THE REGULATION AND FUNCTION OF THE OVARIAN-DERIVED
INSULIN-LIKE GROWTH FACTOR SYSTEM IN ZEBRAFISH

(Danio rerio)

By
David A. Irwin

A thesis
presented to
The University of Guelph

In partial fulfillment of requirements
for the degree of
Master of Science
in
Integrative Biology

Guelph, Ontario, Canada

© David A. Irwin, December, 2011
ABSTRACT

Insulin-like growth factors (IGF) are known paracrine/autocrine regulators of ovarian development in teleosts. Initial studies investigated the hormonal and intracellular signal cascades involved in regulating the expression of ovarian-derived IGFs in zebrafish (Danio rerio). Quantitative real-time PCR was used to quantify the expression of \textit{igf3}, \textit{igf2a}, and \textit{igf2b} in full grown immature (FG; 0.57-0.65 mm) and mid-vitellogenic (MV; 0.45-0.56 mm) follicles. Addition of the gonadotropin analogue human chorionic gonadotropin (hCG) and the adenylate cyclase activator forskolin increased \textit{igf3} expression in FG and MV follicles, but had no effect on \textit{igf2a} or \textit{igf2b} expression. The effects of hCG were blocked by the addition of the protein kinase A inhibitor H-89. Pituitary adenylate cyclase activating peptide stimulated a small increase in \textit{igf3} expression in FG follicles, while growth hormone and salmon gonadotropin releasing hormone had no effect on \textit{igf3}, \textit{igf2a}, or \textit{igf2b} expression. Treatment with melittin, prostaglandin F$_2$a, and prostaglandin E$_2$ inhibited \textit{igf3} and \textit{igf2b} expression in FG follicles whereas the protein kinase C activators, PMA and A23187, significantly inhibited \textit{igf3}, \textit{igf2a}, \textit{igf2b} expression in FG and MV follicles. Secondary studies investigated the involvement of ovarian-derived IGFs in mediating the ovarian actions of gonadotropins on cell survival and steroidogenesis. Treatment of FG follicles with recombinant human IGF-I, hCG, or forskolin inhibited the induction of caspase-3/7 activity, which was used as a measure of apoptosis. The effects of hCG and forskolin on caspase-3/7 were attenuated by co-treatment with NVP-AEW54, an IGF-I receptor antagonist. hCG increased production of the maturation-inducing steroid 17\alpha, 20\beta-dihydroxy-4-pregnen-3-one and co-treatment with NVP-AEW54 had no effect. These results suggest there is a high degree of hormonal specificity in regulating IGFs in the zebrafish ovary and the ovarian-
derived IGFs, presumably IGF-III, are downstream mediators of gonadotropin-dependent cell survival, but are not involved in gonadotropin-induced steroidogenesis.
ACKNOWLEDGEMENTS

I would like to take the time to acknowledge those people that have helped me throughout my graduate studies. First of all, I would like to express my gratitude to my advisor Dr. Glen Van Der Kraak for all of the guidance throughout this journey. Next, I would like to thank my advisory committee Dr. Chun Peng and Dr. Todd Gillis for their useful advice and the addition of their expertise to my research. I would express my appreciation to Jacquie Matsumoto for showing me around the lab and I would like to thank the past and present members of the Van Der Kraak lab for allowing me to use them as a sounding board for my ideas as well as helping maintain the fish and assisting with in vitro experiments. Finally, I would like to thank my family and friends for their continued support and listening to all aspects of my research the past couple of years.
# TABLE OF CONTENTS

Abstract ........................................................................................................................................... ii  
Acknowledgements........................................................................................................................ iv  
Table of Contents ............................................................................................................................ v  
List of Tables ................................................................................................................................... vi  
List of Figures ............................................................................................................................... vii  
List of Abbreviations ................................................................................................................... viii  

CHAPTER 1 – General Introduction.............................................................................................. 1  
  Hormonal Control of Ovarian Development................................................................................. 2  
  Local Protein Factors.................................................................................................................. 4  
  The IGF System........................................................................................................................ 5  
  Thesis Objectives and Outline................................................................................................. 11  

CHAPTER 2 – Hormonal and cellular regulation and actions of the ovarian-derived insulin-like growth factors (IGF) in the zebrafish (*Danio rerio*) ............................................................................ 12  
  Introduction ........................................................................................................................... 12  
  Materials and Methods ......................................................................................................... 15  
  Results .................................................................................................................................. 23  
  Discussion.............................................................................................................................. 47  

CHAPTER 3 – General Discussion .............................................................................................. 54  
  Further Questions ................................................................................................................ 56  
  Conclusions ........................................................................................................................ 58  

References ..................................................................................................................................... 61
LIST OF TABLES

CHAPTER 2

Table 1: List of treatments and dosages used in Experimental Series #1 .............................................. 21

Table 2: List of forward and reverse qPCR primers ............................................................................. 22

Table 3: Summary of Figures 1 – 8 ......................................................................................................... 42
# LIST OF FIGURES

## CHAPTER 2

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>In vitro</em> effects of hCG and H-89 on IGF-III, IGF-IIa, and IGF-IIb mRNA expression in FG and MV follicles</td>
<td>26</td>
</tr>
<tr>
<td>2</td>
<td><em>In vitro</em> effects of forskolin on IGF-III, IGF-IIa, and IGF-IIb mRNA expression in FG and MV follicles</td>
<td>28</td>
</tr>
<tr>
<td>3</td>
<td><em>In vitro</em> effects of PACAP on IGF-III, IGF-IIa, and IGF-IIb mRNA expression in FG and MV follicles</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td><em>In vitro</em> effects of cGH on IGF-III, IGF-IIa, and IGF-IIb mRNA expression in FG and MV follicles</td>
<td>32</td>
</tr>
<tr>
<td>5</td>
<td><em>In vitro</em> effects of sGnRH on IGF-III, IGF-IIa, and IGF-IIb mRNA expression in FG and MV follicles</td>
<td>34</td>
</tr>
<tr>
<td>6</td>
<td><em>In vitro</em> effects of melittin on IGF-III, IGF-IIa, and IGF-IIb mRNA expression in FG and MV follicles</td>
<td>36</td>
</tr>
<tr>
<td>7</td>
<td><em>In vitro</em> effects of prostaglandin E&lt;sub&gt;2&lt;/sub&gt; and prostaglandin F&lt;sub&gt;2α&lt;/sub&gt; on IGF-III, IGF-IIa, and IGF-IIb mRNA expression in FG and MV follicles</td>
<td>38</td>
</tr>
<tr>
<td>8</td>
<td><em>In vitro</em> effects of PMA and A23187 on IGF-III, IGF-IIa, and IGF-IIb mRNA expression in FG and MV follicles</td>
<td>40</td>
</tr>
<tr>
<td>9</td>
<td>Effects of recombinant human IGF-I on caspase-3/7 activity in FG follicles following 10 h incubation in serum-free media <em>in vitro</em></td>
<td>43</td>
</tr>
<tr>
<td>10</td>
<td><em>In vitro</em> effects of hCG and NVP-AEW541 on caspase-3/7 activity in serum-free media</td>
<td>44</td>
</tr>
<tr>
<td>11</td>
<td><em>In vitro</em> effects of forskolin and NVP-AEW541 on caspase-3/7 activity in serum-free media</td>
<td>45</td>
</tr>
<tr>
<td>12</td>
<td><em>In vitro</em> effects of hCG and NVP-AEW541 on 17α, 20β-P production from whole ovary tissue</td>
<td>46</td>
</tr>
</tbody>
</table>

## CHAPTER 3

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Proposed model of IGF-III regulation and function in zebrafish ovarian follicles</td>
<td>59</td>
</tr>
</tbody>
</table>
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Proper Name</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>17α, 20β-P</td>
<td>17α, 20β-dihydroxy-4-pregnen-3-one</td>
<td>Steroid hormone</td>
</tr>
<tr>
<td>20β-S</td>
<td>17, 20β-21-trihydroxy-4-pregnen-3-one</td>
<td>Steroid hormone</td>
</tr>
<tr>
<td>A23187</td>
<td>Calcium ionophore A23187</td>
<td>Calcium ionophore</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid assay</td>
<td>Protein assay</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
<td>Growth factor</td>
</tr>
<tr>
<td>cAMP</td>
<td>3'-5'-cyclic adenosine monophosphate</td>
<td>Signalling molecule</td>
</tr>
<tr>
<td>Caspase</td>
<td>Cysteine aspartic acid protease</td>
<td>Protease</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
<td>Vehicle solution</td>
</tr>
<tr>
<td>E2</td>
<td>17β-estradiol</td>
<td>Steroid hormone</td>
</tr>
<tr>
<td>EF-1α</td>
<td>Elongation factor 1α</td>
<td>Normalization gene</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
<td>Growth factor</td>
</tr>
<tr>
<td>EIA</td>
<td>Enzyme Immunoassay</td>
<td>Hormone Assay</td>
</tr>
<tr>
<td>EP</td>
<td>Prostaglandin E series receptor</td>
<td>Receptor</td>
</tr>
<tr>
<td>ERBB</td>
<td>Epidermal growth factor receptor</td>
<td>Receptor</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
<td>Vehicle solution</td>
</tr>
<tr>
<td>FG</td>
<td>Full grown immature follicles</td>
<td>Follicle size class</td>
</tr>
<tr>
<td>Forskolin</td>
<td>Forskolin</td>
<td>Adenylyl cyclase activator</td>
</tr>
<tr>
<td>FP</td>
<td>Prostaglandin F series receptor</td>
<td>Receptor</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle-stimulating hormone</td>
<td>Hormone</td>
</tr>
<tr>
<td>(c)GH</td>
<td>(carp) Growth hormone</td>
<td>Hormone</td>
</tr>
<tr>
<td>(s)GnRH</td>
<td>(salmon) Gonadotropin-releasing hormone</td>
<td>Hormone</td>
</tr>
<tr>
<td>GVBD</td>
<td>Germinal vesicle breakdown</td>
<td>Marker of oocyte maturation</td>
</tr>
<tr>
<td>H-89</td>
<td>H-89</td>
<td>PKA inhibitor</td>
</tr>
<tr>
<td>HPG axis</td>
<td>Hypothalamus-pituitary-gonad axis</td>
<td>Reproductive axis</td>
</tr>
<tr>
<td>hCG</td>
<td>Human chorionic gonadotropin</td>
<td>Hormone</td>
</tr>
<tr>
<td>IBMX</td>
<td>3-isobutyl-1-methylxanthine</td>
<td>Phosphodiesterase inhibitor</td>
</tr>
<tr>
<td>(rh/rz)IGF</td>
<td>(recombinant human/zebrafish) Insulin-like growth factor</td>
<td>Growth factor</td>
</tr>
<tr>
<td>IGFBP</td>
<td>Insulin-like growth factor binding protein</td>
<td>Binding protein</td>
</tr>
<tr>
<td>IGF-IR</td>
<td>Insulin-like growth factor-I receptor</td>
<td>Receptor</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
<td>Signalling molecule</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
<td>Hormone</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
<td>Protein kinase</td>
</tr>
<tr>
<td>Melittin</td>
<td>Melittin</td>
<td>Phospholipase A₂ activator</td>
</tr>
<tr>
<td>MIS</td>
<td>Maturation-inducing steroid</td>
<td>Steroid hormone</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
<td>Protease</td>
</tr>
<tr>
<td>MPF</td>
<td>Maturation promoting factor</td>
<td>Cell cycle initiator</td>
</tr>
<tr>
<td>MV</td>
<td>Mid-vitellogenic follicles</td>
<td>Follicle size class</td>
</tr>
<tr>
<td>NVP-AEW541</td>
<td>NVP-AEW541</td>
<td>IGF-IR inhibitor</td>
</tr>
<tr>
<td>OMC</td>
<td>Oocyte maturation competence</td>
<td>Ability to respond to MIS</td>
</tr>
<tr>
<td>PACAP</td>
<td>Pituitary adenylate cyclase activating peptide</td>
<td>Growth factor</td>
</tr>
<tr>
<td>PGE₂</td>
<td>Prostaglandin E₂</td>
<td>Lipid hormone</td>
</tr>
<tr>
<td>PGF₂α</td>
<td>Prostaglandin F₂α</td>
<td>Lipid hormone</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
<td>Category</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>PI3-K</td>
<td>phosphoinositol-3-kinase</td>
<td>Protein kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
<td>Protein kinase</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
<td>Protein kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
<td>Protein kinase</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
<td>PKC activator</td>
</tr>
<tr>
<td>(m)PR</td>
<td>(membrane) Progestin receptor</td>
<td>Receptor</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
<td>Gene expression assay</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
<td>Growth factor</td>
</tr>
</tbody>
</table>
CHAPTER 1

