The Involvement of the Insulin-like Growth Factor System during the Oocyte Maturation and Early Development of Zebrafish

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Abstract of thesis entitled:

The Involvement of the Insulin-like Growth Factor System during Oocyte Maturation and Early Development of Zebrafish

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As a functional unit involved in both maintaining endocrine homeostasis and also producing mature eggs, the ovary plays a central role in female reproduction. The development and function of the ovarian follicles are controlled by gonadotropins released from the pituitary. It is widely accepted that the action of gonadotropins on ovarian follicles is mediated by paracrine/autocrine factors produced by the somatic cells surrounding the oocyte. Increasing evidence indicates that the Igf system is involved in mediating the action of gonadotropins in the ovary. Previously, we identified a gonad-specific Igf subtype (Igf3) distinct from Igf1 and Igf2. This finding further highlights the importance of the Igf system in the fish ovary. In this thesis, efforts were made to understand the role of the Igf system in ovary using zebrafish as the model organism, and attention was focused on Igf3.

We first examined the gene expression patterns of Igf3 in the ovary. The *igf3* gene in zebrafish was found to be alternatively spliced into two transcripts, with *transcript variant 1* exclusively expressed in the gonads and *transcript variant 2* only expressed during early development. Using specific antibodies developed for zebrafish Igf3, both the prepropeptide and the mature peptide forms of Igf3 were found to be predominantly expressed in the zebrafish ovary. Real-time PCR and *in situ* hybridization revealed that *igf3* mRNA levels were relatively low in the early follicles but significantly increased after the mid-vitellogenic stage (midstage III) and were high in the full grown follicles. In the full grown follicles, *igf3* mRNA was

detected primarily in the somatic follicular cells, with a low level of expression in the oocytes. Igf3 immunoreactivity was confined to the follicular cells alone.

Because the expression of Igf3 is correlated with the LH receptor in zebrafish follicles, the regulation of *igf3* by gonadotropins was subsequently studied in the ovary. The expression of *igf3* was significantly up-regulated in both ovarian fragments and isolated follicles upon treatment with hCG in dose- and time-dependent manners. Treatment with 8-Br-cAMP or IBMX mimicked the effects of hCG on the expression of *igf3* in follicles of different stages.

To study the function of Igf3, bioactive recombinant Igf3 proteins were prepared using a bacterial expression system. Incubation of follicles with recombinant zebrafish Igf3 significantly enhanced oocyte maturation in time-, dose- and stage-dependent manners. The potential mechanisms of Igf3-induced oocyte maturation were then investigated. Igf3 stimulated oocyte maturation via a steroid-independent manner. Igf3 induced oocyte maturation through Igf1rs and the PI3 kinase, PDE3 and MAP kinase were necessary for Igf3-mediated oocyte maturation in zebrafish.

Four Igfs are present in zebrafish, and our results show that all four *igfs* are expressed in the ovary of zebrafish and exhibit the differential expression profiles during folliculogenesis. Using a primary culture of zebrafish follicle cells, we demonstrated that hCG stimulated *igf2b* and *igf3* expression but suppressed *igf2a* expression. Moreover, the effect of gonadotropin could be mimicked by IBMX, which increased the intracellular levels of cAMP, suggesting the possible involvement of cAMP in the gonadotropin-based regulation and differential expression of *igf2a*, *igf2b* and *igf3*. These results also show that the Igf3 is the Igf subtype most sensitive to gonadatropin and cAMP.

In addition, the expression patterns of *igf1*, *igf2a*, *igf2b*, *igf3*, *igf1ra* and *igf1rb* were also studied during zebrafish embryogenesis. The unique temporal and spatial

expression patterns of *igf1*, *igf2a*, *igf2b*, *igf3*, *igf1ra* and *igf1rb* were revealed by both real-time PCR and whole mount *in situ* hybridization, the results suggest divergent functions for these Igfs in early zebrafish development.

Taken together, the present studies provide substantial information about the Igf system, especially that of Igf3 in the zebrafish ovary. Data were gathered regarding Igf3 expression, regulation and functions, which is not only helpful for the understanding of the role of the Igf system in fish reproduction, but also contributes toward uncovering the ovarian signaling network involved in oocyte maturation across vertebrates. This study of *igfs* gene expression provides direct information to the study of Igf signaling in zebrafish.

摘要

卵巢作為負責產生成熟卵子以及維持內分泌的功能器官，在雌性生殖中起着核心作用。大量研究已表明卵巢的生長及其功能是受腦垂體分泌的促性腺激素調控。促性腺激素的作用可由卵巢的體細胞中產生的旁分泌/自分泌因子所調控，該觀點已被普遍接受。越來越多的證據表明，胰島素樣生長因子（Igf）系統在卵巢中參與促性腺激素的功能調控。在之前的研究中，我們發現了除了 Igf1 和 Igf2 外，一種新的胰島素樣生長因子（Igf3），其表達部位僅限於硬骨魚類的性腺。該發現也進一步提示胰島素樣生長因子系統在魚類卵巢中的重要性。在本研究中，我們利用斑馬魚作為研究對象，對胰島素樣生長因子系統特別是 Igf3 在卵巢中的功能進行了研究。

首先我們對 Igf3 在卵巢中的表達模式進行了研究。我們發現 *igf3* 基因在斑馬魚中有兩個不同的轉錄本，轉錄本-1 在性腺中高表達，轉錄本-2 在早期發育過程中表達。利用針對斑馬魚 Igf3 的特異抗體，我們發現 Igf3 的前體肽和成熟肽都主要在斑馬魚卵巢中表達。實時定量 PCR 和原位雜交的結果顯示，*igf3* mRNA 的表達在早期卵泡中相對較低，卵黃發生中期開始增加，卵黃成熟期最高。在卵黃成熟期的卵泡中，*igf3* mRNA 在濾泡細胞中的表達量相對於卵母細胞較高。Igf3 蛋白的表達也集中在濾泡細胞中。

鑑於 Igf3 在卵巢中表達與黃體生成素受體表達有相關性，提示 Igf3 可能被垂體促性腺激素所調控。在人絨毛膜促性腺激素的處理下，*igf3* 的表達被顯著上調並呈劑量和時間依賴性。該刺激作用可被增加胞內環磷酸腺苷濃度的藥物所模擬，提示垂體促性腺激素的作用可被環磷酸腺苷所介導。

為研究 Igf3 的功能，我們在細菌系統中成功製備了有生物活性的斑馬魚 Igf3 重組蛋白。將斑馬魚 Igf3 的重組蛋白與卵泡進行孵育，可以顯著促進卵母細胞

成熟並呈時間，劑量和卵泡時期依賴性。之後我們進一步探討了 Igf3 促進卵母細胞成熟的分子機制，結果顯示 Igf3 誘導卵母細胞成熟並不依賴卵泡中類固醇生成。我們證明了 Igf3 通過激活 Igf1rs 誘導卵母細胞成熟，同時也證明了 PI3 激酶，PDE3 以及 MAP 激酶為 Igf3 誘導卵母細胞所必需。

目前斑馬魚中存在四種胰島素樣生長因子，我們的結果表明四種胰島素樣生長因子的都在斑馬魚的卵巢中表達，其表達模式在卵泡生長過程中不同。利用原代培養的斑馬魚卵泡細胞，我們發現絨毛膜促性腺激素刺激 *igf2b* 和 *igf3* 的表達，卻抑制 *igf2a* 的表達，該刺激或抑制所用也可被增加胞內環磷酸腺苷濃度的藥物所模擬，提示環磷酸腺苷參與促性腺激素調控 *igf2a*，*igf2b* 和 *igf3* 表達的可能性。通過比較這四種 *igf* 的表達和調控，我們也發現 *igf3* 是卵泡生長過程中表達量變化最為劇烈，對促性腺激素以及環磷酸腺苷最為敏感的一種胰島素類生長因子。

此外，我們也分析了四种胰島素類生長因子配體 *igf1*，*igf2a*，*igf2b*，*igf3* 和兩種受體 *igf1ra* 以及 *igf1rb* 在斑馬魚胚胎發育中的時空表達模式。利用實時定量 PCR 和原位雜交技術，我們發現四種胰島素類生長因子在胚胎發育過程中獨特的表達，該結果也提示在早期發育過程中，四種胰島素類生長因子可能發揮的不同角色。

本文對胰島素類生長因子特別是 Igf3 在斑馬魚卵巢中的表達，激素調控及其功能進行了充分的研究。這些結果不僅有助於對更深入的了解胰島素類生長因子在魚類卵巢中的生理作用，也有助於揭示調控脊椎動物卵母細胞成熟的分子信號機制。此外，對早期發育過程中胰島素類生長因子的表達研究，為其後研究其功能提供了直接參考。

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LI Jianzhen

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List of abbreviations

3β-HSD	3β-hydroxysteroid dehydrogenase
17-HP	17α-hydroxyprogesterone
17β-HSD	17β-hydroxysteroid dehydrogenase
20β-HSD	20β-hydroxysteroid dehydrogenase
aa	Amino acid
ActRII	Activin type II receptor
ActRI	Activin type I receptor
AD	Actinomycin D
BCIP	5-bromo-4-chloro-3-inolyl phosphate-4-toluidine salt
BMPs	Bone morphological proteins
BSA	Bovine serum albumin
bp	Base pairs
cAMP	Cyclic adenosine monophophate
CH	Cycloheximide
CRE	cAMP response element
Cyp19	Cytochrome P450 aromatase
DHP	17α,20β-dehydroxy-4-pregen-3-one
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle's medium
dpf	Day post fertilization
E.coli	*Escherichia coli*
E2	Estradiol
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
EV	Early vitellogenic
FG	Full-grown
FP	Floor plate
FSH	Follicle-stimulating hormone
GDF-9	Growth differentiation factor-9
GtH	gonadatropin
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
HC	Hypochord
hCG	Human chorionic gonadatropin
hpf	Hour post fertilization
IBMX	3-isobutyl-1-methyl-xanthine
IGF	Insulin-like growth factor

