetic proteins in the zebrafish ovary. *Biology of reproduction*, *89*(3), 55-1.
23. Lin, S. Y., Phillips, D. J., & de Kretser, D. M. (2003). Regulation of ovarian function by the TGF-beta superfamily and follistatin. *Reproduction*, *126*(2), 133-148.
24. Lonze, B. E., & Ginty, D. D. (2002). Function and regulation of CREB family transcription factors in the nervous system. *Neuron*, *35*(4), 605-623.
25. Maheshwari, A., Hamilton, M., & Bhattacharya, S. (2008). Effect of female age on the diagnostic categories of infertility. *Human reproduction*, *23*(3), 538-542.
26. Maitra, S., Das, D., Ghosh, P., Hajra, S., Roy, S. S., & Bhattacharya, S. (2014). High cAMP attenuation of insulin-stimulated meiotic G2-M1 transition in zebrafish oocytes: interaction between the cAMP-dependent protein kinase (PKA) and the MAPK3/1 pathways. Molecular and cellular endocrinology, 393(1-2), 109-119.
27. Martin, A. C. L., & Cooper, D. M. F. (2006). Layers of organization of cAMP microdomains in a simple cell.
28. Mascarenhas, M. N., Flaxman, S. R., Boerma, T., Vanderpoel, S., & Stevens, G. A. (2012). National, regional, and global trends in infertility prevalence since 1990: a systematic analysis of 277 health surveys. *PLoS medicine*, *9*(12), e1001356.

29. Mayr, B., & Montminy, M. (2001). Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nature reviews Molecular cell biology*, *2*(8), 599.
30. Mazón, M. J., Gómez, A., & Zanuy, S. (2011). Follicle stimulating hormone activates the steroidogenic process via cAMP/PKA and MAP-kinase pathways in the ovary of European sea bass (Dicentrarchus labrax). *Indian Journal of Science and Technology*, *4*(S8), 190-191.
31. McClintock, B. (1950). The origin and behavior of mutable loci in maize. *Proceedings of the National Academy of Sciences*, *36*(6), 344-355.
32. McKenna, S. D., Pietropaolo, M., Tos, E. G., Clark, A., Fischer, D., Kagan, D., ... & Palmer, S. (2005). Pharmacological inhibition of phosphodiesterase 4 triggers ovulation in follicle-stimulating hormone-primed rats. *Endocrinology*, *146*(1), 208-214.
33. Meyer, T. E., & Habener, J. F. (1993). Cyclic adenosine 3′, 5′-monophosphate response element binding protein (CREB) and related transcription-activating deoxyribonucleic acid-binding proteins. *Endocrine reviews*, *14*(3), 269-290.
34. Michael, M. D., Michael, L. F., & Simpson, E. R. (1997). A CRE-like sequence that binds CREB and contributes to cAMP-dependent regulation of the proximal promoter of the human aromatase P450 (CYP19) gene. Molecular and cellular endocrinology, 134(2), 147-156.
35. Molina, C. A., Foulkes, N. S., Lalli, E., & Sassone-Corsi, P. (1993). Inducibility and negative autoregulation of CREM: an alternative promoter directs the expression of ICER, an early response repressor. *Cell*, *75*(5), 875-886.
36. Mosher, W. D. (1988). Fecundity and infertility in the United States. *American journal of public health*, *78*(2), 181-182.
37. Muñiz, L. C., Yehia, G., Ratnakar, P. V., & Molina, C. A. Hyperovulation in an ovarian-specific transgenic mouse model. *Unpublished manuscript.*
38. Nagahama, Y., & Yamashita, M. (2008). Regulation of oocyte maturation in fish. *Development, Growth & Differentiation*, *50*(s1).
39. Park, J. Y., Richard, F., Chun, S. Y., Park, J. H., Law, E., Horner, K., ... & Conti, M. (2003). Phosphodiesterase regulation is critical for the differentiation and pattern of gene expression in granulosa cells of the ovarian follicle. *Molecular Endocrinology*, *17*(6), 1117-1130.
40. Poon, S. K., So, W. K., Yu, X., Liu, L., & Ge, W. (2009). Characterization of inhibin α subunit (inha) in the zebrafish: evidence for a potential feedback loop between the pituitary and ovary. *Reproduction*, *138*(4), 709-719.
41. Popovics, P., Rekasi, Z., Stewart, A. J., & Kovacs, M. (2011). Regulation of pituitary inhibin/activin subunits and follistatin gene expression by GnRH in female rats. *Journal of Endocrinology*, *210*(1), 71-79.
42. Sands, W. A., & Palmer, T. M. (2008). Regulating gene transcription in response to cyclic AMP elevation. *Cellular signalling*, *20*(3), 460-466.
43. Sassone-Corsi, P. (1998). Coupling gene expression to cAMP signalling: role of CREB and CREM. *The international journal of biochemistry & cell biology*, *30*(1), 27-38.