GENERAL INTRODUCTION

Ovarian development in vertebrates is traditionally thought to be controlled through the hypothalamus-pituitary-gonad (HPG) axis by means of the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Hillier, 2001; Ge, 2005a). A number of ovarian-derived growth factors in mammals and fish including epidermal growth factor (EGF), pituitary adenylate cyclase activating peptide (PACAP), and several members of the transforming growth factor-β (TGF-β) superfamily play an active role in mediating and modulating the effects of endocrine hormones during ovarian development (Hillier, 2001; Ge, 2005b; Clelland and Peng, 2009; Sirotkin, 2011). The insulin-like growth factor (IGF) system is well known for its role in regulating somatic growth and recent studies suggest that the IGF system also has an important function during teleost ovarian development (Reinecke, 2010b). The objective of this thesis was to further examine the hormonal and cellular mechanisms involved in regulating the ovarian-derived IGF system using zebrafish (Danio rerio) as a model species. Additionally, this thesis investigates the function of the IGF system in ovarian development as well as the possibility that the locally-derived IGFs mediate the actions of the gonadotropins.

This chapter will take an in-depth look at ovarian development in fish and summarize the existing literature. Hormonal control of ovarian development will be examined with emphasis on regulation of folliculogenesis, steroidogenesis, oocyte maturation, and ovulation. The potential paracrine/autocrine function of a number of local factors will be examined with the primary focus being on the IGF system. In particular, the IGF system will be reviewed including what is known of their actions as regulators of ovarian development and function in fish. The objectives and outline for the rest of the thesis will also be discussed.
Hormonal Control of Zebrafish Ovarian Development

The growth and development of oocytes in teleosts are primarily under the control of the gonadotropins. Gonadotropin-releasing hormone (GnRH) from the hypothalamus stimulates the release of FSH and LH from the pituitary initiating ovarian development (Levavi-Sivan et al., 2010). Five stages of oocyte development have been characterized in zebrafish by Selman et al. (1993) and FSH and LH have differential effects depending on the stage of development. During stage I, primary growth oocytes become arrested during prophase I and start to form a follicular layer comprised of fibroblasts, granulosa, and theca cells (Lubzens et al., 2010). Stage II, the cortical alveolus stage, marks the appearance of yolk vesicles in the oocyte as well as the formation of a vitelline envelope (Selman et al., 1993). The hormonal regulation of the first two stages of oocyte development is not well understood in zebrafish and at this time the roles of the gonadotropins are unclear.

Stage III or vitellogenesis, is where the majority of growth occurs and is where the actions of the gonadotropins have been well established. FSH is suggested to be the key hormone regulating follicular growth and this is due to high levels of FSH receptor mRNA levels in ovarian follicles during vitellogenesis (Kwok et al., 2005). FSH stimulates production of 17β-estradiol (E₂) in the follicle, which in turn stimulates the production of vitellogenin from the liver (Patino and Sullivan, 2002). The oocyte starts forming yolk from the accumulation of vitellogenin and the follicle increases in size. LH, on the other hand, is the primary hormone involved in Stage IV or oocyte maturation since the expression of LH receptors begins to increase during vitellogenesis and exhibit peak expression in maturing follicles (Kwok et al., 2005). LH-dependent maturation occurs in two stages. First, LH stimulates oocyte maturation competence (OMC) which is the ability of oocytes to respond to the progestin-based maturation-
inducing steroid (MIS) (Patino et al., 2001; Clelland and Peng, 2009). Next, LH initiates follicles to switch from producing E₂ to the production of MIS which is required for oocyte maturation (Nagahama and Yamashita, 2008).

Multiple progestin hormones have the ability to stimulate oocyte maturation in fish. In most teleosts including zebrafish the MIS is 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-P); however, 17, 20β-21-trihydroxy-4-pregnen-3-one (20β-S) has been shown to be the MIS in some teleosts (Selman et al., 1994; Clelland and Peng, 2009). MIS stimulates oocyte maturation through non-genomic actions mediated by membrane progestin receptors (mPR) (Clelland and Peng, 2009). mPRs are coupled to G \textsubscript{i} proteins that inhibit cAMP production in the oocyte and lead to the \textit{de novo} synthesis of cyclin B1 and cdc2; together these proteins comprise the maturation promoting factor (MPF) (Patino and Sullivan, 2002; Nagahama and Yamashita, 2008; Clelland and Peng, 2009). Activation of MPF leads to germinal vesicle breakdown (GVBD) and resumption of meiosis. GVBD is associated with a change from an opaque to a transparent appearance in the oocyte resulting from the proteolysis of yolk proteins which breaks down the crystalline structure of the yolk droplets (Lubzens et al., 2010).

After oocyte maturation, the follicular layer is digested by proteolytic enzymes resulting in Stage V, the ovulated egg (Clelland and Peng, 2009). Ovulation is a MIS-dependent event that unlike oocyte maturation requires transcriptional events to proceed (Patino and Sullivan, 2002). Nuclear progestin receptors have been characterized in the ovaries of spotted seatrout (\textit{Cynoscion nebulosus}) (Pinter and Thomas, 1995), Atlantic salmon (\textit{Salmo salar}) (Chen et al., 2011), and zebrafish (Hanna et al., 2010) and may be involved in regulating the transcriptional events involved in MIS-dependent ovulation. Evidence suggests that elevated prostaglandins levels are associated with ovulation (Berndtson et al., 1989; Goetz, 1997; Lister and Van Der
Kraak, 2008; Fujimori et al., 2011) and prostaglandins have also been shown to stimulate in vitro ovulation in the ovarian follicles of goldfish (Carassius auratus) (Goetz, 1993) and Atlantic croaker (Micropogonias undulates) (Patino et al., 2003). The exact mechanism behind prostaglandin-stimulated ovulation in fish is unknown because the pathway has received little attention; however, prostaglandin signalling has been described in mammals where a number of prostaglandin receptors and signalling mechanisms have been characterized (Negishi et al., 1993; Narumiya et al., 1999; Bos et al., 2004; Sugimoto and Narumiya, 2007). An important event associated with ovulation in zebrafish is the synthesis of prostaglandins which is coupled to increased transcription of phospholipase A₂ and cyclooxygenase-2 (Lister and Van Der Kraak, 2009).

Local Protein Factors

There is growing evidence that a number of local factors are involved in regulating follicular growth and oocyte maturation. The TGF-β superfamily is a collection of regulatory proteins that includes the activin/inhibin subfamily, bone morphogenic proteins (BMP), and TGF-β1 (Kohli et al., 2003). Studies in zebrafish have shown that ovarian activin expression is stimulated by the gonadotropin analogue human chorionic gonadotropin (hCG) and goldfish pituitary extract, suggesting that activins are downstream mediators of gonadotropin signalling (Pang and Ge, 2002c). Further evidence that indicates the activins are activated downstream of gonadotropin action is that follistatin, an activin binding protein, blocked hCG-induced oocyte maturation (Pang and Ge, 1999; Pang and Ge, 2002b). Other studies with zebrafish showed that BMP-15 (Clelland et al., 2006) and TGF-β1 (Kohli et al., 2003) are produced in the oocyte and the surrounding follicular layers and have an inhibitory role in oocyte maturation.
EGF has also been shown to be a local regulatory factor of ovarian development in zebrafish. EGF mRNA expression is localized to the oocyte and its receptors are found in both oocyte and surrounding follicular layers (Tse and Ge, 2010). Multiple EGF receptors have been characterized in the zebrafish ovary and it has been suggested EGF can have stimulatory and inhibitory actions on oocyte maturation depending on the receptor that is activated. A recent study by Peyton and Thomas (2011) demonstrated that the EGF receptor ERBB1 is associated with E2 inhibition of oocyte maturation, whereas activation of a second EGF receptor ERBB2 is involved in MIS-induced maturation. EGFs have also been shown to stimulate the expression of activin subunits and inhibit the expression of follistatin in the zebrafish ovary (Wang and Ge, 2004).

PACAP is a neuropeptide member of the vasoactive intestinal peptide family that is known to stimulate cAMP production (Wang et al., 2003). Two forms of PACAP along with three receptors have been characterized in the zebrafish ovarian follicle. PACAP expression is up-regulated by gonadotropins and it is believed to act as a local amplifier of the gonadotropin signal cascade (Zhou et al., 2011). Interestingly, PACAP stimulates oocyte maturation in intact follicles, but inhibits GVBD in denuded follicles (Zhou et al., 2011).

The IGF System

The IGF system is comprised of ligands, receptors, and binding proteins that are highly conserved in vertebrates. Initially, the IGF system was believed to play an important role in somatic growth by mediating the actions of growth hormone (GH) at various sites within the body. IGFs have now been shown to be involved in multiple processes including development, cell survival, and mitosis (Wood et al., 2005). The majority of circulating IGFs are produced in the liver upon GH stimulation; however, it is well accepted that IGFs are produced from extra-
hepatic sources independent of GH stimulation (Duan, 1997). The gonads of mammals (Silva et al., 2009) and fish (Reinecke, 2010b) have been shown to produce IGFs suggesting that the IGF system may have important reproductive functions.