IGFBP	Insulin-like growth factor binding protein
IGF1R	Insulin-like growth factor type 1 receptor
IGF2R	Insulin-like growth factor type 2 receptor
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IRS-1	Insulin-receptor substrate-1
kDa	Kilo-dalton
LB	Luria-Brertani
LH	Luteinizing hormone
MAPK	Mitogen-activated protein kinases
MB	Midbrain
DHB	Dorsal middle brain
MIH	Maturation-inducing hormone
MIS	Maturation-inducing steroids
MPF	Maturation-promoting factor
MV	Mid-vitellogenic
mRNA	Messenger ribonucleic acid
NBT	4-nitro blue tetrazolium
OV	Otic vesicle
P450arom	Aromatase
P450c17	17α-hydroxylase/17,20-lyase
PA	Pharyngeal arch
PDE	Phosphodiesterase
PFA	Paraformaldehyde
PGC	Primordial germ cell
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PTU	1-phenyl-2-thiourea
PV	Pre-vitellogenic
RACE	Rapid amplification of cDNA ends
RT-PCR	Reverse Transcription–Polymerase chain reaction
SDS	Sodium dodecyl sulfate
ST	Sternohyodieus
StAR	Steroidogenic acute regulator
TG	Trigeminal ganglia
TGF	Transforming growth factor
UTR	Untranslated region

List of fish names mentioned in the thesis

Common name	Scientific names
Amago salmon	*Oncorhynchus rhodurus*
Atlantic croaker	*Micropogonias undulatus*
Atlantic salmon	*Salmo salar*
chub mackerel	*Scomber japonicus*
Coho salmon	*Oncorhynchus keta*
Common Carp	*Cyprinius carpio*
Goldfish	*Carassius auratus*
Gilthead seabream	*Sparus aurata*
Japanese eel	*Anguilla japonica*
Killifish	*Fundulus heteroclitus*
Medaka	*Oryzias latipes*
Rainbow trout	*Oncorhynchus mykiss*
Red seabream	*Pagrus major*
White seabass	*Cynoscion nobilis*
Striped bass	*Morone saxatilis*
Tilapia	*Orechromis niloticus*
Yellowtail	*Seriola quinqueradiata*
Zebrafish	*Danio rerio*

Chapter 1 General Introduction

The word "ovary" is derived from the Latin word "ovum," meaning egg. The ovary is not only the female gonad, producing mature oocytes for fertilization and successful propagation of the species, but is also the female reproductive gland, controlling much of female development and physiology. The ovary is generated from primordial germ cells when these cells migrate into the urogenital ridge. The immature gonad is differentiated along a female-specific pathway, and the newly formed oocytes proliferate, grow and subsequently enter meiosis, eventually reaching the stage of maturation and ovulation. All of these processes are under the precise, coordinated control of hundreds of interacting factors including gonadotropins (follicle-stimulating hormone [FSH] and luteinizing hormone [LH]) and a variety of local ovarian factors (Edson et al., 2009; McGee and Hsueh, 2000).

1.1 Life cycle of ovarian follicles

The ovarian follicle is the basic functional and structural unit of the vertebrate ovary. The development of oocytes is inevitably correlated with the surrounding somatic layers. An oocyte and the surrounding layers of specialized somatic cells including inner granulose cells and outer layers of theca cells, constitute an ovarian follicle (Babin et al., 2007; Nagahama et al., 1995; Peters and McNatty, 1980). In its broadest sense, the life history of an ovarian follicle is the process by which primordial germ cells (PGCs) become ova that are ready to be fertilized. This process can be classified into six major steps: (1) PGC specification (germline segregation); (2) PGC migration; (3) transformation of PGC into oogonia, (4) transformation of oogonia into oocytes (onset of meiosis) and growth of oocytes while under meiotic arrest (follicle development), (5) resumption of meiosis (maturation), and (6) expulsion of the ovum from its follicle (ovulation). In this section, this process will

be briefly reviewed as below.

1.1.1 Ovary formation

1.1.1.1 PGCs specification

All metazoans contains two kinds of diploid cell types: cells forming the somatic cells which remain diploid and germline cells that give rise to the haploid reproductive cells or gametes. The diploid cells from the germline are alled PGCs during early development stages (Babin et al., 2007).

Studies from *Caenorhabditis elegans*, *Drosophila* and *Xenopus* have demonstrated the origin of PGCs in a defined area of the egg (referred to as germ plasm) (Houston and King, 2000; Saffman and Lasko, 1999; Williamson and Lehmann, 1996). Germ plasm can be identified morphologically by the presence of germ granules, conspicuous membrane-free organelles with an electron-dense granulofibrillar appearance. These electron-dense structures are associated with mitochondria and contain RNA and protein molecules (Matova and Cooley, 2001). Evidence from studies of *Drosophila* mutants has supported the hypothesis that these organelles carry the germline "determinants" (Williamson and Lehmann, 1996). In zebrafish, several recent studies have also indicated that germline specification depends on maternally provided material (Raz, 2003). In contrast, the specification of PGCs in mammals follows a different strategy. The available information indicates that the PGCs in mammals are induced through cellular interactions during the gastrulation stage, and not by the way of inheriting maternally provided cytoplasmic determinants (Bendel-Stenzel et al., 1998; McLaren, 1999; Wylie, 1999). Recent studies of knockout mice have defined members of the BMP family as major factors that are essential for development of PGC precursors (Chang et al., 2002; Edson et al., 2009; Hayashi et al., 2007).

1.1.1.2 PGCs migration

In all systems, PGCs form far from the site of the developing gonads and migrate long distances to the site of developing gonad. The identification of the PGC marker genes in different species, such as the alkaline phosphatase in the mouse (Chiquoine, 1954; Ginsburg et al., 1990), *vasa* in *Drosophila* (Hay et al., 1988) and zebrafish (Olsen et al., 1997; Yoon et al., 1997), has allowed for PGCs to be visualized in the embryo. In zebrafish, PGCs marked by *vasa* or *nanos1* are segregated into four clusters in the blastrula stage. These PGC clusters then migrate dorsally and align at the border between the head and trunk mesoderm or align within the lateral mesoderm. Both lines of cells then move towards an intermediate target within the lateral mesoderm. At the 8-somite stage, PGCs leave this intermediate target and migrate posteriorly to colonize the developing gonad. At the prim-5 stage, PGCs have formed clusters between the 8th and 10th somites. The entire migration process takes approximately 20 hours in zebrafish (Raz, 2003).

1.1.1.3 From PGCs to oogonium

After the migration of PGCs into the site of the developing gonad, PGCs associate with and are surrounded by several special somatic cells. These cells have many special morphological features, such as a high electron density, and exhibit irregular outlines similar to the irregular shapes of the PGCs. These somatic cells in the presumptive gonad have an embryological origin different from PGCs. The external somatic layer is primarily derived from the genital ridge epithelium, and some of these specialized cells develop from the mesenchyme (Devlin and Nagahama, 2002). The presence of these somatic cells surrounding the PGCs in the developing female gonad indicates the start of follicle differentiation. The gradual transformation of PGCs into oogonia accompanies the change of cell structure and morphology. The oogonia proliferate rapidly in the ovary through mitosis before the beginning of

meiosis. An oogonium enters into the next stage after the initiation of meiotic division (Babin et al., 2007).

1.1.2 Ovarian development, oocyte maturation and ovulation

1.1.2.1 Ovarian development

Following the onset of meiosis and formation of the ovarian follicle (folliculogenesis), significant growth of the oocyte begins. The ovarian follicle consists of the oocyte and its envelope, which is composed of cells and outer membrane. During folliculogenesis, the morphology changes as the oocyte and the surrounding cells differentiate. Varying terms and classification systems have been used to describe the different stages of development. In mammals, the follicles are divided into four main stages: primary, preantral, antral, and preovulatory (Fig. 1-1A) (McGee and Hsueh, 2000). In primary-stage follicles, the small follicle consists of a small oocyte, a few flat granulosa cells and a basement membrane and its appearance and size change little with advancing age. The preantral follicle is characterized by an oocyte that is in the growth phase with one or more layers of cuboidal granulosa cells surrounding it. As the granulosa cells multiply, there is a concomitant increase in the production and accumulation of fluid, which leads to the formation of a follicular cavity (antrum). The number of large follicles that become preovulatory and ovulate varies widely in different species. Many changes involving the surface epithelium, the follicle envelope and the oocyte occur in the preovulatory follicle. A marked expansion of the entire follicle takes place shortly before follicular rupture and the increase in size is considerable (Peters and McNatty, 1980).