44. Segner, H. (2009). Zebrafish (Danio rerio) as a model organism for investigating endocrine disruption. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *149*(2), 187-195.
45. Selman, K., Wallace, R. A., Sarka, A., & Qi, X. (1993). Stages of oocyte development in the zebrafish, Brachydanio rerio. *Journal of morphology*, *218*(2), 203-224.
46. So, W. K., Kwok, H. F., & Ge, W. (2005). Zebrafish gonadotropins and their receptors: II. Cloning and characterization of zebrafish follicle-stimulating hormone and luteinizing hormone subunits—their spatial-temporal expression patterns and receptor specificity. Biology of reproduction, 72(6), 1382-1396.
47. Stehle, J. H., Foulkes, N. S., Molina, C. A., Simonneaux, V., Pévet, P., & Sassone-Corsi, P. (1993). Adrenergic signals direct rhythmic expression of transcriptional represser CREM in the pineal gland. *Nature*, *365*(6444), 314.
48. Steward, R. G., Lan, L., Shah, A. A., Yeh, J. S., Price, T. M., Goldfarb, J. M., & Muasher, S. J. (2014). Oocyte number as a predictor for ovarian hyperstimulation syndrome and live birth: an analysis of 256,381 in vitro fertilization cycles. *Fertility and sterility*, *101*(4), 967-973.
49. Tanaka, M., Fukada, S., Matsuyama, M., & Nagahama, Y. (1995). Structure and promoter analysis of the cytochrome P-450 aromatase gene of the teleost fish, medaka (Oryzias latipes). *The Journal of Biochemistry*, *117*(4), 719-725.
50. Thisse, B., Heyer, V., Lux, A., Alunni, V., Degrave, A., Seiliez, I., ... & Thisse, C. (2004). Spatial and temporal expression of the zebrafish genome by large-scale in situ hybridization screening. In *Methods in cell biology* (Vol. 77, pp. 505-519). Academic Press.
51. Tong, S. K., & Chung, B. C. (2003). Analysis of zebrafish cyp19 promoters. *The Journal of steroid biochemistry and molecular biology*, *86*(3-5), 381-386.
52. Vaegter, K. K., Lakic, T. G., Olovsson, M., Berglund, L., Brodin, T., & Holte, J. (2017). Which factors are most predictive for live birth after in vitro fertilization and intracytoplasmic sperm injection (IVF/ICSI) treatments? Analysis of 100 prospectively recorded variables in 8,400 IVF/ICSI single-embryo transfers. *Fertility and sterility*, *107*(3), 641-648.
53. Vezzosi, D., & Bertherat, J. (2011). Phosphodiesterases in endocrine physiology and disease. *European journal of endocrinology*, *165*(2), 177-188.
54. Walker, W. H., & Habener, J. F. (1996). Role of transcription factors CREB and CREM in cAMP-regulated transcription during spermatogenesis. *Trends in Endocrinology & Metabolism*, *7*(4), 133-138.
55. Wang, Y., & Ge, W. (2004). Developmental profiles of activin βA, βB, and follistatin expression in the zebrafish ovary: evidence for their differential roles during sexual maturation and ovulatory cycle. *Biology of reproduction*, *71*(6), 2056-2064.
56. Wang, Z., Shi, F., Jiang, Y. Q., LU, L. Z., Wang, H., Watanabe, G., & Taya, K. (2007). Changes of cyclic AMP levels and phosphodiesterase activities in the rat ovary. *Journal of Reproduction and Development*, *53*(4), 717-725.
57. Willoughby, D., & Cooper, D. M. (2007). Organization and Ca2+ regulation of adenylyl cyclases in cAMP microdomains. *Physiological reviews*, *87*(3), 965-1010.

58. Woods, I. G., Kelly, P. D., Chu, F., Ngo-Hazelett, P., Yan, Y. L., Huang, H., ... & Talbot, W. S. (2000). A comparative map of the zebrafish genome. *Genome research*, *10*(12), 1903-1914.
59. Yan, C., Miller, C. L., & Abe, J. (2007). Regulation of Phosphodiesterase 3 and Inducible cAMP Early Repressor in the Heart. *Circulation Research*, *100*(4), 489–501. http://doi.org/10.1161/01.RES.0000258451.44949.d7
60. Yehia, G., Razavi, R., Memin, E., Schlotter, F., & Molina, C. A. (2001). The expression of inducible cAMP early repressor (ICER) is altered in prostate cancer cells and reverses the transformed phenotype of the LNCaP prostate tumor cell line. *Cancer research*, *61*(16), 6055-6059.
61. Zhang, Z., Zhu, B., & Ge, W. (2015). Genetic analysis of zebrafish gonadotropin (FSH and LH) functions by TALEN-mediated gene disruption. *Molecular Endocrinology*, *29*(1), 76-98.
62. Zon, L. I., & Peterson, R. T. (2005). In vivo drug discovery in the zebrafish. *Nature reviews Drug discovery*, *4*(1), 35.