**Ligands**

Two IGF ligands, IGF-I and IGF-II, are found in mammals and are members of the insulin superfamily. Members of the vertebrate insulin family share a similar tertiary structure and are synthesized as preprohormones. The preprohormone consists of six biochemically distinct domains; which following proteolytic cleavage of the signal and E domain results in the functionally mature IGF peptide (Wood et al., 2005). In mammals, the IGFs have been shown to have effects on reproduction including actions on steroidogenesis, oocyte maturation, follicle growth and survival (Wang and Chard, 1999; Sirotkin, 2011).

Similar to mammals, two IGFs have been identified in a number of teleosts (Wood et al., 2005); however, four IGFs including, IGF-I, duplicate IGF-IIs, and IGF-III, have been characterized in the zebrafish (Wang et al., 2008). All the zebrafish IGFs have distinct temporal and spatial expression in most tissues including liver, brain, gill, heart, intestine, and gonad suggesting distinct actions for each ligand. Zebrafish IGF-I and IGF-IIa/b are believed to be orthologous to human IGF-I and IGF-II, respectively (Zou et al., 2009). Human and zebrafish IGF-I share 82% sequence identity and IGF-IIa and IGF-IIb share similar sequence identity between each other (70%) and their human counterparts (76% and 75%, respectively) (Zou et al., 2009). IGF-III is a gonad-specific IGF that was recently discovered in zebrafish and Nile tilapia (Oreochromis niloticus) suggesting its importance in teleost reproductive function (Wang et al., 2008). IGF-III has low sequence identity with zebrafish and human IGF-I (50% and 51%, respectively), but it has a similar tertiary structure suggesting that it has similar biological actions.
to the IGF-I and IGF-II (Zou et al., 2009). The evolution of IGF-III in zebrafish remains unknown; however, there are two competing hypotheses on its origin. IGF-III is proposed to be a novel IGF that has arisen over time (Wang et al., 2008). Alternatively, it may be a duplicate of IGF-I that diverged from the other IGFs due to its restricted expression in the gonads (Zou et al., 2009).

IGFs are found in the oocyte and its surrounding follicular layers in the ovary of rainbow trout (Oncorhynchus mykiss), common carp (Cyprinus carpio), red sea bream (Pagrus major), and Mozambique tilapia (Oreochromis mossambicus) (Reinecke, 2010b). However, other studies have shown that IGF-III mRNA and protein is found exclusively in the follicular layer of zebrafish ovarian follicle (Nelson and Van Der Kraak, 2010a; Li et al., 2011). Interestingly, igf3 expression was relatively low during the early stages of folliculogenesis and starts to increase through vitellogenesis reaching peak expression in the full grown immature follicle (Li et al., 2011). Further in vitro experiments have demonstrated that treatment with hCG increased igf3 expression in mid-vitellogenic and full grown zebrafish ovarian follicles (Nelson and Van Der Kraak, 2010a).

Receptors

Two types of IGF receptors, IGF-IR and IGF-IIR, exist in mammals. IGF-IR is a transmembrane receptor that belongs to the tyrosine kinase family of receptors which also includes the insulin receptor (Wood et al., 2005). The IGF-IR and insulin receptor share a similar heterotetrameric α2β2 structure which includes the tyrosine kinase domain as well as an insulin receptor substrate (IRS) binding site, ATP binding site and a phosphoinositol-3-kinase (PI3-K) binding site (Adams et al., 2000). IGF-IR uses a number of signal cascades to propagate its signal including the activation of mitogen-activated protein kinase (MAPK), PI3-K, and protein
kinase B (PKB/Akt); all of which may lead to alterations in gene expression and protein synthesis (Adams et al., 2000; Wood et al., 2005). IGF-IIR is a transmembrane mannose 6-phosphate receptor. It has no catalytic domains and is believed to be involved in sequestering IGF ligands which will lead to their eventual degradation in the lysosome (Wood et al., 2005). IGF-IR activation regulates the biological actions of the IGFs due to its ability to bind IGFs and propagate a signal; whereas the biological function of IGF-IIR is unclear.

IGF-IR has been characterized in teleosts and two duplicate IGF-IRs, IGF-IRa and –IRb, have been identified in zebrafish (Maures et al., 2002). Zebrafish IGF-IRa and IGF-IRb share high structural similarity with each other; however, they share low amino acid sequence homology with each other (69.8%) and the human receptor (63.2% and 59.6, respectively) (Wood et al., 2005). Both receptors activate similar signal transduction pathways as the mammalian receptor. The duplicate IGF-IR genes encode fully functioning proteins with differential expression suggesting distinct biological functions (Maures et al., 2002). The IGF-IR receptors are found throughout the body of zebrafish including but not limited to brain, eyes, gills, liver, muscle, and gonads. A recent study by Nelson and Van Der Kraak (2010a) found igf1ra and igf1rb expression in zebrafish ovarian follicles suggesting that IGF signalling is important for ovarian development.

**Binding Proteins**

Six IGF binding proteins (IGFBPs) have been characterized in mammals. The majority of circulating IGFs are bound to IGFBPs and it has been shown that the binding proteins have a greater affinity for IGFs than the IGF receptors (Wood et al., 2005). IGFBPs were traditionally thought to sequester the IGFs in an inactive complex to prevent their biological actions. Recent studies suggest that IGFBPS have expanded actions including regulating the rate of turnover,
transportation, and tissue distribution of the IGFs (Duan and Xu, 2005). It has also been
demonstrated that some of the IGFBPs have biological effects that are independent of the IGFs
(Duan and Xu, 2005).

Similar to mammals, six IGFBPS have been identified in rainbow trout (Kamangar et al.,
2006); however, in other teleosts including zebrafish there are duplicate forms of the IGFBPs
suggesting the existence of twelve IGFBPs (Zhou et al., 2008). The actions of the IGFBPs in fish
are not well understood, but the literature suggests they have similar functions to those seen in
mammals. Additionally, IGFBPs have been localized to the ovary of rainbow trout (Kamangar et
al., 2006), gilthead sea bream (Sparus aurata) (Funkenstein et al., 2002), orange spotted grouper
(Epinephelus coioides) (Chen et al., 2010), and zebrafish (Chen et al., 2004). Kamangar et al.
(2006) demonstrated that the IGFBPs have distinct expression profiles throughout ovarian
development and that the IGFBPs are differentially regulated by gonadotropins, 17α, 20β-P, and
E2. For instance, IGFBP-3 mRNA expression is down-regulated in intact ovarian follicles by
partially purified gonadotropin and 17α, 20β-P and E2 stimulates its expression, whereas IGFBP-
2 expression is unaffected by the same hormonal treatments (Kamangar et al., 2006).

Ovarian Actions of the IGF system

There are fewer studies that have examined the reproductive effects of the IGFs in fish,
but the literature suggests that IGFs function in a similar manner as mammals. Recombinant
human IGF-I (rhIGF-I) stimulated steroidogenesis in coho salmon (Oncorhynchus kisutch)
(Maestro et al., 1997), red sea bream (Kagawa et al., 2003), common carp (Cyprinus carpio)
(Paul et al., 2010), and zebrafish (Nelson and Van Der Kraak, 2010b). One of the earliest studies
examining the role of IGFs in the ovary of fish found that rhIGF-I and rhIGF–II induced OMC as
well as oocyte maturation and did so independently of steroid production in the red sea bream
RhIGF-I and rhIGF-II also induce oocyte maturation in striped bass (*Morone saxatilis*) (Weber and Sullivan, 2000), common carp (Paul et al., 2009), and zebrafish (Nelson and Van Der Kraak, 2010b). However, in the white bass (*Morone chrysops*) (Weber and Sullivan, 2005) and white perch (*Morone Americana*) (Weber et al., 2007) only OMC was achieved through treatment with rhIGF-I and rhIGF-II. Studies investigating the ovarian actions of IGF-III are limited. A recent study by Li et al. (2011) demonstrated that recombinant zebrafish IGF-III (rzIGF-III) induces oocyte maturation in zebrafish follicles.

Although there has been extensive work on investigating the actions of the IGFs around oocyte maturation; the possible actions of IGFs on other reproductive endpoints remain unexplored. Teleost ovarian follicles have been shown to undergo apoptosis *in vitro* following serum-starvation and it is likely that several hormones promote cell growth and survival during ovarian development (Janz and Van Der Kraak, 1997; Wood and Van Der Kraak, 2001). IGFs have been shown to have anti-apoptotic properties in the mammalian ovary (Chun et al., 1994); however, treatment with rhIGF-I had no effect on apoptosis in the rainbow trout follicles (Wood and Van Der Kraak, 2002). In the zebrafish, the IGF system has been shown to promote cell survival during development (Schlueter et al., 2007a; Schlueter et al., 2007b), but it is unknown if IGFs promote cell survival in the zebrafish ovary.

Previous studies in mammals have demonstrated that IGFs mimic the actions of the gonadotropins and it has been suggested that IGFs are downstream mediators of gonadotropin action (Chun et al., 1994; Yu et al., 2003; Sirotkin, 2011). Similar trends have also been observed in fish where treatment with hCG and rhIGF-I both stimulate steroidogenesis (Kagawa et al., 2003; Nelson and Van Der Kraak, 2010b; Paul et al., 2010) and oocyte maturation (Kagawa et al., 1994; Mukhejee et al., 2006). Ovarian-derived IGFs are potentially acting as
local mediators of gonadotropin signalling in the fish ovary; however, very few studies have
directly examined this interactive relationship.

**Thesis Objectives and Outline**

The goal of this thesis was to further investigate the regulation and actions of the ovarian
IGF system in zebrafish. Chapter 2 examines the hormonal and cellular mechanisms involved in
the regulation of the *igfβ, igf2a*, and *igf2b* expression in ovarian follicles. Investigating the
regulation of the ovarian-derived IGFs provides valuable insight into potential novel actions of
the IGF system in the teleost ovary. Additional research was done to investigate the novel
function of the IGFs in preventing apoptosis in ovarian follicles as well as examine the
involvement of the ovarian-derived IGFs in mediating the actions of the gonadotropins on
apoptosis and steroidogenesis.