Unlike that of mammals, the ovary of fish is highly variable due to the wide range of reproductive patterns including oviparity, ovoviviparity and viviparity. Three major ovarian types have been classified based on the pattern of oocyte development (Nagahama, 1983; Wallace and Selman, 1980). The synchronous ovary contains

oocytes that are all at the same stage of development; the group-synchronous ovary consists of at least two populations of oocytes at different developmental stages, and the asynchronous ovary contains oocytes at all stages of development (Nagahama et al., 1995). Generally, the development of the ovarian follicles in teleosts can be divided into four stages: stage I (primary growth stage); stage II (pre-vitellogenesis stage); stage III (vitellogenesis stage); and stage IV (maturation stage) (Fig. 1-1B). In the primary growth stage, the cellular structure of young primary oocytes is similar to that of oogonia. The granulosa cells proliferate and form a layer of flattened cells. In the pre-vitellogenesis stage, the size of the oocyte increases as the nucleus enters the diplotene stage of prophase in the first meiotic division. In addition, the oocytes are surrounded by several cell layers, i.e., the basement lamina, theca, and granulosa cells. The ovarian follicle enlarges during the vitellogenesis stage, and the oocyte accumulates the yolk containing nutritional reserves from the blood stream. The granulosa cells and oocytes are connected by heterologous gap junctions during oocyte development. These cellular structures provide a mechanism for the transfer of small molecules including inorganic ions, secondary messengers such as cAMP and nutrients, from one cell to another (Kidder and Mhawi, 2002).

1.1.2.2 Oocyte maturation

During maturation, the fully grown oocyte exits the diplotene stage and restarts meiotic division after receiving an activation signal. In fish, after the oocyte is fully grown upon completion of vitellogenesis, the follicle becomes ready for the oocyte maturation or the resumption of meiosis, which is accompanied by several maturational processes in the nucleus and cytoplasm of the oocyte. Fully grown fish oocytes arrest in the late prophase I of meiosis I and must progress to the second meiotic metaphase before fertilization. The nucleus of fish immature oocytes (the germinal vesicle, GV) is generally inconspicuous because of the opaque cytoplasm.

The GV at this stage is typically located centrally or halfway between the center and the oocyte periphery. Initially, the GV migrates to the animal pole, where the micropyle is located. A significant reorganization of cytoplasmic microtubules distribution occurs during the early phase of hormonally induced oocyte maturation. After migration is completed, the membrane undergoes GV breaks down (GVBD), and its contents become intermingled with the surrounding cytoplasm. After GVBD, the oocytes enter into metaphase II of meiosis II by extruding the first polar body and then arrest in this stage. Fertilization releases this arrest, and the oocytes complete the second meiotic division by extruding the second polar body and initiate a series of embryonic divisions (Fig. 1-2) (Babin et al., 2007; Nagahama et al., 1995; Verlhac and Villeneuve, 2009). The entire course of maturation involves a series of events, including polar body extrusion, chromosome condensation and the spindle formation. In most studies, GVBD is usually regarded as a hallmark of the progress of oocyte maturation. A detailed discussion of oocyte maturation processes and regulation is found in section 1.4 separately.

1.1.2.3 Ovulation

After oocyte maturation, the mature oocyte is released from the surrounding follicle cells to be fertilized, in a process also known as ovulation. Following the disruption of microvillar connections between the follicular wall and the oocyte that occurs during oocyte maturation, the ovulation process progresses through a series of events including the opening of the follicular wall surrounding the oocyte and the active expulsion of the oocyte through the opening (Bolamba et al., 2003; Cerda et al., 1999). These processes are regulated by a number of molecules including proteases, protease inhibitors and vasoactive peptides (Goetz and Garczynski, 1997).

1.2 Regulation of ovarian follicles

It is well established that the development and function of the ovarian follicles are controlled by gonadotropins (LH and FSH), both of which are released from the pituitary, and that their coordinated actions are essential for the fine-tuning of ovarian function. In addition, a network of locally produced growth factors such as Igfs, Egf, and activin is also involved in the regulation of ovarian follicles. These growth factors serve as mediators of gonadotropic signal transduction from the follicle cells, in which gonadotropin receptors are located, to the oocyte and also regulate these signals through interactions with other factors from the follicle cells and/or the oocyte itself (Babin et al., 2007).

1.2.1 Gonadotropins - LH and FSH

1.2.1.1 Structure

It has been demonstrated that two gonadotropins, FSH and LH, directly control many aspects of gonadal development and function across vertebrates. These gonadotropins are secreted form the anterior pituitary gland. Both are heterodimeric glycoproteins formed by two subunits. A common α subunit is non-covalently but specifically linked to a specific β subunit, determining the biological activity the gonadatropin. All three subunits including α, FSHβ and LHβ are encoded by distinct genes (Levavi-Sivan et al., 2010). In fish, a single gonadotropin (LH) was originally believed to control ovarian function (Donaldson et al., 1972; Sundararaj et al., 1972; Sundararaj et al., 1971). However, a pair of gonadotropins, initially termed GtH1 and GtH2, were later identified in different fish species including coho salmon (Swanson et al., 1991), *Fundulus heteroclitus* (Lin et al., 1992), tuna (Okada et al., 1994), striped bass (Hassin et al., 1995), gilthead seabream (Elizur et al., 1996), goldfish (Kobayashi et al., 1997; Yoshiura et al., 1997), rainbow trout (Govoroun et al., 1997) and sea bass (Moles et al., 2008).

1.2.1.2 Receptors

Similar to the situation with GtHs, it was believed for many years that only one type of GtH receptor (GtHR) existed in fish. Early binding studies indicated that only one type of GtH receptor could be indentified (Breton et al., 1986; Salmon et al., 1984). The presence of two distinct GtH receptors in fish species was initially demonstrated in salmon (Miwa et al., 1994). So far, two GtH receptors have been cloned in a number of fish species, including salmon (Maugars and Schmitz, 2006; Oba et al., 1999a; Oba et al., 1999b), catfish (Bogerd et al., 2001; Kumar et al., 2001), zebrafish (Kwok et al., 2005; So et al., 2005), sea bass (Rocha et al., 2007), eel (Jeng et al., 2007) and rainbow trout (Sambroni et al., 2007). Both FSH and LH receptors are G-protein-coupled receptors (GPCR) of the rhodopsin-like receptors family. Like other GPCRs, each GtH receptor has an N-terminal extracellular domain for ligand binding, seven hydrophobic helices inserted in the plasma membrane, and a C-terminal intracellular domain mediating intracellular signal transduction. One of the key unique feature of the GtHRs is their large extracellular domains that constitue more than half of the length of the protein. This extracellular domain contains a central region with several leucine-rich repeats (LRRs) lacked by amino-terminal and carboxy-terminal cysteine-rich regions (the NCR and CCR, respectively). Fish GtH receptors follow the same general structure as mammals, but the extracellular domains of fish FSH receptors are distinct from those of mammals. Fish FSH receptors do not contain a typical NCR domain, and 30 amino acids found between the third and fourth cysteine in mammalian receptors are not present in the fish CCR domains (Levavi-Sivan et al., 2010).

1.2.1.3 Signal transduction

Both FSH and LH receptors activate the membrane-associated adenylate cyclase

(AC), causing an increase in intracellular cAMP. Upon activation by ligand binding, the receptors associate with Gs which then dissociates into the G-α and G-βγ subunits and transmits different signals by interacting with various effectors. G-α activates AC which catalyzes the conversion of ATP into cAMP, thereby stimulating intracellular cAMP levels and leading to the activation of cAMP-dependent protein kinase A (PKA) in the cytoplasm (Babin et al., 2007; Bogerd et al., 2001; Vischer et al., 2003). The catalytic subunit of activated PKA then translocates into the nucleus and phosphorylates structural proteins, enzymes and transcriptional factors, such as the cAMP responsive element. In addition to stimulating cAMP production, GtHs are also able to promote the activation of phospholipase C (PLC). PLC activation results in the rapid metabolism of phospatidlyinositol-4,5-bisphosphate (PtdINs(4,5)P_2), leading to the formation of inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3) and diacylglycerol (DAG). Ins(1,4,5)P3 triggers the release of calcium from intracellular stores (Furuichi et al., 1989). DAG regulates the activity of the protein kinase C (PKC) family of calcium and phospholipid-dependent enzymes (Nishizuka, 1984). In addition to these transduction pathways, considerable evidence also suggests that GtHRs can also interact with various cytoplasmic scaffold and adapter proteins, which can link the receptors to a range of signaling intermediates and intracellular effectors (Babin et al., 2007; Hall and Lefkowitz, 2002).

1.2.1.4 Actions on steroidogenesis

In mammals, it is clear that FSH and LH are necessary for the appropriate regulation of steroidogenesis in granulosa and theca cells. GtHs stimulate the production of estradiol-17β (E2) and progesterone (P4), which play important roles in ovarian function and control of the reproductive cycle. The expression and activity of P450arom in granulosa cells is increased by FSH, whereas P450scc expression is stimulated by LH in luteal cells, thus, the synthesis of E2 and P4 is increased,

respectively (Leung and Steele, 1992). After the LH surge, the level of P450arom mRNA declines and the granulosa cells change from proliferating, E2-producing cells to differentiated cells that secrete P4. In the theca cells of a growing follicle, expression of both P450scc and P45017α increases in a LH-dependent manner, leading to augmented androgen synthesis. It has also been well demonstrated that GtHs regulate steroidogenic acute regulatory (StAR) protein. Therefore, both FSH and LH are essential for generating the appropriate environment for steroid synthesis within the growing follicle (Misrahi et al., 1998; Wood and Strauss, 2002).

In fish, both GtHs can stimulate steroid production by the salmonid ovarian tissue (Montserrat et al., 2004; Planas et al., 2000; Planas et al., 1997; Suzuki et al., 1988). However, their steroidogenic potencies vary depending on the state of gonadal development (Montserrat et al., 2004; Planas et al., 2000; Planas et al., 1997). In coho salmon, LH but not FSH stimulates 17α,20β-dihydroxy-4-pregnen-3-one (DHP) production in granulose layers, whereas both LH and FSH stimulate steroid production in theca-interstitial layers, with LH showing a higher activity (Maestro et al., 1997). In red seabream, only LH can stimulate both aromatase activity and the expression of P450arom (Kagawa et al., 2003), the authors therefore suggested that the physiological functions of FSH may be redundant in the ovary. These results also indicate that the effects of teleost gonadotropin on steroidogenesis exhibit a wide range of variations.

1.2.1.5 Actions on folliculogenesis

In mammals, it is believed that FSH and LH are unlikely to be involved in the regulation of primordial follicles because functional gonadotropin receptors have not yet developed in them (Dunkel et al., 1994; O'Shaughnessy et al., 1997; Oktay et al., 1997; Rannikki et al., 1995; Sokka and Huhtaniemi, 1990). The growth of preantral follicles was believed to be gonadotropin independent because follicles still develop

to the antral stage in several animal models as well as humans with minimal circulating FSH or defective FSH receptors (Aittomaki et al., 1995; Dierich et al., 1998; Fauser and Van Heusden, 1997; Hillier, 1994; Kumar et al., 1997). However, studies of rodents suggested that the development of early follicles is under the regulation of gonadotropins (Cain et al., 1995; Dahl et al., 1988). Available information has demonstrated that gonadotropin can regulate cell divison and differentiation in the preantral follicles (Boland et al., 1993; Roy and Greenwald, 1989). In the later stages of follicle development, both FSH and LH have been demonstrated to be essential for the proliferation and survival of follicular somatic cells and the cyclic recruitment of antral follicles. Decreasing the concentration of serum gonadotropins causes atresia and apoptosis in developing follicles (Nahum et al., 1996). Corresponding to the trophic actions of gonadotropins, FSH and LH receptors are highly expressed in the follicular somatic cells suggesting that ovarian follicles are responsive to gonadotropins. In fish, similar to other vertebrates, FSH is the primary factor responsible for the regulation of follicle growth (vitellogenesis) (Nagahama et al., 1995).

1.2.2 Growth factors

Growth factors are a large and heterogeneous group of peptide signaling molecules. Increasing amounts of evidence have shown that they act as both hormonal and local paracrine/autocrine regulators and are involved in many aspects of ovarian development.

1.2.2.1 Insulin-like growth factor system

As the significance of putative intraovarian regulators has been became increasingly recognized, much of the attentions has been drawn to insulin-like growth factors (Igfs). Igfs are low molecular weight, mitogenic peptides that are structurally related to proinsulin. The initial step in the cellular action of Igfs is

ligand-receptor binding, and both Igf1 and Igf2 possess specific high-affinity receptors (Giudice, 1992). Both Igf1 and Igf2 are bound in plasma and other biological fluids by a family of proteins known as Igf-binding proteins (Igfbps). The Igfbp family consists of six distinct proteins (Jones and Clemmons, 1995; Rechler, 1993), which both inhibit Igf activity by preventing binding to the Igf receptor and promote Igf activity through aiding in delivery to the receptor and increasing the Igf half-life by preventing Igf degradation. Many experimental results have indicated an essential role for Igfs in ovary (Yoshimura, 2003). Most recently, our lab has identified a novel Igf (Igf3) from the teleost; the expression of Igf3 is restricted to the gonads, which further highlights the role of the Igf system in fish reproduction (Wang et al., 2008).

1.2.2.1.1 Structure of Igf1 and Igf2

Like proinsulin, both Igf1 and Igf2 are single-chain polypeptides containing three intrachain disulfide bonds. Both Igf1 and Igf2 encode preprohormones, containing a putative signal peptide that is removed during secretion to yield the prohormone composed of five distinct domains (B-C-A-D-E). Subsequent proteolytic release of the E domain yields the mature IGF peptide (domains B-C-A-D). However, proinsulin contains three domains (B-C-A). Regions of structural homology exist in the A and B domains of proinsulin, and the connecting region (C domain) between the A and B domains shares weak homology. Three-dimensional models indicate that Igf1 and Igf2 have identical hydrophobic cores and identical conformation of the amino acid residues corresponding to the A and B chains of insulin (Giudice, 1992; Wood et al., 2005).

1.2.2.1.2 Receptors

Both Igf1 and Igf2 exert their physiological actions by interacting with specific

cell-surface membrane receptors including Igf type 1 and type 2 receptors. The Igf type 1 receptor preferentially binds Igf1 and is commonly called the Igf1r. This receptor is a member of the tyrosine kinase superfamily of transmembrane receptors. Igf1r is recognized as the major mediator of Igf signaling in mammals. The Igf1r is bisected into two subunits, the α subunit and β subunit. The α and β subunits are first linked by disulfide bonds to form an αβ hemireceptor, which subsequently associates with another αβ hemireceptor to form the mature $\alpha_2\beta_2$ holoreceptor. The extracellular α subunit is responsive to ligand binding, whereas the intracellular portion located within the β subunit contains the substrate-binding site, ATP-binding site and tyrosine kinase activity (Giudice, 1992; Wood et al., 2005) (Fig. 1-3). The Igf type 2 receptor exhibits greater affinity for Igf2 than for Igf1, which is identically to mannose-6-phosphate receptors. The absence of catalytic domains suggests that Igf2r does not stimulate downstream signaling pathways (Tong et al., 1988). In fish, the properties of Igf1rs, including binding specificity and enzyme activities, are similar to those of the mammalian proteins as has been demonstrated in various fish species (Wood et al., 2005).

1.2.2.1.3 Signal transduction and cellular signaling

Activation of the Igf1r through ligand binding is known to trigger several signal transduction pathways. Ligand binding triggers tyrosine phosphorylation of the β domain, leading to the recruitment of multiple endogenous substrates such as insulin-receptor substrate to specific phosphotyrosine docking sites within the β subunit. After the recruitment of insulin-receptor substrate and other substrate molecules, a cascade of additional phosphorylation events involving multiple second-messenger molecules is activated. For instance, the recruitment of SH2 domain-containing proteins can activate the monomeric G protein Ras, which in turn can activate the mitogen-activated protein kinase (MAPK) signaling pathway.

Receptor phosphorylation can also activate the phosphoinositide 3-kinase (PI3-k) pathway and promote phosphorylation of its major component, protein kinase B (PKB/Akt). These signaling pathways ultimately induce alterations in target gene expression (Chitnis et al., 2008; Dupont and Holzenberger, 2003)(Fig. 1-4).

1.2.2.1.4 Expression in ovary

There is some diversity in the localization of Igf expression in the ovary of different species. In rodents, *igf1* mRNA is expressed in the granulosa cells of growing follicles, whereas *igf2* mRNA is located in the granulosa cells of atretic follicles (Zhou et al., 1996). However, the expression level of *igf1* is low in ovine and bovine ovaries (Perks et al., 1995). Instead, *igf2* expression has been detected in the thecal cells of both species, and *igf2* expression levels are high in developing early-stage follicles (Armstrong and Webb, 1997; Perks et al., 1995). In contrast, in the human ovary, granulosa cells are a site of Igf2 rather than Igf1 gene expression (el-Roeiy et al., 1993; Zhou and Bondy, 1993). These results suggest that in humans, Igf2 is the primary Igf, whereas in rodents, Igf1 plays the larger role in the control of reproductive functions. Moreover, the expression of *igf1r* is increased during the early growth of follicles in sheep as well as in cattle (Monget et al., 1989; Wandji et al., 1992). In ovine follicles, the *igf1r* transcript is present in both the theca and granulosa layers, but the concentration of *igf1r* mRNA decreases with increased follicle diameter (Perks et al., 1995). The *igf1r* mRNA is present in human granulosa and theca layers (Poretsky et al., 1999).

Igf1 peptide and/or mRNA has been detected in granulosa cells in different stage of follicles in gilthead seabream (Perrot et al., 2000), red seabream (Kagawa et al., 1995) and tilapia (Berishvili et al., 2006; Schmid et al., 1999a). In both gilthead seabream and tilapia, *igf1* mRNA was observed infrequently in oocytes at later stages of development (Berishvili et al., 2006). In contrast to *igf1*, both *igf2* mRNA and

peptide were detected only in the granulosa cells of the late follicle stages in tilapia (Schmid et al., 1999a).