The final chapter of this thesis discusses the current state of knowledge of the IGF system
and ovarian function and how the results of the current study fit into the field of reproductive
endocrinology. Additionally, chapter 3 will provide a commentary on future research
opportunities to further our understanding of the function of the IGF system in fish reproductive
endocrinology.
CHAPTER 2

Hormonal and cellular regulation and actions of the ovarian-derived insulin-like growth factors (IGF) in the zebrafish (*Danio rerio*)

INTRODUCTION

Progress has been made in identifying the components of the insulin-like growth factor (IGF) system in the ovary of fish, but the literature describing the hormonal regulation and actions of the IGF system in ovarian function remains incomplete. IGF ligands have been characterized in the ovary of several teleosts including rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*), red sea bream (*Pagrus major*), and Mozambique tilapia (*Oreochromis mossambicus*) (Wood *et al*., 2005; Reinecke, 2010b). The zebrafish (*Danio rerio*) ovary contains four IGFs including IGF-I, duplicate forms of IGF-II (IGF-IIa and IGF-IIb) and IGF-III. IGF-III has been considered to be the primary IGF in the ovary due to its restricted distribution and high levels of mRNA expression (Wang *et al*., 2008; Zou *et al*., 2009). IGF-III mRNA and protein were localized to the follicular cells (Nelson and Van Der Kraak, 2010a; Li *et al*., 2011) and *igf3* expression increases during vitellogenesis reaching a peak at the full grown immature follicle stage in zebrafish (Li *et al*., 2011). Previous studies demonstrated that human chorionic gonadotropin (hCG), a gonadotropin analogue, was a strong regulator of *igf3* expression in zebrafish (Nelson and Van Der Kraak, 2010a); however, the cellular mechanism behind the regulation is unknown. Gonadotropins use a cAMP/PKA-dependent cascade to regulate steroidogenesis (Iwamatsu *et al*., 1987; Srivastava and Van Der Kraak, 1994; Mendez *et al*., 2003) and ovarian gene expression (Pang and Ge, 2002c; Wang *et al*., 2003; Ings and Van Der Kraak, 2006) and it is likely that *igf3* expression is controlled in a similar fashion.
Aside from the gonadotropins, little is known about other endocrine or paracrine/autocrine regulators of the ovarian IGF system. IGF-I is primarily produced in the liver under the control of growth hormone (GH) (Wood et al., 2005); however, it remains unclear whether GH is involved in the regulation of ovarian-derived IGFs. GH does not have an effect on ovarian igf1 expression in Coho salmon (*Oncorhynchus kisutch*) (Duguay et al., 1994), but igf1 and igf2 expression were elevated in the testis of transgenic Nile tilapia (*Oreochromis niloticus*) over-expressing GH (Eppler et al., 2009). Interestingly, in the ovary of the adult gilthead seabream (*Sparus aurata*), GH stimulated igf1 expression during the pre-reproductive period, but had no effect during the reproductive period (Gioacchini et al., 2005).

The effects of the ovarian-derived hormones, local growth factors, and their signalling cascades on the IGF system have been ignored. Previous studies have shown that the maturation-inducing steroid (MIS), 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-P), inhibits igf3 expression in the zebrafish ovary (Nelson and Van Der Kraak, 2010a). Gonadotropin-releasing hormone (GnRH) (Pati and Habibi, 1998; Yu et al., 1998) and its receptors (Pati and Habibi, 1993) are produced in the teleost ovary where it is thought to play a role in oocyte maturation and apoptosis. Given that IGFs also stimulate oocyte maturation, a possible interactive connection between the peptide hormones could exist. Additionally, pituitary adenylate cyclase activating peptide (PACAP) is a powerful stimulator of cAMP production and is produced in the ovary of zebrafish (Wang et al., 2003) suggesting a possible function in regulating the ovarian IGF system. Activation of PKC stimulated the production of prostaglandins in goldfish (*Carassius auratus*) ovarian follicles (Kellner and Van Der Kraak, 1992) and both PKC activation (Goetz, 1993; Patino et al., 2003) and prostaglandin production (Berndtson et al., 1989; Lister and Van Der Kraak, 2008) have been linked to ovulation in teleosts. Although
prostaglandins and PKC are active in the teleost ovary, their involvement in the regulation of the ovarian IGF system has not been considered. However, in cows it has been shown that prostaglandin F$_{2\alpha}$-induced luteolysis down-regulates IGF-II mRNA (Neuvians et al., 2003).

Interestingly, it has been observed that many of the actions of the IGFs on ovarian development in fish overlap with actions of the gonadotropins which raises the question of whether the ovarian-derived IGFs mediate some of the actions of the gonadotropins. Addition of both hCG and recombinant human IGF-I (rhIGF-I) induced 17β-estradiol (E$_2$) production in red sea bream (Kagawa et al., 2003) and common carp (Paul et al., 2010) and stimulated production of 17α, 20β-P in zebrafish (Nelson and Van Der Kraak, 2010b). Additionally, treatment with hCG or rhIGF-I induced oocyte maturation in red sea bream (Kagawa et al., 1994) and common carp (Mukhejee et al., 2006); however in zebrafish only rhIGF-I was a potent stimulator of oocyte maturation (Nelson and Van Der Kraak, 2010b). In mammals, gonadotropins and IGFs have been shown to promote cell survival in ovarian follicles (Quirk et al., 2004); however, in rainbow trout, only salmon gonadotropin and not rhIGF-I prevented apoptosis in ovarian follicles (Wood and Van Der Kraak, 2002). In vitro incubation of zebrafish ovarian follicles in hCG prevented DNA fragmentation and blocked the activity of the executioner protein caspase-3 (Eykelbosh and Van Der Kraak, Unpublished). The IGF system has also been shown to promote cell survival during embryonic development in zebrafish (Schlueter et al., 2007a), but it remains unknown whether IGFs affect follicle cell survival in the zebrafish ovary.

The first objective of this study was to evaluate the involvement of endocrine and local hormones and activators of different signal transduction pathways in regulating igf3, igf2a, and igf2b expression in the zebrafish ovary. A second objective was to determine whether locally-derived IGFs mediate the effects of the gonadotropins on follicle cell survival and steroid
production. The actions of the IGFs, including IGF-III, are mediated through IGF-I receptors (IGF-IR) (Maures et al., 2002; Wood et al., 2005) and an IGF-IR inhibitor was used in combination with hCG to determine possible mediatory actions of ovarian-derived IGFs on gonadotropin signalling.

**MATERIALS AND METHODS**

**Animals**

Adult zebrafish were purchased from Aquality (Mississauga, ON) and held in the Hagen Aqualab (University of Guelph, Guelph, ON) for at least one month prior to experimentation. Fish were maintained in an environmental chamber in an Aquatic Habitat containment unit (Aquatic Habitats, Apopka, FL) with re-circulated well-water maintained at 28°C. The photoperiod of the environmental chamber was set to a 12 hrs light: 12 hrs dark cycle. Fish were fed commercial salmon fry pellets (Martin Mills, Elmira, ON) on a daily basis with occasional supplementation of frozen brine shrimp (San Francisco Bay Brand, Inc., Newark, CA).

**Chemicals**

Human chorionic gonadotropin (hCG), H-89, forskolin, salmon gonadotropin releasing hormone (sGnRH), melittin, phorbol 12-myristate 13-acetate (PMA), A23187, and recombinant human IGF-I (rhIGF-I) were obtained from Sigma (St. Louis, Missouri). Prostaglandin $E_2$ ($PGE_2$) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) were obtained from Cayman Chemical (Ann Arbor, Michigan). Pituitary adenylate cyclase activating peptide (PACAP) was purchased from Enzo Life Sciences (Plymouth Meeting, PA). Carp growth hormone (cGH) was purchased from Cedarlane Laboratories (Burlington, Ontario). The IGF-1R specific inhibitor, NVP-AEW541, was generously donated by Novartis Pharmaceuticals (Basel, Switzerland).
Follicle Collection and Incubations

Gravid female zebrafish were euthanized using an overdose of MS-222 (Syndel Laboratories Inc, Vancouver, BC). The ovaries were removed and placed in 60% Lebovitz’s L-15 Medium (Invitrogen, Carlsbad, CA) containing penicillin and streptomycin (200 IU/L and 200 µg/L; Invitrogen) to prevent bacterial growth. Using fine forceps, mid-vitellogenic (MV; 0.45-0.56 mm) and full grown immature (FG; 0.57-0.65 mm) follicles were isolated and pooled from approximately 10-15 fish. These follicle size classes were chosen due to their high expression levels of the IGF ligands (Li et al., 2011) and hormonal treatments induce detectable changes in gene expression within 3 h (Nelson and Van Der Kraak, 2010a).

Regulation of ovarian IGFs

A series of in vitro experiments were performed to determine the hormones and intracellular signal cascades involved in the regulation of the IGF system in FG and MV follicles. Approximately, forty follicles were randomly distributed into each well of a 24-well polystyrene plate and incubated in their respective treatments 3 h in the dark at 28°C. Hormonal and modulators of intracellular signalling pathway treatments and their respective concentrations are listed in Table 1. Control follicles were incubated in media containing only the vehicle solution. These experiments included tests with hCG and the PKA inhibitor H-89 to determine the role of PKA in the hCG signalling pathway. For these experiments, follicles were incubated with the inhibitor for 30 min prior to the addition of hCG. At the end of the incubation period, follicles were collected and stored at -80°C until RNA extraction. Each treatment was run in at least triplicate and each experiment was repeated three times with follicles obtained from different pools of fish. Dimethyl sulfoxide (DMSO) and ethanol (EtOH) were used as vehicle solutions and never exceed a concentration of 0.5% in the wells.
Role of ovarian-derived IGFs

Treatment with gonadotropins inhibited apoptosis in rainbow trout (Janz and Van Der Kraak, 1997) and zebrafish (Eykelbosh and Van Der Kraak, Unpublished), but it remains unknown if IGFs are involved in preventing ovarian follicle apoptosis zebrafish. The first experiment examined the effect of rhIGF-I on activity of caspase-3/7. Caspase-3/7 are members of the cysteine aspartic acid protease family that act as executioner proteins in the apoptotic pathway. There is a significant induction of caspase-3/7 activity in vitro following an 8 h incubation of zebrafish follicles in serum-free media (Eykelbosh and Van der Kraak, 2010) and this can be used as a measure of apoptosis. Approximately, 45-50 FG follicles were distributed in each well of a 24-well plate and were incubated in the dark for 10 h at 28°C in rhIGF-I (100 nM). Additional experiments were conducted to determine if locally-derived IGFs mediate the anti-apoptotic actions of hCG and forskolin in zebrafish follicles. For these experiments, follicles were incubated with the IGF-IR inhibitor NVP-AEW541 (10 μM in DMSO) for 30 min prior to the addition of hCG (20 IU/ml) or forskolin (2 μM in DMSO). Follicles were incubated for 10 h in the dark at 28°C. Control follicles were incubated in media containing only the vehicle solution. All follicles were saved at -80°C prior to testing for caspase-3/7 activity. Each treatment was run in quadruplicate and each experiment was repeated three times with tissue obtained from separate fish. The amount of DMSO in the wells never exceeded 0.5%.

Previous studies have shown that hCG and rhIGF-I stimulate the in vitro production of 17α, 20β-P after a 6 h incubation (Nelson and Van Der Kraak, 2010b). A second set of experiments were designed to determine if ovarian-derived IGFs mediate the actions of hCG on 17α, 20β-P production. Whole ovary pieces pooled from 5-6 fish were weighed and 25 ± 2 mg of ovarian tissue was randomly distributed to each well of a 24-well plate. Ovarian fragments were
incubated for 30 min with NVP-AEW541 prior to addition of hCG; control ovarian fragments were incubated in media containing only the vehicle solution. Tissue was incubated in the dark for 6 h at 28°C. Media was saved at -80°C to examine the levels of 17α, 20β-P.