1.2.2.1.5 Actions on steroidogenesis

In the ovary of mammals, both Igf1 and Igf2 are believed to exert effects by synergizing with FSH and LH in their effects on many aspects of ovary development including steroidogenesis in granulosa and theca cells, respectively (Khamsi et al., 2001). In porcine granulosa cells, synergism between Igfs and gonadotropins has been observed with regard to genes encoding proteins responsible for the uptake of cholesterol substrate and the induced expression of the receptor of low-density lipoprotein, StAR and P450 cytochrome side-chain cleavage (P450scc). Sekar and colleagues demonstrated that StAR mRNA expression could not be induced by LH and Igfs individually and that LH alone minimally stimulated P450scc and LDL-R mRNA expression. However, the expression of these genes was significantly up-regulated by LH in combination with Igfs (Sekar et al., 2000). In rodent granulosa cells, the effects of FSH and Igf on the expression of steroidogenic enzyme are specific. A synergistic action with Igf1 is required for FSH induction of P450scc expression (Eimerl and Orly, 2002). Evidence from different species of fishes indicates common and distinct actions of Igf1 on ovarian steroidogenesis. It has been reported that Igf1 exerts no effects on steroidogenesis in ovarian follicles of mummichog (Negatu et al., 1998). However, Igf1 in the white perch increases testosterone and E2 production but decreases the production of MIH in ovary fragments (Weber et al., 2007). In coho salmon, Igf1 up-regulates E2 and MIH production by the granulosa cells but inhibits the production of testosterone and 17α-hydroxyprogesterone by the theca cells in preovulatory ovary (Maestro et al., 1997). Similarly, Igf1 increases E2 and MIH production and decreases testosterone levels in the culture medium of ovarian fragments from striped bass (Weber and

Sullivan, 2000). In red seabream, Igf stimulates both aromatase activity and P450 aromatase gene expression in ovarian follicles and likely increases the responsiveness of E2 production to LH through enhancing the expression of the LH receptor (Kagawa et al., 2003). In contrast, in trout, Igf1 suppresses the effects of LH on steroid production by inhibiting the activity of cAMP-dependent protein kinase (Mendez et al., 2005). Therefore, Igf1 is highly involved in fish ovarian steroidogenesis.

1.2.2.1.6 Actions on folliculogenesis

The involvement of the Igf system in intraovarian regulation of folliculogenesis has been extensively studied in mammals. In rhesus monkeys, Igf1 and its receptor may mediate the primordial-to-primary follicle transition, as androgen treatment of females resulted in a significant increase in the number of primary follicles, concomitant with a significant increase in the expression of *igf1* and *igf1r* mRNA in primordial follicles (Vendola et al., 1999). However, the available information from most mammals indicates that Igfs are not required for the growth of early follicles or for their gonadotropin-independent development. Fortune and colleagues reported that in cows, the earliest stages of follicular development are regulated, at least in part, by Igf1; however, Igf1 did not stimulate bovine primordial-to-primary follicle transition in a culture of bovine ovarian cortex (Fortune et al., 2004). In the rat, it has been demonstrated that insulin, but not Igf1, promotes the primordial-to-primary follicle transition (Kezele et al., 2002). However, Igfs are involved in the increase in FSH-responsiveness of granulosa cells after the follicles enter into gonadaotropin-dependent stages. Direct evidence was provided using the Igf1 knockout mouse, in which it appears that the follicular population is not affected through the early antral stage. However, the ovaries do not contain any antral follicles and are unable to ovulate, even after treatment with exogenous

gonadotropins, suggesting that Igf1 is essential for antral follicle development (Baker et al., 1996).

Many studies have also indicated that Igf1 stimulates the differentiation or proliferation of granulosa cells, depending on the stage of development of the follicle (Silva et al., 2009). In cattle and mice, Igf1 is essential for increasing the sensitivity of small antral follicles to gonadotropin and is required for their transition into the gonadotropin-dependent follicular stage. Furthermore, Igf1 stimulates the growth of cultured small antral follicles and improves oocyte viability (Walters et al., 2006). In mice, the expression level of Igf2 is low in the ovary, and evidence from knockout mice showed no fertility defects, suggesting that the Igf2 is not likely to be involved in folliculogenesis (DeChiara et al., 1990; Wandji et al., 1998). In contrast, Igf2 is the principal Igf in human ovarian follicles and acting in an autocrine fashion in thecal cells and in a paracrine fashion in granulosa cells (el-Roeiy et al., 1993).

Moreover, the action of Igf1 synergizes with FSH to enhance the proliferation of cultured rat granulosa cells (Giudice, 1992). In the presence of FSH, LH receptor synthesis was stimulated by Igf1 in rat theca-interstitial cells and granulosa cells (Magoffin and Weitsman, 1994; Tsuchiya et al., 1999).

1.2.2.1.7 Identification of Igf3 in teleosts

Two types of Igfs (Igf1 and Igf2) have been identified in various vertebrates. We identified a novel Igf (Igf3) was indentified in several teleost species (Wang et al., 2008). So far, Igf3 has been identified from several fish species including zebrafish, tilapia, medaka and southern catfish, which suggests that Igf3 is widespread among in teleosts. Zebrafish Igf3 is encoded by a gene that is distinct from those that encode for Igf1 and Igf2. Phylogenetic analysis also revealed that Igf3 forms a separate clade distinct from Igf1 and Igf2 (Fig. 1-5). Like the other Igfs, Igf3 contains five domains: B, C, A, D and E (Fig. 1-6). The predicted tertiary protein structure of Igf3 is similar

to that of Igf1 and Igf2 (Fig. 1-7). Interestingly, previous analysis has revealed the gonad-specific expression of Igf3 in tilapia and zebrafish (Wang et al., 2008; Zou et al., 2009).

The B and A domains of Igf have been previously demonstrated to be important for receptor binding and the subsequent biological activity of the growth factor (Bayne et al., 1989; Shooter et al., 1996). Domain B contains two conserved cysteines, both of which form disulfide bonds in Igf1, and three conserved glycines resdues that are important for the tertiary structure of Igf. All of the Igf3 sequences indentified from teleosts possess these essential residues. Together with the similar tertiary structure exhibited by the three types of Igf, these features suggest that certain aspects of the biological functions of Igf3 are akin to those of the other Igfs. On the other hand, Igf1 and Igf2 possess a highly conserved ETLCG motif. This motif is changed to KVRCG, RARCG or RLRCG in Igf3. Although the significance of this difference is not known at the moment, it is likely that Igf3 does possess some specific biological activities of its own. Furthermore, the C domain of Igf1 is involved in maintaining a high affinity for the Igf1r, but the C domain is not required for Igf2r binding. Previously, Wang and colleagues proposed that the diversity in the C domain of Igf3 suggests the presence of a specific receptor for Igf3 (Wang et al., 2008). However, the role of Igf3 in the fish gonad is still largely unknown.

1.2.2.2 Epidermal growth factor family

The epidermal growth factor family includes epidermal growth factor (EGF), heparin-binding EGF-like growth factor, transforming growth factor-α (TGF-α), amphiregulin, epiregulin, epeigen, betacellulin, and other factors (Sirotkin, 2010). Most EGF-family peptides are synthesized as transmembrane precursors that are proteolytically cleaved to release the soluble form of the hormone or function as membrane-anchored hormones in juxtacrine signaling. These factors exert effects through four types of structurally related transmembrane receptors, including ErbB1

(EGFR), ErbB2, ErbB3 and ErbB4 (Lafky et al., 2008). Each of these receptors consists of a cysteine-rich extracellular domain, a membrane-spanning domain, and a large cytoplasmic domain containing a tyrosine kinase domain. Ligand binding stimulates receptor dimerization and tyrosine phosphorylation at several sites that then serve to dock effector proteins and induce physiological responses (Riese and Stern, 1998).

In addition to their potent mitogenic effect in a variety of tissues, the importance of EGFs in the ovary is also drawing attention. The expression of EGF, TGF-α and EGF receptors has been demonstrated in the ovary of mammals (Almahbobi et al., 1998; Fukumatsu et al., 1995; Goritz et al., 1996; Scaramuzzi and Downing, 1995; Scurry et al., 1994), birds (Onagbesan et al., 1994; Van Nassauw et al., 1995; Yao and Bahr, 2001) and fishes (MacDougall and Van Der Kraak, 1998; Pati et al., 1996; Wang and Ge, 2004a). A body of recent studies demonstrates a role for both EGF and such EGF-like growth factors, such as amphiregulin, epiregulin, and betacellulin in the control of ovarian functions. In rodents and humans, the production of these factors is dramatically up-regulated by GnRH and LH in granulosa cells (Motola et al., 2006; Panigone et al., 2008). These results indicate the involvement of the EGF network in mediating the effects of GnRH and gonadotropin on the ovary (Conti et al., 2006; Hsieh et al., 2009).

Stimulatory effects of EGF on the proliferation of ovarian granulosa and theca cells have been demonstrated (May et al., 1992; May et al., 1988; Peddie et al., 1994), along with increased release of progesterone, cAMP and cGMP and regulation of the production of E2. EGF also stimulates the proliferation of cumulus oophorus cells (Lorenzo et al., 1994) and inhibits their apoptosis (Sirotkin, 2010). Furthermore, many studies have demonstrated that EGF activates the oocyte maturation in a variety of mammalian species (Dekel and Sherizly, 1985; Downs, 1989; Grupen et al., 1997; Lonergan et al., 1996; Lorenzo et al., 1993). Although the role of EGF has

been extensively studied in the ovary of mammals, there is little information about EGF and its receptor in lower vertebrates. In fish, studies have implicated EGF in the regulation of vitellogenic follicle DNA synthesis (Kumar Srivastava and Van Der Kraak, 1995), follicle survival (Janz and Van Der Kraak, 1997), follicle steroidogenesis (MacDougall and Van Der Kraak, 1998) and oocyte maturation (Wang and Ge, 2004a).

1.2.2.3 Transforming growth factor-β superfamily

The members of the transforming growth factor-β (TGF-β) superfamily and their roles as local regulators of ovarian function and fertility have been demonstrated in recent years (Juengel and McNatty, 2005). The TGF-β superfamily consists of three major families that include the TGF-β family, the inhibin/activin family and the bone morphogenetic protein (BMP) family (Chang et al., 2002; Massague, 1990).