**RNA Extraction and Quantification**

RNA was extracted from the treated follicle samples using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Briefly, follicles were incubated in TRIzol reagent and homogenized using a 3 ml syringe with a 21 G needle. Samples were incubated for 10 min at room temperature prior to the addition of chloroform (Sigma) and incubated for another 3 min. Samples were centrifuged for 15 min at 12 000 G. The aqueous phase was removed and added to tubes of isopropanol (Sigma) and incubated for 10 min; samples were then centrifuged for 10 min at 12 000 G to collect the RNA pellet. The pellet was washed once in 70% ethanol and reconstituted in 10 µl of Gibco Ultrapure DNase/RNase free distilled water (Invitrogen).

RNA subsamples were diluted 1:10 in Gibco water and total RNA was quantified from the diluted samples using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA). The purity of the RNA was determined using the 260/280 absorbance ratio; all samples had a ratio of 1.8-2.2. RNA samples were diluted to a final concentration of 1 µg/µl.

**Reverse Transcription**

The methods for reverse transcription (RT) were based on those described in Nelson and Van Der Kraak (2010a).

**Quantitative Real-Time PCR**

Relative gene expression was measured using real-time quantitative polymerase chain reaction (qPCR) with methods modified from Ings and Van Der Kraak (2006). Primer sequences
for *igf2a*, *igf2b*, *igf3*, and *ef1α* (Table 2) were based on those used in Nelson and Van Der Kraak (2010a). cDNA was diluted 10x and 3.75 µl of diluted sample was added to 96-well PCR plate along with forward and reverse primers (1.6 µM, Sigma) and Perfecta SYBR Green Fastmix, ROX (Quanta Biosciences, Gaithersburg, MD) for a total volume of 15 µl. Samples were run in duplicate. Using ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) or the StepOnePlus Real-Time PCR system (Applied Biosystems), samples were incubated at 95°C for 5 min. After the initial program, the detection system ran a pre-programmed cycle of 1 s at 95°C and 30 s at 60°C, for 40 cycles. Expression levels are normalized to the housekeeping gene, elongation factor 1α (*EF-1α*). A six point standard curve for each gene was run with the samples from every experiment to quantify gene expression.

**Caspase-3/7 Activity**

Caspase-3/7 activity was measured using the SensoLyte Homogeneous AMC Caspase-3/7 assay kit (Anaspec, Fremont, CA). Samples were incubated for 5 min on ice in 1x lysis buffer. Samples were sonicated with 5 x 1 s bursts and then centrifuged for 10 min at 10 000 G to remove insoluble debris. On ice, 50 µl of sample was loaded into an opaque 96-well plate with 100 µl 1x lysis buffer, and 50 µl of substrate solution (Assay buffer, DTT, and Ac-DEVD-AMC substrate). All samples were run in duplicate. Plates were read on an Flx Microplate Fluorescence Reader (Bio-Tek Instruments, Winooski, VT) at 28°C. The excitation/emission of the fluorescent probe was measured at 354 nm/442 nm. Measurements were made every 5 min for 100 min and caspase-3/7 activity was expressed as the maximum slope of the linear curve relative to the total amount of protein loaded in each well. Protein concentration was determined using the bicinchonic acid (BCA) assay (Pierce Biotechnology, Rockford, IL).
**Steroid Measurements**

17α, 20β-P levels were measured from media using enzyme immunoassay (EIA) from Cayman Chemical according to the manufacturers guidelines. The absorbance was read at 420 nm using a SpectraMax 190 Absorbance Microplate Reader (Molecular Devices, Sunnyvale, CA).

**Statistical Analysis**

Data from replicate experiments were pooled. A Levene’s test was completed to test for the homogeneity of variances and where needed, the data were log transformed. An analysis of variance (ANOVA) was used to determine if there are differences between treatment groups. Tukey’s Honestly Significant Difference test was used to determine significance between treatment groups. The significance will be set at $\alpha = 0.05$ for all tests.
<table>
<thead>
<tr>
<th>Chemical</th>
<th>Description</th>
<th>Dosage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human chorionic gonadotropin (hCG)</td>
<td>Gonadotropin analogue</td>
<td>20 IU/ml</td>
</tr>
<tr>
<td>H-89</td>
<td>PKA inhibitor</td>
<td>25 µM in DMSO</td>
</tr>
<tr>
<td>Forskolin</td>
<td>Adenylate cyclase activator</td>
<td>10 µM in DMSO</td>
</tr>
<tr>
<td>Pituitary adenylate cyclase activating peptide (PACAP)</td>
<td>Peptide hormone</td>
<td>10, 100 nM</td>
</tr>
<tr>
<td>Carp growth hormone (cGH)</td>
<td>Hormone</td>
<td>10, 100 ng/ml</td>
</tr>
<tr>
<td>Salmon gonadotropin releasing hormone (sGnRH)</td>
<td>Hormone</td>
<td>100 nM</td>
</tr>
<tr>
<td>Melittin</td>
<td>Phospholipase activator</td>
<td>50, 100 nM</td>
</tr>
<tr>
<td>Prostaglandin E₂ (PGE₂)</td>
<td>Hormone</td>
<td>50, 200 ng/ml in DMSO</td>
</tr>
<tr>
<td>Prostaglandin F₉α (PGF₂α)</td>
<td>Hormone</td>
<td>50, 200 ng/ml in DMSO</td>
</tr>
<tr>
<td>Phorbol 12-myristate 13-acetate (PMA)</td>
<td>PKC activator</td>
<td>400 nM in EtOH</td>
</tr>
<tr>
<td>A23187</td>
<td>Calcium ionophore</td>
<td>10 µM in EtOH</td>
</tr>
</tbody>
</table>
**Table 2**: List of forward and reverse qPCR primers.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-IIa</td>
<td>F – CGCCTGCCATGGATGATTAC</td>
</tr>
<tr>
<td></td>
<td>R – TCAGTGAGCGCATCGTTGTT</td>
</tr>
<tr>
<td>IGF-IIb</td>
<td>F – AACCTGCCAAGTTCAGAGAGGG</td>
</tr>
<tr>
<td></td>
<td>R – GGACCTCCTGTTTTAATGCGG</td>
</tr>
<tr>
<td>IGF-III</td>
<td>F – ACGCTGCAGCAGAAGAATAG</td>
</tr>
<tr>
<td></td>
<td>R – GCTGCTCCAGGTTTGCTAT</td>
</tr>
<tr>
<td>EF-1α</td>
<td>F – GATCACTGGTACTTCTCAGGCTGA</td>
</tr>
<tr>
<td></td>
<td>R – GGTGAAAGCCAGGAGGAG GC</td>
</tr>
</tbody>
</table>
RESULTS

Regulation of IGF system

Addition of hCG (20 IU/ml) induced a significant increase in igf3 expression in FG and MV follicles (Fig. 1A). Treatment with the PKA inhibitor H-89 (25 µM, in DMSO) reduced basal levels of igf3 expression as well as abolished the effects of hCG on igf3. hCG had no effect on igf2a or igf2b expression, however, H-89 reduced basal levels of igf2a in FG and MV follicles and igf2b in FG follicles (Fig. 1B, 1C).

Addition of forskolin (10 µM), an adenylate cyclase activator, increased igf3 expression in FG and MV follicles (Fig. 2A). Forskolin had no effect on igf2a or igf2b expression in FG follicles, but reduced igf2a and igf2b expression in MV follicles (Fig. 2B, 2C).

PACAP (10 and 100 nM) increased igf3 in a dose-dependent manner in FG follicles, but had no effect on igf3 expression in MV follicles (Fig. 3A). PACAP had no effect on gene igf2a or igf2b expression in either follicle size class (Fig. 3B, 3C).

cGH (Fig. 4) had no effect on igf2a, igf2b, and igf3 expression in either FG or MV follicles. sGnRH (Fig. 5C) significantly decreased igf2b expression in FG follicles, but had no effect on igf2b expression in MV follicles. sGnRH had no effect on igf3 or igf2a expression in FG or MV follicles (Fig. 5A, 5B).

Melittin (50 and 100 nM), a phospholipase activator and stimulator of arachidonic acid release, reduced igf3 and igf2b expression in only FG follicles (Fig. 6A, 6C). There was no effect on igf2a expression in either FG or MV follicles (Fig. 6B). The effects of prostaglandins in FG follicles were similar to that observed in response to melittin. PGE$_2$ (50 and 200 nM) and PGF$_{2a}$ (50 and 200 nM) significantly reduced igf3 expression (Fig. 7A). Igf2b expression was
significantly reduced with the highest dose of PGE$_2$ and PGF$_{2\alpha}$ (Fig. 7C). Prostaglandins had no effect on $igf2a$ expression (Fig. 7B).

$Igf3$ expression was markedly reduced in FG follicles following the addition of the phorbol ester, PMA (400 nM) and the calcium ionophore A23187 (10 µm); however, there was no additive effect (Fig. 8A). In MV follicles, PMA reduced $igf3$ expression whereas A23187 had no effect. Expression levels of $igf2a$ and $igf2b$ decreased in FG and MV follicles treated with PMA and A23187 (Fig. 8B, 8C).

The results of Fig. 1 – 8 are summarized in Table 3.

**Caspase-3/7 Activity**

There was very low caspase-3/7 activity in follicles collected from fish at the start of the incubation period (time zero). Follicles incubated in media alone for 10 h had a significant induction of caspase-3/7 activity (Fig. 9). The addition of rhIGF-I significantly decreased caspase-3/7 activity at 10 h compared to the untreated follicles.

Follicles incubated with hCG (20 IU/ml) for 10 h have a significant reduction in the caspase-3/7 activity when compared to untreated follicles (Fig. 10). Addition of the IGF-IR antagonist, NVP-AEW541 (10 µM), attenuated the anti-apoptotic actions of hCG on caspase-3/7 activity. NVP-AEW541 had no effect on caspase-3/7 activity.