1.2.2.3.1 Transforming growth factor-β family

In mammals, the TGF-β subfamily is comprised of TGF-β1, TGF-β2 and TGF-β3. The production of these factors has been observed in granulosa and thecal cells as well as in oocytes in several species (Bristol and Woodruff, 2004; Chegini and Flanders, 1992; Juneja et al., 1996; Nilsson et al., 2003; Schmid et al., 1994; Teerds and Dorrington, 1992). The ALK5 (also known as TGF-βr I) and TGF-βR II were believed to be the receptors of the TGF-βs. Expression of ALK5 mRNA and protein has been demonstrated in the ovary of many species (Juengel et al., 2004; Juneja et al., 1996; Qu et al., 2000; Roy and Kole, 1998). The receptor-mediated second messenger system for TGF-βs, known as the Smad2/Smad3 pathway, appears to be present in granulosa and theca cells as well as in the oocytes of follicles throughout all stages of folliculogenesis. Both Smad2 and 3 proteins have been localized to the oocytes, granulosa and theca cells of both preantral and antral follicles in rats

(Drummond et al., 2002; Xu et al., 2002) and mice (Gueripel et al., 2004).

In rodents, TGF-βs can stimulate the proliferation of granulosa cells (Dorrington et al., 1988; Roy, 1993; Saragueta et al., 2002), however, in other species such as cattle (Skinner et al., 1987), sheep (Juengel et al., 2004) and pigs (Gangrade and May, 1990), TGF-βs have only a weak stimulatory ability or even inhibitory effects on the proliferation of granulosa cells. Similarly, TGF-βs stimulate progesterone synthesis in rodent granulosa cells (Dodson and Schomberg, 1987; Hutchinson et al., 1987; Knecht et al., 1987) whereas inhibitory effects are observed in granulosa cells in cattle (Gilchrist et al., 2003), sheep (Fabre et al., 2003) and pigs (Mondschein et al., 1988). The effects of TGF-βs on theca/interstitial cells with respect to steroidogenesis appear similar among these species. TGF-βs likely suppress steroidogenesis in theca cells from most species (Juengel and McNatty, 2005). For example, TGF-βs suppressed LH or forskolin-stimulated androgen production in theca cells from different species including rat (Hernandez et al., 1990), pig (Caubo et al., 1989), cow (Demeter-Arlotto et al., 1993) and human (Attia et al., 2000).

1.2.2.3.2 Activin/inhibin family

The activin/inhibin system includes activin, inhibin, activin receptors, and the activin-binding protein follistatin (Knight and Glister, 2001). Inhibins, activin and follistatin were first purified from the ovarian follicular fluid based on their ability to modulate the secretion of FSH from the pituitary; inhibin and follistatin suppress FSH secretion, whereas activin enhances FSH secretion (Ling et al., 1986a; Ling et al., 1986b; Miyamoto et al., 1985; Rivier et al., 1985; Robertson et al., 1987; Ueno et al., 1987; Vale et al., 1986). Subsequently, increasing evidence has demonstrated that activins are involved in many ovarian cell functions.

Both inhibin and activin belong to the TGF-β superfamily. Inhibins (inhibin A and inhibin B) are heterodimers of a unique α subunit linked to either a βA or βB subunit

to generate inhibin A (α-βA) or inhibin B (β-βB). Activins are either hetero- or homodimers of the β subunits (βAβA, βBβB, βAβB) forming activin A, activin B and activin AB (Ying, 1988). Follistatin is structurally unrelated to the TGF-β superfamily but is linked functionally through its role as a high-affinity binding protein for activins (Phillips and de Kretser, 1998). The biological functions of activins are mediated by two classes of activin receptors, activin type I and type II receptor (ActRI and ActRII). ActRII has two subtypes called ActRIIA and ActRIIB (Attisano et al., 1992).

Many studies have demonstrated that activins upregulate FSH receptor or FSH-induced LH receptor levels as well as inhibin production in rat granulosa cells (Findlay, 1993; Kishi et al., 1998; Minegishi et al., 1999; Minegishi et al., 1995; Nakamura et al., 1995). Activins regulate basal and gonadotropin-induced estradiol and progesterone production in cultured granulosa cells, and in undifferentiated rat and primate granulosa cells, activins enhance FSH-induced estradiol and progesterone production (Miro and Hillier, 1992; Miro et al., 1991; Xiao et al., 1990). However, the inhibitory effects were observed in the fully differentiated granulosa cells from the humans, rats and cattle (Miro and Hillier, 1992; Miro et al., 1991; Rabinovici et al., 1992; Shukovski and Findlay, 1990). Moreover, effects of activins on follicle growth has been found in immature animals (Liu et al., 1998), and activins also promote the reorganization of follicular structures in the presence of FSH in vitro (Li et al., 1995). Thus, these results indicate that the contribution of activins to the increasing responsiveness of developing follicles to gonadotropins (Ethier and Findlay, 2001; Knight and Glister, 2001).

1.2.2.3.3 Bone morphogenetic protein family

The BMP family consists of several bone morphogenetic proteins (identified as BMP-2 through BMP-15) and growth differentiating factor (GDF) ligands, receptors

and binding proteins. Two members of this family, GDF-9 and BMP-15 (also known as GDF-9B), have received increasing attention, as both of them have been found to be selectively expressed by oocytes in rodents, cattle and sheep (Jaatinen et al., 1999; Knight and Glister, 2006; McGrath et al., 1995; McNatty et al., 2001). Recently, Liu and Ge also observed the specific expression of GDF-9 mRNA in the oocytes of zebrafish (Liu and Ge, 2007). Direct evidence from GDP-9 null mice indicateds GDF-9 is essential for folliculogenesis (Dong et al., 1996). This conclusion is also supported by other studies both *in vivo* and *in vitro* in rats (Nilsson and Skinner, 2002; Vitt et al., 2000). However, there is some controversy regarding whether GDF-9 affects the primordial-to-primary follicle transition (Knight and Glister, 2006).

BMP-15 is highly similar to GDF-9 at the level of nucleotide and protein sequences, and it has been shown to stimulate downstream pathways through BMPRIB (or ALK6) as a type I receptor and BMPRII as a type II receptor (Moore et al., 2003). Both BMP-15 and GDF-9 bind to the type II receptor, but each appears to activate a different downstream Smad signaling pathways (Kaivo-Oja et al., 2003; Roh et al., 2003). BMP-15 has been shown to play integral roles in the regulation of ovarian folliculogenesis and follicular function (Shimasaki et al., 2003). The importance of BMP-15 in the sheep ovary was exemplified by the demonstration that BMP-15 mutation causes an increase in the ovulation rate in heterozygotes (Galloway et al., 2000). Mice with targeted deletions in the BMP-15 gene show decreased ovulation and fertilization rates, further demonstrating the importance of BMP-15 in ovarian function (Yan et al., 2001). Peng and colleagues also demonstrated in zebrafish that BMP-15 modulates follicular growth and prevents premature oocyte maturation in zebrafish (Clelland et al., 2006; Clelland et al., 2007; Peng et al., 2009). The involvement of other members of the BMP family including BMP-4, BMP-6 and BMP-7 in ovarian development has also been demonstrated for example, null mutations in BMP-7 or BMP-4 in mice are peri-natally lethal,

precluding comparison of postnatal ovarian follicle development (Knight and Glister, 2006).

1.3 Regulation of oocyte maturation

Vertebrate oocytes, which grow within the ovarian follicles, are arrested at the dipotene stage of the first meiotic division. Upon the stimulation of with FSH and LH, developed oocytes resume meiosis, followed by the completion of meiosis I and another arrest in metaphase of meiosis II. Oocytes arrested in meiosis II are ready for ovulation and subsequent fertilization (Fig. 1-2). Morphologically, the resumption of meiosis is characterized by the disappearance of the nuclear membrane of the oocyte in a process also called GVBD, accompanied by chromosome condensation, assembly of the meiotic spindle and formation of the first polar body (Nagahama and Yamashita, 2008; Zhang et al., 2007). In vertebrates, the regulation of oocyte maturation involves both pituitary gonadotropins and local intraovarian factors.

1.3.1 Gonadaotropins

The oocyte maturation is triggered by gonadotropin surge was well demonstrated. LH is the primary stimulus for oocyte maturation in vertebrate oocytes. In mice and rat, GVBD occurs approximately two or four hours after exposure of isolated follicles to LH (Park et al., 2004; Tsafriri, 1985), or after injection of the animal with the LH receptor agonist, human chorionic gonadotropin (hCG) (Larsen et al., 1986; Schultz et al., 1983). Pharmacological blockade of the LH surge with Nembutal, a GnRH antagonist, or hypophsectomy prevents oocyte maturation, while injection of hCG or LH can effectively resume oocyte meiosis (Zelinski-Wooten et al., 1992; Zelinski-Wooten et al., 1991). In frogs and fish, gonadotropin has also been demonstrated to be the major regulator of oocyte maturation (Nagahama et al., 1995; Wasserman and Masui, 1974).