FG follicles incubated for 10 hours with forskolin (2 µM) also have a significant reduction in caspase-3/7 activity when compared to the untreated follicles (Fig. 11). Similar to the results with hCG, addition of the NVP-AEW541 blocked the anti-apoptotic effects of forskolin on caspase-3/7 activity.
17α, 20β-P Production

NVP-AEW541 (10 µM) stimulated a small increase in 17α, 20β-P production in whole ovary pieces, whereas treatment with hCG (20 IU/ml) stimulated an 8-fold increase in 17α, 20P production (Fig. 12). Whole ovary pieces co-treated with hCG and NVP-AEW541 stimulated 17α, 20β-P production to levels similar to hCG treatment alone.
A) IGF-III Gene Expression (Fold Change)

- Control
- H-89
- hCG
- hCG + H-89

B) IGF-IIa Gene Expression (Fold Change)

C) IGF-IIb Gene Expression (Fold Change)
**Figure 1:** *In vitro* effects of hCG (20 IU/ml) and H-89 (25 µM, in DMSO) on IGF-III (A), IGF-IIa (B), and IGF-IIb (C) mRNA expression in full grown immature (FG; 0.57-0.65 mm) and mid-vitellogenic (MV; 0.45-0.56 mm) follicles. Follicles were pre-treated with H-89 for 30 min prior to 3 h incubation in hCG and H-89. Results were obtained using RT-qPCR. Data were normalized to EF-1α and presented as a fold change relative to the control for each size class. Size classes were analyzed separately. Values represent the mean ± SE (n=3) of three pooled experiments with 3-4 replicate wells per experiment. Different letters indicate a significant difference between treatments (p < 0.05; ANOVA, Tukey’s).
A) IGF-III Gene Expression (Fold Change)

- Control
- Forskolin

B) IGF-IIa Gene Expression (Fold Change)

- Control
- Forskolin

C) IGF-IIb Gene Expression (Fold Change)

- Control
- Forskolin
Figure 2: *In vitro* effects of forskolin (10 µM; in DMSO) on IGF-III (A), IGF-IIa (B), and IGF-IIb (C) mRNA expression in full grown immature (FG; 0.57-0.65 mm) and mid-vitellogenic (MV; 0.45-0.56 mm) follicles incubated for 3 h. Results were obtained using RT-qPCR. Data were normalized to EF-1α and presented as a fold change relative to the control for each size class. Size classes were analyzed separately. Values represent the mean ± SE (n=3) of three pooled experiments with 3-4 replicate wells per experiment. Asterisks indicate a significant difference compared to control (p < 0.05; ANOVA).
A) IGF-III Gene Expression (Fold Change)

B) IGF-IIa Gene Expression (Fold Change)

C) IGF-IIb Gene Expression (Fold Change)
**Figure 3**: *In vitro* effects of PACAP (10 and 100 nM) on IGF-III (A), IGF-IIa (B), and IGF-IIb (C) mRNA expression in full grown immature (FG; 0.57-0.65 mm) and mid-vitellogenic (MV; 0.45-0.56 mm) follicles incubated for 3 h. Results were obtained using RT-qPCR. Data were normalized to EF-1α and presented as a fold change relative to the control for each size class. Size classes were analyzed separately. Values represent the mean ± SE (n=3) of three pooled experiments with 3-4 replicate wells per experiment. Different letters indicate a significant difference between treatments (p < 0.05; ANOVA, Tukey’s).
A) IGF-III Gene Expression (Fold Change)

Control
10 ng/ml cGH
100 ng/ml cGH

FG MV

B) IGF-IIa Gene Expression (Fold Change)

Control
10 ng/ml cGH
100 ng/ml cGH

FG MV

C) IGF-IIb Gene Expression (Fold Change)

Control
10 ng/ml cGH
100 ng/ml cGH

FG MV
Figure 4: *In vitro* effects of cGH (10 and 100 ng/ml) on IGF-III (A), IGF-IIa (B), and IGF-IIb (C) mRNA expression in full grown immature (FG; 0.57-0.65 mm) and mid-vitellogenic (MV; 0.45-0.56 mm) follicles incubated for 3 h. Results were obtained using RT-qPCR. Data were normalized to EF-1α and presented as a fold change relative to the control for each size class. Size classes were analyzed separately. Values represent the mean ± SE (n=3) of three pooled experiments with 3-4 replicate wells per experiment. Different letters indicate a significant difference between treatments (p < 0.05; ANOVA, Tukey’s).
A) IGF-III Gene Expression (Fold Change)

B) IGF-IIa Gene Expression (Fold Change)

C) IGF-IIb Gene Expression (Fold Change)
**Figure 5**: *In vitro* effects of sGnRH (100 nM) on IGF-III (A), IGF-IIa (B), and IGF-IIb (C) mRNA expression in full grown immature (FG; 0.57-0.65 mm) and mid-vitellogenic (MV; 0.45-0.56 mm) follicles incubated for 3 h. Results were obtained using RT-qPCR. Data were normalized to EF-1α and presented as a fold change relative to the control for each size class. Size classes were analyzed separately. Values represent the mean ± SE (n=3) of three pooled experiments with 3-4 replicate wells per experiment. Different letters indicate a significant difference between treatments (p < 0.05; ANOVA, Tukey’s).
Figure 6: *In vitro* effects of melittin (50 and 100 nM) on IGF-III (A), IGF-IIa (B), and IGF-IIb (C) mRNA expression in full grown immature (FG; 0.57-0.65 mm) and mid-vitellogenic (MV; 0.45-0.56 mm) follicles incubated for 3 h. Results were obtained using RT-qPCR. Data were normalized to EF-1α and presented as a fold change relative to the control for each size class. Size classes were analyzed separately. Values represent the mean ± SE (n=3) of three pooled experiments with 3-4 replicate wells per experiment. Different letters indicate a significant difference between treatments (p < 0.05; ANOVA, Tukey’s).
A) IGF-III Gene Expression (Fold Change)

B) IGF-IIa Gene Expression (Fold Change)

C) IGF-IIb Gene Expression (Fold Change)
Figure 7: *In vitro* effects of prostaglandin E₂ (PGE₂; 50 and 200 ng/ml; in DMSO) and prostaglandin F₂α (PGF₂α; 50 and 200 ng/ml; in DMSO) on IGF-III (A), IGF-IIa (B), and IGF-IIb (C) mRNA expression in full grown immature (FG; 0.57-0.65 mm) follicles incubated for 3 h. Results were obtained using RT-qPCR. Data were normalized to EF-1α and presented as a fold change relative to the control for each size class. Size classes were analyzed separately. Values represent the mean ± SE (n=3) of three pooled experiments with 3-4 replicate wells per experiment. Different letters indicate a significant difference between treatments (p < 0.05; ANOVA, Tukey’s).
A) IGF-III Gene Expression (Fold Change)

B) IGF-IIa Gene Expression (Fold Change)

C) IGF-IIb Gene Expression (Fold Change)
**Figure 8:** *In vitro* effects of PMA (400 nM; in ethanol) and A23187 (10 µM; in ethanol) on IGF-III (A), IGF-IIa (B), and IGF-IIb (C) mRNA expression in full grown immature (FG; 0.57-0.65 mm) and mid-vitellogenic (MV; 0.45-0.56 mm) follicles incubated for 3 h. Results were obtained using RT-qPCR. Data were normalized to EF-1α and presented as a fold change relative to the control for each size class. Size classes were analyzed separately. Values represent the mean ± SE (n=3) of three pooled experiments with 3-4 replicate wells per experiment. Different letters indicate a significant difference between treatments (p < 0.05; ANOVA, Tukey’s).
Table 3: Summary of Figures 1 – 8. Values represent fold change in gene expression in full grown immature (FG; 0.57-0.65 mm) and mid-vitellogenic (MV; 0.45-0.56 mm) follicles.

<table>
<thead>
<tr>
<th>Figure</th>
<th>Treatment</th>
<th>Fold Change in Gene Expression (Relative to Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Igf3</td>
</tr>
<tr>
<td>1</td>
<td>H-89</td>
<td>FG</td>
</tr>
<tr>
<td></td>
<td>hCG</td>
<td>MV</td>
</tr>
<tr>
<td></td>
<td>hCG + H-89</td>
<td>FG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MV</td>
</tr>
<tr>
<td>2</td>
<td>Forskolin</td>
<td>FG</td>
</tr>
<tr>
<td></td>
<td>10 nM PACAP</td>
<td>MV</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MV</td>
</tr>
<tr>
<td>3</td>
<td>100 nM PACAP</td>
<td>FG</td>
</tr>
<tr>
<td></td>
<td>10 ng/ml GH</td>
<td>MV</td>
</tr>
<tr>
<td>4</td>
<td>100 ng/ml GH</td>
<td>FG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MV</td>
</tr>
<tr>
<td>5</td>
<td>GnRH</td>
<td>FG</td>
</tr>
<tr>
<td></td>
<td>50 nM Melittin</td>
<td>MV</td>
</tr>
<tr>
<td>6</td>
<td>50 ng/ml PGF_2α</td>
<td>FG</td>
</tr>
<tr>
<td></td>
<td>200 ng/ml PGF_2α</td>
<td>MV</td>
</tr>
<tr>
<td>7</td>
<td>50 ng/ml PGF_2α</td>
<td>FG</td>
</tr>
<tr>
<td></td>
<td>200 ng/ml PGF_2α</td>
<td>MV</td>
</tr>
<tr>
<td>8</td>
<td>PMA</td>
<td>FG</td>
</tr>
<tr>
<td></td>
<td>A23187</td>
<td>MV</td>
</tr>
<tr>
<td></td>
<td>PMA + A23187</td>
<td>FG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MV</td>
</tr>
</tbody>
</table>

* represents p < 0.05
** represents p < 0.01
*** represents p < 0.001
Figure 9: Effects of recombinant human IGF-I (rhIGF-I; 100 nM) on caspase-3/7 activity in full grown immature follicles (FG; 0.57-0.65 mm) following 10 h incubation in serum-free media in vitro. Activity measurements (relative fluorescence units/min) were obtained from the caspase-3/7 fluorometric assay kit and normalized to the amount of total protein in the sample. Values represent the mean ± SE (n=3) of three pooled experiments with 4 replicate wells per experiment. Different letters indicate a significant difference between treatments (p < 0.05; ANOVA, Tukey’s).
**Figure 10**: *In vitro* effects of hCG (20 IU/ml) and NVP-AEW541 (10 µM; in DMSO) on caspase-3/7 activity in serum-free media. Full grown immature follicles (FG; 0.57-0.65 mm) were pre-incubated in NVP-AEW541 for 30 min prior to 10 h incubation in hCG and NVP-AEW541. Activity measurements (relative fluorescence units/min) were obtained from the caspase-3/7 fluorometric assay kit and normalized to the amount of total protein in the sample. Values represent the mean ± SE (n=3) of three pooled experiments with 4 replicate wells per experiment. Different letters indicate a significant difference between treatments (p < 0.05; ANOVA, Tukey’s).
**Figure 11:** *In vitro* effects of forskolin (2 µM) and NVP-AEW541 (10 µM; in DMSO) on caspase-3/7 activity in serum-free media. Full grown immature follicles (FG; 0.57-0.65 mm) were pre-incubated in NVP-AEW541 for 30 min prior to 10 h incubation in forskolin and NVP-AEW541. Activity measurements (relative fluorescence units/min) were obtained from the caspase-3/7 fluorometric assay kit and normalized to the amount of total protein in the sample. Values represent the mean ± SE (n=3) of three pooled experiments with 4 replicate wells per experiment. Different letters indicate a significant difference between treatments (p < 0.05; ANOVA, Tukey’s).
Figure 12: *In vitro* effects of hCG (20 IU/ml) and NVP-AEW541 (10 µM; in DMSO) on 17α, 20β-P production from whole ovary tissue. Ovary pieces were pre-incubated in NVP-AEW541 for 30 min prior to 6 h incubation in hCG and NVP-AEW541. Results were obtained from the 17α, 20β-P EIA and normalized to total amount of ovarian tissue (approximately 25 ± 2 mg). Values represent the mean ± SE (n=3) of three pooled experiments with 4 replicate wells per experiment. Different letters indicate a significant difference between treatments (p < 0.05; ANOVA, Tukey’s).
DISCUSSION

Regulation of Ovarian IGFs

The current studies confirm that hCG is a potent stimulator of \textit{igf3} expression in FG and MV follicles (Nelson and Van Der Kraak, 2010a). Interestingly, hCG had no effect on \textit{igf2a} or \textit{igf2b} expression providing further evidence that IGF-III is the major IGF ligand in the ovary. These studies also demonstrated the stimulatory effect of hCG on \textit{igf3} expression can be abolished by blocking PKA activity with H-89 suggesting that gonadotropins use a cAMP/PKA signalling cascade to modulate ovarian IGF expression. Constitutive expression levels of \textit{igf3}, \textit{igf2a}, and \textit{igf2b} also appear to be regulated by PKA. Treatment with forskolin induced a similar increase in \textit{igf3} expression as hCG providing further evidence supporting the involvement of cAMP/PKA in gonadotropin signalling. These results are consistent with the recent work of Li et al. (2011) who found treatment with dibutyl cAMP and IBMX mimic the hCG effects on \textit{igf3} expression in zebrafish. They also suggest that the promoter region of \textit{igf3} contains a putative cAMP response element.

Despite the fact that gonadotropins stimulate \textit{igf3} expression using cAMP as a second messenger, not all compounds that stimulate cAMP production are strong regulators of \textit{igf3}. In the current study, treatment with PACAP stimulated an increase in \textit{igf3} expression in FG follicles; however, when compared to hCG treatment it had a weak effect. Previous studies have demonstrated that treatment with PACAP stimulated increases in follistatin mRNA expression similar to treatment with hCG in cultured zebrafish follicle cells (Wang and Ge, 2003; Wang et al., 2003). The inconsistencies between the effects of hCG and PACAP on \textit{igf3} expression in the current study and effects on follistatin mRNA expression in previous studies may be a result of differences in the experimental design. The previous studies were based on the use of follicle cell
culture, which has been shown to promote an increase in PACAP receptors (Zhou et al., 2011) perhaps leading to a distorted effect of PACAP treatment on gene expression.

GH is generally thought to be involved in regulating growth and metabolism, but it has also been demonstrated to be involved in reproduction. In goldfish, GH potentiates the effects of the gonadotropins on steroidogenesis (Van Der Kraak et al., 1990) and GH alone can stimulate steroidogenesis in the spotted seatrout (Cynoscion nebulosus) (Singh and Thomas, 1993). GH receptors have been characterized in the ovary of rainbow trout (Gomez et al., 1999), Nile tilapia (Ma et al., 2007), and Japanese flounder (Paralichthys olivaceus) (Nakao et al., 2004). In the Mozambique tilapia, GHr and IGF-I mRNA co-localize within the ovary suggesting the presence of a functional GH/IGF axis in the ovary of fish (Kajimura et al., 2004). However, the results of the current study showed that GH had no effect on \( \text{igf3} \), \( \text{igf2a} \), or \( \text{igf2b} \) expression in the ovary of zebrafish. This finding was consistent with previous studies examining \( \text{igf3} \) expression in the gonads of Nile tilapia (Berishvili et al., 2010). Interestingly, the same study showed that GH had a stimulatory effect on ovarian \( \text{igf1} \) expression, which is consistent with another study that showed bovine GH stimulated IGF-I production in the gonads of rainbow trout (Biga et al., 2004). We did not examine \( \text{igf1} \) expression levels in the current study due to its low levels of expression levels in the zebrafish ovary (Nelson and Van Der Kraak, 2010a).

GnRH ligands and receptors have been characterized in the ovary of goldfish (Pati and Habibi, 1993; Pati and Habibi, 1998) and rainbow trout (von Schalburg et al., 1999; Uzbekova et al., 2002) suggesting possible paracrine/autocrine effects on ovarian function. GnRH has been shown to regulate steroidogenesis and oocyte maturation (Pati and Habibi, 2000). The results of the current study showed that sGnRH was not a regulator of the ovarian IGF system.
Zebrafish metabolize arachidonic acid leading to the production of multiple metabolites including prostaglandins (Lister and Van Der Kraak, 2008). Prostaglandins regulate steroidogenesis in goldfish ovarian follicles (Mercure and Van Der Kraak, 1996) and are thought to be important regulators of ovulation (Patino et al., 2003). The current study was the first to show that liberation of arachidonic acid through treatment with melittin or addition of PGE$_2$ or PGF$_{2\alpha}$ inhibit the IGF system in zebrafish FG follicles. These results were consistent with studies in cows where it has been shown that PGF$_{2\alpha}$ inhibits IGF-I signalling in the corpus luteum (Arvisais et al., 2010) and that PGF$_{2\alpha}$-induced luteolysis inhibits IGF-I mRNA and protein expression (Berisha et al., 2010). Prostaglandin inhibition of the IGF system may be part of negative feedback system to shut off the IGF system prior to ovulation. Prostaglandins activate matrix metalloproteinases (MMPs) which are involved in the digestion of the extracellular matrix in sheep corpus luteum (Towle et al., 2002). Previous studies have shown that treatment of bovine granulosa cells with IGF-I inhibited MMPs (Portela et al., 2009) and suggest that IGFs might need to be down-regulated for ovulation to occur. In fish, MMPs are also thought to be involved in breaking down the follicular layer during ovulation leading to follicular rupture (Berndtson and Goetz, 1990; Ogiwara et al., 2005; Thome et al., 2010) and it raises the possibility that inhibition of the ovarian IGF system by the prostaglandins may be a necessary step for ovulation to proceed.

Production of prostaglandins was stimulated by PKC activators in yellow perch (Perca flavescens) (Berndtson et al., 1989), goldfish (Kellner and Van Der Kraak, 1992), and Atlantic croaker (Micropogonias undulates) (Patino et al., 2003). The current study demonstrated that PKC activators, PMA and A23187, strongly down-regulate $igf3$, $igf2a$ and $igf2b$ expression in FG follicles, which suggests that production of prostaglandins by PKC may be the driving force
behind the down-regulation of the IGFs. Interestingly, only PMA inhibited the gene expression of IGFs in MV follicles suggesting that there might be different isoforms of PKC between the follicle size classes or MV follicles have lower overall levels of PKC. Additionally, PKC activation and melittin inhibited expression of the IGFs in FG follicles, but only PKC activation reduced the expression of IGFs in MV follicles suggesting that PKC may also be acting downstream of prostaglandin receptor signalling. A number of prostaglandin receptors have been characterized in mammals including four prostaglandin E series receptors (EP) and a single prostaglandin F series receptor (FP) (Narumiya et al., 1999; Bos et al., 2004); however, very few studies have examined the receptor subtypes in fish. Recent papers suggest the presence of two E series receptors, EP2 and EP4 receptors, in Eurasian perch (Perca fluviatilis) (Henrotte et al., 2011) and medaka (Oryzias latipes) (Fujimori et al., 2011), respectively, but the current results suggest that these receptors may be in low abundance in zebrafish ovarian follicles. EP2 and EP4 receptors are coupled to G_s protein that upon stimulation activates adenylate cyclase leading to an increase in intracellular cAMP (Sugimoto and Narumiya, 2007). An increase in the production of cAMP, which would lead to at least a minimal increase in igf3, but the opposite effect results from PGE_2 treatment in zebrafish. FP receptors are coupled to G_q protein that results in PKC activation (Bos et al., 2004) and studies in mammals have demonstrated that both PGF_2α and PGE_2 are able to bind and activate the FP receptor (Narumiya et al., 1999). Alternatively, the EP receptors may be localized to a specific cell type in the zebrafish follicle that may not be involved in the production of IGF-III. The results of the current study provide evidence in support of the presence of FP receptor in FG zebrafish follicles; however, more work is needed to examine the presence and localization of prostaglandin receptors in the zebrafish ovarian follicle.
Role of ovarian-derived IGFs

Teleost ovarian follicles are susceptible to apoptosis in vitro following incubation in serum-free media and it is believed that cell survival is a necessary prerequisite for normal ovarian growth. In contrast, activation of apoptotic pathways in vivo may play a role in post-ovulatory regression of follicle cells or in the loss of growing follicles due to atresia (Wood and Van Der Kraak, 2001). In zebrafish, the IGF system has been implicated in promoting cell survival and growth during development (Schlueter et al., 2007a) and fin regeneration (Chablais and Jazwinska, 2010). In the current studies, treatment with rhIGF-I inhibited caspase-3/7 activity in ovarian follicles indicating that the IGFs block apoptotic pathways in the ovary of zebrafish. These results are consistent with studies in mammals where IGFs reduced DNA fragmentation in pre-ovulatory follicles (Chun et al., 1994) and purified theca-interstitial cells (Spaczynski et al., 2005). In contrast, IGFs had no effect on in vitro apoptosis in rainbow trout ovarian follicles (Wood and Van Der Kraak, 2002). These results suggest that IGFs in the zebrafish ovary promote follicle cell survival similar to mammals and that mechanistic differences in preventing ovarian apoptosis exist between fish species. Species differences in the susceptibility of ovarian follicles to apoptosis have been previously reported between rainbow trout and goldfish (Wood and Van Der Kraak, 2001). Similar to the inhibitory effects of rhIGF-I on caspase-3/7 activity, hCG also significantly reduced caspase-3-like activity in zebrafish ovarian follicles (Eykelbosh and Van Der Kraak, Unpublished) suggesting that IGFs may be acting downstream of the gonadotropins to promote cell survival.

The IGF system has already been shown to have multiple effects on ovarian function; however, these studies used rhIGF-I to study the actions of the IGFs. Although using rhIGF-I is an effective way to investigate the actions of the IGFs it may not be the most accurate method.
due to low sequence identity between human and fish IGFs (Zou et al., 2009). The actions of the endogenous IGFs can be investigated by creating species-specific recombinant protein and recent studies have shown that recombinant zebrafish IGF-III induces oocyte maturation in zebrafish follicles similar to rhIGF-I (Li et al., 2011). An alternative approach to investigating the actions of the IGFs would be to block their signalling at the level of the receptor using an IGF-IR specific inhibitor, such as NVP-AEW54, which inhibits autophosphorylation of the IGF-IR (Garcia-Echeverria et al., 2004) and this approach was used to examine the “loss of function” effects. Although NVP-AEW541 was designed as anti-cancer treatment for use in mammals, it has been useful in investigating IGF signalling in vertebrates including teleosts. Chablais and Jaźwińska (2010) demonstrated that inhibiting IGF-IR signalling with NVP-AEW541 had a similar impact as morpholino antisense oligonucleotide knock-down of IGF-II, -IRa, and –IRb on fin regeneration in zebrafish.