The LH receptor is a well-characterized G-protein-coupled receptor, which are not found on the oocyte itself, but rather rather on the somtic cells surrounding the oocyte; both the outer layers of mural granulosa cells and the inner layer of thec cells have these receptors (Amsterdam et al., 1975), so the mechanisms by which LH stimulates oocyte maturation is indirect. Most studies of LH stimulation of oocyte maturation have focus on the action of LH signaling in the mural granulosa cells (Richards et al. 2002). It was established that LH binding to LH receptor causes the production of cAMP in the granulosa cells (Conti et al.; 2002; Dekel, 1988; Dekel et al., 1988; Schultz et al., 1983; Tsafriri, 1985), increasing cAMP in the follicle, using forskolin (Dekel and Sherizly, 1983) or a PDE4 inhibitor (Tsafriri et al., 1996), is sufficient to cause oocyte maturation in the oocyte. Furthermore, lines of evidence also suggest that the paracrine/autocrine factors produced by the surrounding somatic cells are involved LH signaling on oocyte maturation (Danforth, 1995; Ge, 2005; Hillier, 2001).

1.3.2 Maturation inducing-hormone (MIH)

How the gonadotropin action on the follicular somatic cells translates to a change in signaling molecules into the oocyte to initiate oocyte maturation remains to be elucidated. However, the notion that the action of gonadotropin on oocyte maturation is mediated by the production of MIH is widely accepted in lower vertebrates (Maller, 1985a; Maller, 1985b; Nagahama and Yamashita, 2008; Nagahama et al., 1995). MIH is a steroid that interacts with a membrane-bound receptor on the oocyte surface. Since the first identification of 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) as an MIH in the amago salmon (Nagahama and Adachi, 1985), this steroid has been identified in many other fishes, such as killifish (Petrino et al., 1993), yellowtail (Rahman et al., 2002) and chub mackerel (Matsuyama et al., 2005). A derivative of 17α,20β-DP (17α,20β,21-trihydroxy-4-pregnen-3-one (20β-S)) is also believed to be

a natural MIH in several fishes, such as spotted seatrout (Trant and Thomas, 1989) and striped bass (King et al., 1994). A complex series of enzymes are responsible for the biosynthesis of sex steroids in fish gonads. This process is illustrated in Fig. 1-8. Initially the steroid precursor pregnenolone is synthesized through side-chain-cleavage of cholesterol by cholesterol side-chain cleavage cytochrome P450 (P450scc). Pregnenolone is converted to progesterone by 3β-HSD or to 17α-hydroxypregnenolone by 17α-hydroxylase activity of cytochrome P450c17. Progesterone and 17α-hydroxypregnenolone are then converted to 17α-hydroxyprogesteone which is followed by the production of 17α,20β-DP by 20β-hydroxysteroid dehydrogenase (20β-HSD) (Nagahama and Yamashita, 2008).

Two-cell type model Studies on the ovarian follicles of salmonid fishes led to the proposal of two-cell type model (Fig. 1-8) (Nagahama, 1994). Like other vertebrates, two major cell layers including an outer thecal cell layer and an inner granulosa cell layer are found in fish ovarian follicles. In two-cell type model, the thecal cell layer produces 17α-hydroxyprogesteone that transverses the basal lamina and is converted to 17α,20β-DP in the granulosa cell layer where gonadaotropin acts to enhance the activity of 20β-HSD, which is the key enzyme involved in the conversion of 17α-hydroxypregnenolone to 17α,20β-DP (Nagahama and Yamashita, 2008).

MIH receptor The presence of the MIH receptor on the fish oocyte surface had long been suggest, but its biochemical entity was not characterized until the recent breakthrough made by Zhu and colleagues in 2003 (Zhu et al., 2003a; Zhu et al., 2003b), they reported a distinct family of membrane-bound progestin receptors (mPR). mPR can be classified into three subtypes, mPRα, mPRβ and mPRγ, all of them are G-protein-coupled receptor and seem to mediate non-genomic actions of steroids. Recently, the presence of mPR has been reported in other fish species,

including Atlantic croaker, puffer fish, catfish, goldfish and rainbow trout (Braun and Thomas, 2004; Kazeto et al., 2005a; Kazeto et al., 2005b; Thomas et al., 2004; Tokumoto et al., 2006).

1.3.3 Maturation promoting factor (MPF)

The MIH signal received on the oocyte surface is transduced to the cytoplasm, finally resulting in the formation and activation of MPF. MPF was first purified from mature oocytes of the African clawed frog *Xenopus laevis* as a 200 kDa complex containing 32 and 45 kDa proteins (Lohka et al., 1988). The 32 kDa protein is a *Xenopus* homolog of cdc2, a serine/threonine protein kinase , and the 45 kDa protein is a *Xenopus* counterpart of Cyclin B. Later, MPF was also purified from mature oocyte of starfish (Labbe et al., 1989a; Labbe et al., 1989b) and the carp (Yamashita et al., 1992). In contrast to gonadatropin and MIH, the action of MPF is universal among species (Babin et al., 2007).

In fish, MPF has been highly purified from eggs of several species, such as the carp, catfish and perch, where it exhibits the universal molecular structure of a complex of Cdc2 and cyclin B (Fig. 1-9) (Balamurugan and Haider, 1998; Basu et al., 2004; Yamashita et al., 1992). Unlike the case of starfish, mouse and *Xenopus* which have pre-MPF in oocytes, pre-MPF is absent in immature oocytes of fish (Verlhac and Villeneuve, 2009). Cyclin B is not expressed at the protein level in the prophase-blocked oocytes of many fish species including goldfish, carp, catfish, zebrafish and lamprey (Verlhac and Villeneuve, 2009). All Cdc2 molecules exist as a monomer, and the protein content of Cdc2 is constant during oocyte maturation. In response to the cellular signal, cyclin B is synthesized from its stored mRNA and binds to pre-existing Cdc2 (Hirai et al., 1992). It has been well demonstrated that cyclin B is the only protein whose translation is necessary and sufficient to promote MPF activation in goldfish oocyte (Katsu et al., 1993). However, it is still not clear

how the signal received on the oocyte surface is transmitted to cytoplasm and lead to the activation of MPF. Some evidences suggested that a decrease in the activity of protein kinase A caused by the decrease in intracellular cAMP level plays an important role in activation of MPF and induction of oocyte maturation in fish (Babin et al., 2007).

1.3.4 Growth factors

The regulation of oocyte maturation in all studied vertebrates, from fish to mammals, involves a number of intraovarian growth factors besides gonadotropin and maturation-inducing hormone. Factors such as Igfs, Egf, and members of the TGF-β superfamily have also been demonstrated to play a role in regulating oocyte maturation.

Either LH or FSH increases the secretion of Igf1 in porcine granulosa cells (Hsu and Hammond, 1987). Exposure to FSH also increases the ability of Igf1 to bind rat granulosa cells (Adashi et al., 1986a). Igf1 augments the ability of FSH to promote binding of LH by granulosa cells (Adashi et al., 1985a; Adashi et al., 1985b; Adashi et al., 1986b), providing a potential mechanism for facilitation of LH action on these cells. Furthermore, Igf1 also enhances the activity of FSH on the secretion of both progesterone and oestradiol in rat granulosa cell (Adashi et al., 1985c). The effects of Igf1 on oocyte maturation have been demonstrated for many vertebrate species, including rabbit (Lorenzo et al., 1996), pig (Grupen et al., 1997), sheep (Guler et al., 2000), and cow (Sakaguchi et al., 2000). In fish, the role of Igf1 on oocyte maturation has been demonstrated in red seabream (Kagawa et al., 1994).

Activin and inhibin have also been demonstrated to stimulate the oocyte maturation in many vertebrate species, including rat (Itoh et al., 1990), monkey (Alak et al., 1996), cow (Stock et al., 1997), human (Alak et al., 1998) and zebrafish (Pang and Ge, 1999; Wu et al., 2000). Pang and Ge also provided evidence for the

involvement of the activin system in oocyte maturation in zebrafish. The expression of ovarian activin can be up-regulated by gonadotropin (Pang and Ge, 2002b). The action of gonadotropin can be significantly blocked by the activin-specific binding protein, follistatin, suggesting a role for the activin system in the gonadotropin pathway controlling oocyte maturation (Pang and Ge, 1999).

In recent years, the importance of EGF on oocyte maturation has also attracted considerable attenctions (Conti et al., 2006; Hsieh et al., 2009; Park et al., 2004; Zamah et al., 2010). A role of EGF in the regulation of oocyte maturation has been demonstrated in the mammals as well as in fish (Peyton and Thomas, 2011; Van Der Kraak and Lister, 2011; Wang and Ge, 2003b; Wang and Ge, 2004a).

1.4 Objectives and outlines of the present study

It is well established that the development and functions of the ovary are regulated by pituitary gonadotropins (follicle-stimulating hormone and luteinizing hormone) in vertebrates. There is increasing evidence demonstrating the importance of locally produced growth factors, such as members of the Igf family, Egf family and Tgf-β superfamily, in the regulation of ovarian function by gonadotropins. Most of our knowledge about the role of these factors in ovarian development and function comes from studies in mammals. The role and regulation of these intraovarian factors in fish are still largely unknown.

In addition to Igf1 and Igf2 found in vertebrates, our lab has identified a novel gonad-specific Igf (Igf3) in teleosts. In this thesis, efforts were made to understand the potential role of the Igf system in ovary and embryo development, and attention was particularly focused on Igf3. Zebrafish is not only an excellent experimental model for analyzing the maturation, function, and regulation of the ovary but is also the ideal vertebrate models for developmental studies.