The results of the current studies show that inhibiting IGF signalling blocked the effects of hCG on caspase-3/7 activity suggesting that production of IGFs are necessary to prevent apoptosis in zebrafish. Furthermore, a similar effect was observed from increased cAMP production resulting from treatment with forskolin and this effect was attenuated by the addition of the IGF-IR inhibitor. These results suggest that gonadotropins stimulate the production of local IGFs, with IGF-III being the primary ligand, to act in a paracrine/autocrine manner to prevent follicular apoptosis similar to the mechanism described in mammals. Chun et al. (1994) found that co-treatment of rat pre-ovulatory follicles with hCG and IGF binding protein-3 blocked the inhibitory effects of hCG on apoptosis. Additionally, it has been shown that IGF-I mediates the inhibitory actions of follicle-stimulating hormone (FSH) on apoptosis in granulosa cells cultured from goat ovarian follicles (Yu et al., 2003).
As the follicle develops, luteinizing hormone (LH) receptors increase in abundance allowing LH to stimulate a steroidogenic shift from producing E2 to the production of MIS (Clelland and Peng, 2009; Kwok et al., 2005). Although a number of local factors are thought to augment this process, the driving force behind the production of MIS in fish is thought to be the LH stimulated production of cAMP (Nagahama and Yamashita, 2008). The current study demonstrated that inhibition of ovarian IGF signalling had no effect on gonadotropin-dependent 17α, 20β-P production, although it does not discount previous studies that have found that IGFs have independent actions on steroidogenesis. These results suggest that the gonadotropins stimulate a common cAMP/PKA cascade which promotes steroidogenesis and IGF-dependent cell survival.

Conclusions

In summary, expression of igf3, igf2a, and igf2b are differentially regulated within the ovary. Gonadotropins, utilizing a cAMP/PKA-dependent signalling cascade, are the most potent stimulators of igf3 expression. GH had no effect on the ovarian IGF system providing further evidence of the tissue-specific actions of GH on the IGF system. This study was the first in fish to show the inhibitory effects of prostaglandins and PKC on the mRNA expression of the ovarian IGFs. Unfortunately, the exact mechanism behind this inhibitory effect remains unknown and will be the focus of future studies. The current study provided novel evidence that IGFs prevent apoptosis in vitro in zebrafish ovarian follicles, which suggests that the IGFs promote follicle cell survival. Additionally, ovarian-derived IGFs, presumably IGF-III, mediate the inhibitory actions of gonadotropins and forskolin on apoptotic pathways in zebrafish ovarian follicles; however, they do not act downstream of the gonadotropins to promote 17α, 20β-P production.
CHAPTER 3

GENERAL DISCUSSION

The current study provides an in-depth look at the hormonal and cellular mechanisms involved in regulating the expression of ovarian IGF ligands and demonstrated that ovarian-derived IGFs mediate some of the actions of the gonadotropins in the ovary of zebrafish. These results provide evidence that the ovarian-derived IGF system is an important autocrine/paracrine regulator of ovarian development in the zebrafish.

The gonadotropins are the major hormones involved in initiating the majority of processes in the ovary including follicle cell growth and survival, steroidogenesis, oocyte maturation, and ovulation (Clelland and Peng, 2009). The results of the current study confirmed that hCG is a potent stimulator of \textit{igf3} expression. Additionally, the current study demonstrated that the gonadotropins use a cAMP/PKA-dependent signalling cascade to regulate \textit{igf3} expression in the ovary. Previous studies have reported that PACAP is an amplifier of gonadotropin signalling by increasing cAMP production (Wang et al., 2003) and the current study found that PACAP only stimulated a small increase in \textit{igf3} expression. These results suggest that PACAP is not heavily involved in the gonadotropin regulation of \textit{igf3} and that PACAP may not be as strong an amplifier of gonadotropin signalling as previously reported. Interestingly, GH, which is a potent stimulator of IGF-I production in the liver, had no effect on \textit{igf3}, \textit{igf2a}, or \textit{igf2b} expression suggesting that there is a high degree of hormonal specificity in terms of the regulating the ovarian IGF system.

The current study also demonstrated that prostaglandins and activation of PKC down-regulates the expression of \textit{igf3} and \textit{igf2b} in the ovary, but only activation of PKC inhibited \textit{igf2a} expression. Production of prostaglandins and increased PKC activity is associated with
ovulation in teleosts (Berndtson et al., 1989; Patino et al., 2003; Lister and Van Der Kraak, 2008; Fujimori et al., 2011) and these regulators represent a potential negative feedback system to inhibit the IGF system during ovulation. Unfortunately, the exact mechanisms of how prostaglandins and PKC exert their actions in the ovary are unknown. Interestingly, treatment of zebrafish follicles with the PKC activator PMA stimulated PGE$_2$ and PGF$_{2\alpha}$ production (Oakie et al., Unpublished), while it is suspected that activation of prostaglandin receptors is coupled to PKC activation. Clearly, further studies are required to better understand the roles of PKC and prostaglandins on ovarian $igf3$, $igf2a$, and $igf2b$ expression.

IGFs have been shown to promote steroidogenesis and oocyte maturation in zebrafish (Nelson and Van Der Kraak, 2010b) and the results of the current study have also demonstrated that rhIGF-I also prevents follicles from undergoing apoptosis following serum starvation in vitro. Additionally, this was the first study to examine the role of locally-produced IGFs in mediating the actions of gonadotropins. Blocking IGF signalling with an IGF-IR-specific inhibitor attenuated the inhibitory effects of hCG on caspase-3/7 activity suggesting that local IGFs act downstream of gonadotropins to promote cell survival. In contrast, blocking IGF signalling had no effect on gonadotropin-dependent 17α, 20β-P production. The results of the current studies suggest that gonadotropins stimulate multiple independent processes and that the ovarian-derived IGFs, presumably IGF-III, mediate select actions of the gonadotropins (Fig. 1).

The ovarian-derived IGFs are important growth factors in regulating cell survival, steroidogenesis and oocyte maturation (Nelson and Van Der Kraak, 2010b), and prostaglandin production (Melnyk and Van Der Kraak, Unpublished). While $igf3$, $igf2a$, and $igf3$ expression are readily detected in the ovary only $igf3$ responds to treatment with gonadotropins or cAMP stimulation. Gonadotropins are thought to be the primary regulators of ovarian development, but
these results suggest that IGF system, especially IGF-III, is an important local system that works alongside the gonadotropins to regulate ovarian development.

**Further Questions**

The existence of multiple local signalling systems in the ovary of zebrafish, including the IGFs, suggests the potential for cross-talk to fine tune various processes involved in ovarian development. Previous studies have demonstrated stimulatory effects of EGF treatment on the activin system (Pang and Ge, 2002a) and it is possible that the activins, EGF, and other local peptide factors modulate the expression and actions of the local IGFs. Unfortunately, only the interactions of the IGFs and PACAP were explored in the current study and the interactions between the IGFs and other local systems still need to be investigated.

Currently, the majority of studies that have investigated the actions of the IGFs in teleosts have utilized *in vitro* models. These types of studies provide significant insight into the actions of the IGFs; however, it does not accurately determine the physiological function of the IGF system. Further studies using knock-down or over-expression approaches are required to determine the physiological actions of the IGFs *in vivo*. Additionally, it has been previously demonstrated that treatment with rhIGF-I stimulates oocyte maturation in follicles still undergoing vitellogenesis (Nelson and Van Der Kraak, 2010b). However, follicles do not mature prematurely *in vivo* despite receiving gonadotropin stimulation that has been shown to increase *igf3* expression *in vitro* and it would be interesting to further examine *in vivo* actions of the IGFs across all the follicle size classes.

The teleost IGF system has been shown to respond to a variety of stressors including hypoxia (Rahman and Thomas, 2011) and endocrine disrupting compounds (Shved *et al.*, 2008). The majority of studies that have investigated the actions of environmental stressors on the IGF
system have examined the effects on the hepatic GH-IGF-I axis (Reinecke, 2010a).

Environmental stressors including temperature, hypoxia, and anthropogenic chemicals have been shown to have effects on ovarian function and it would be interesting to investigate the response of the ovarian IGF system and the concurrent effects on reproductive development.

Although the literature is expanding on the role of the IGF system in ovarian function, the IGF binding proteins have been largely ignored. In vertebrates, the IGF binding proteins act as carrier proteins that regulate turnover, transport, and bioavailability of the IGFs and studies have demonstrated that the binding proteins may function independently of the IGFs (Duan and Xu, 2005). Kamangar et al. (2006) characterized six IGFBPs in the ovary of rainbow trout and it is the most extensive study to date on ovarian IGFBPs in fish. Other studies have examined select IGFBPs in the ovary of teleosts; however, studies examining the temporal, spatial, and hormonal regulation of the IGFBPs are limited.

An IGF system, including IGF-III, has been characterized in the testis of teleosts, but few studies have examined this system. For example, igf3 expression is higher in the testis compared to the ovary of the Nile tilapia (Berishvili et al., 2010), but this study does examine the function of the IGF-III in the testis. IGF-I has also been linked to testicular and ovarian development in the Nile tilapia (Berishvili et al., 2006) and the IGF system may potentially play a role in gonadal development in zebrafish. All zebrafish begin with an immature ovary at hatching. During sex differentiation the ovary will either continue to develop to a sexual mature ovary or regress through apoptosis and testis will begin to develop. The current study has already demonstrated the anti-apoptotic properties of the ovarian IGFs and it would be interesting to examine the expression profiles of the IGFs during sex differentiation to determine the importance of the IGF system during this important life history event.
IGF-III was first reported as being a gonad-specific ligand; however, recent studies suggest that it is expressed in extra-gonadal tissue in Nile tilapia (Berishvili et al., 2010). The highest levels of igf3 expression outside the gonads are found in the brain suggesting that igf3 may also have a potential function in regulating the neuroendocrine aspects of reproduction. Additionally, igf3 expression is found in zebrafish during early development indicating that it is maternally derived (Zou et al., 2009). Expression of igf3 remains high throughout the larval stage suggesting that IGF-III plays an important functional role during zebrafish development. These studies suggest that IGF-III functions outside of the gonads of fish and the potential extra-gonadal actions should be the focus of future investigations.

Conclusions

Local growth factors play an important function during zebrafish ovarian development and the results of the current study have further demonstrated the significance of the ovarian IGF system. Collectively, this thesis investigated pathways involved in regulating the ovarian-derived IGFs as well as revealed a novel function of the IGFs in mediating the actions of the gonadotropins. There is strong evidence to suggest that the IGF system is an important paracrine signalling system during ovarian development; however, additional studies are required to further expand our knowledge on the ovarian-derived IGF system in teleosts.
Figure 1: Proposed model of IGF-III regulation and function in zebrafish ovarian follicles. hCG binding to LH receptor (LHR) activates adenylate cyclase (AC) stimulating the cAMP/PKA signal cascade which leads to increased steroidogenesis and induction of igf3 expression and presumably protein. IGF-III binds to IGF-IR which leads to inhibition of caspase-3/7 activity. PACAP also stimulates igf3 expression through AC activation although it is unknown if its small induction of igf3 expression has effects on steroidogenesis or caspase-3/7 activity. PGE2, PGF2α, and PKC inhibit igf3 expression through an unknown mechanism which likely involves the prostaglandin receptors (EPr and FPr). Solid lines represent known pathways and dashed-lines represent suspected pathways.
REFERENCES


Pang, Y., Ge, W., 2002a. Epidermal growth factor and TGFalpha promote zebrafish oocyte maturation in vitro: potential role of the ovarian activin regulatory system. Endocrinology 143, 47-54.