Although Igf3 has been indentified in several fish species, the information about

its functions in fish is lacking. The gonad-specific expression of Igf3 strongly implies the role of Igf3 in the reproduction. The studies described in **Chapter 2** were set out to reveal the detailed gene expression patterns of Igf3 in gonads. Two spliced forms of *igf3* were identified in zebrafish, *igf3 tv1* is highly expressed in the gonad and *igf3 tv2* is predominantly expressed during embryogenesis. Because the expression of Igf3 mRNA and protein is correlated with LHR expression during folliculogenesis, the regulation of Igf3 by gonadotropin and cAMP was then assessed in intact ovarian follicles. Recombinant zebrafish Igf3 was successfully expressed and purified from a bacterial system and the effects of Igf3 on oocyte maturation were then demonstrated.

Another objective of this study was to investigate the potential downstream signaling pathways involved in Igf3-induced oocyte maturation in zebrafish. The studies described in **Chapter 3** aimed to address the requirement for steroidogenesis in Igf3-induced oocyte maturation; that is, whether Igf3 stimulates oocyte maturation through Igf1r and whether the major downstream factors of the Igf pathway (including PI3-k, PDE3 and MAPK) are necessary for Igf3-induced oocyte maturation.

Four Igf ligands, including Igf1, Igf2a, Igf2b and Igf3, have been identified in zebrafish, and all of them are expressed in the ovary. The expression profiles of the four Igfs during folliculogenesis and their regulation by gonadotropin and cAMP in primary cultured follicle cells as well as full grown stage follicles was described in **Chapter 4**.

Furthermore, the studies described in **Chapter 5** aimed to reveal the temporal and spatial gene expression patterns of all four Igf ligands (*igf1*, *igf2a*, *igf2b* and *igf3*), two *igf* receptors (*igf1ra* and *igf1rb*) in the early development of zebrafish. This information provides the direct direct information relevant to the study of Igf signaling in zebrafish and also yields valuable insight into the function of Igf system

in developmental regulation and into the evolution of growth regulatory mechanisms in vertebrates.

In **Chapter 6**, the findings described in this thesis are summarized and discussed.

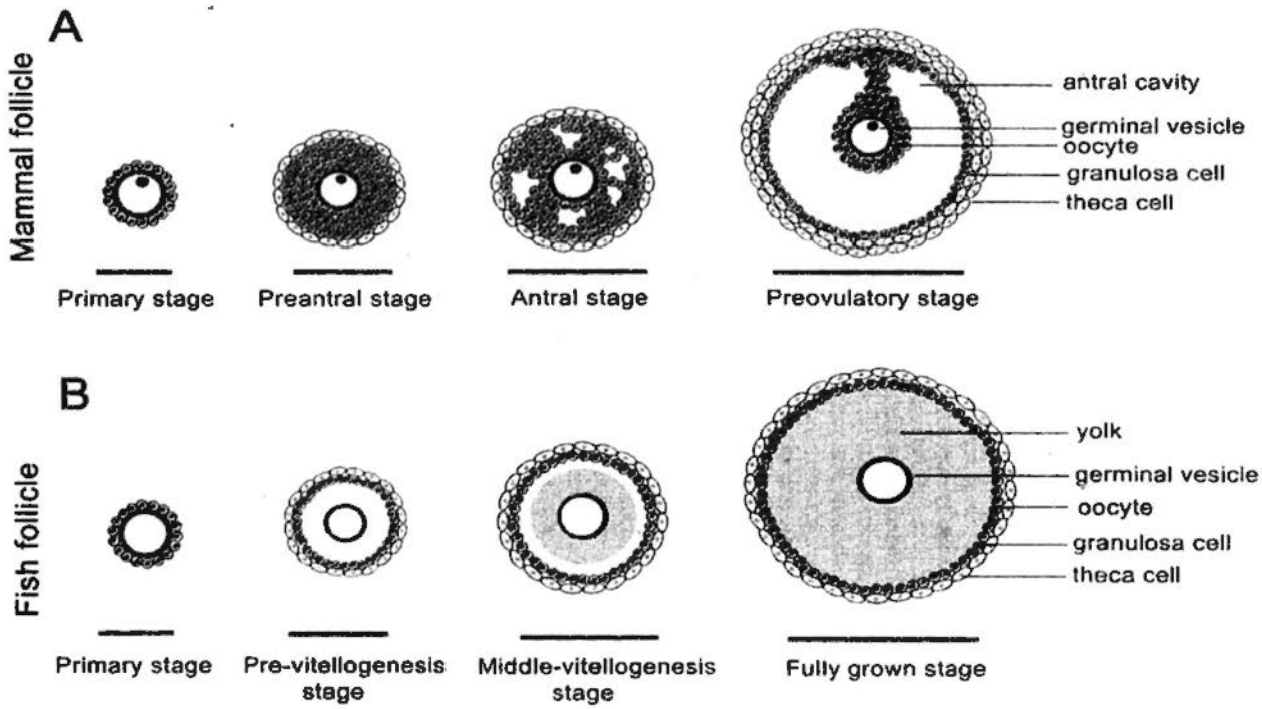

Fig. 1-1 The structure of different major follicle stages in mammals (A) and fish (B).

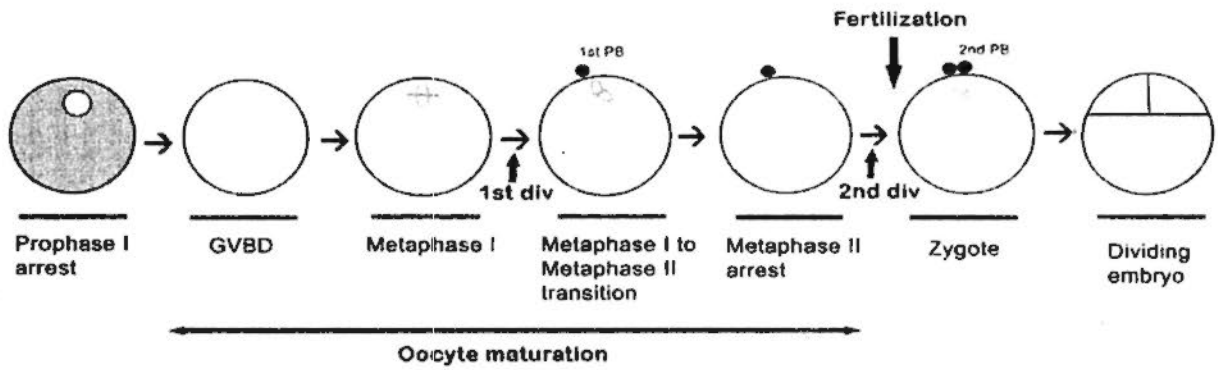

Fig. 1-2 Schematic of oocyte maturation process.

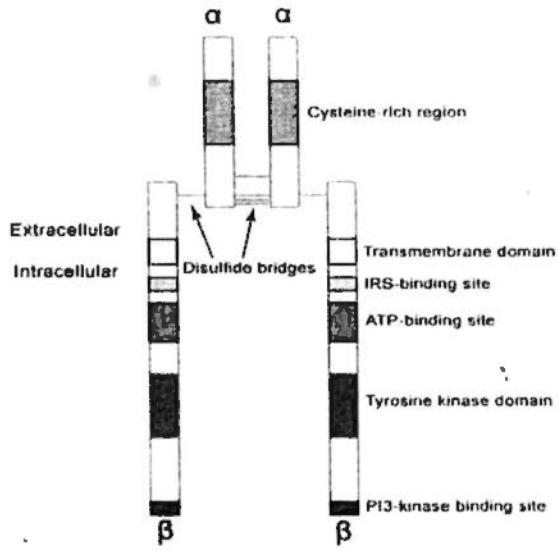

Fig. 1-3 The stucuture of heterotetrameric type 1 Igf receptor.

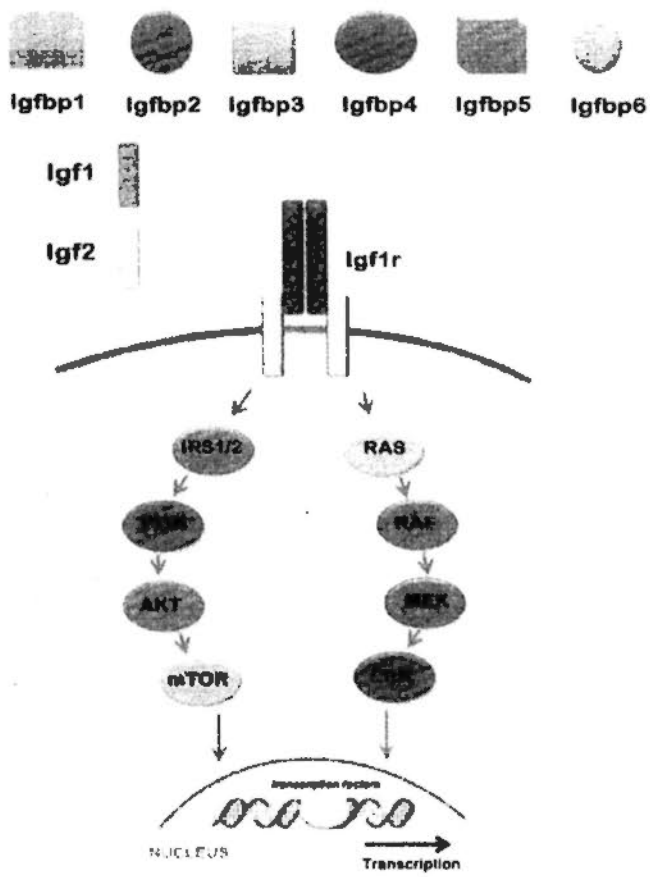

Fig. 1-4 Diagramic model of Igf system and signal transduction.

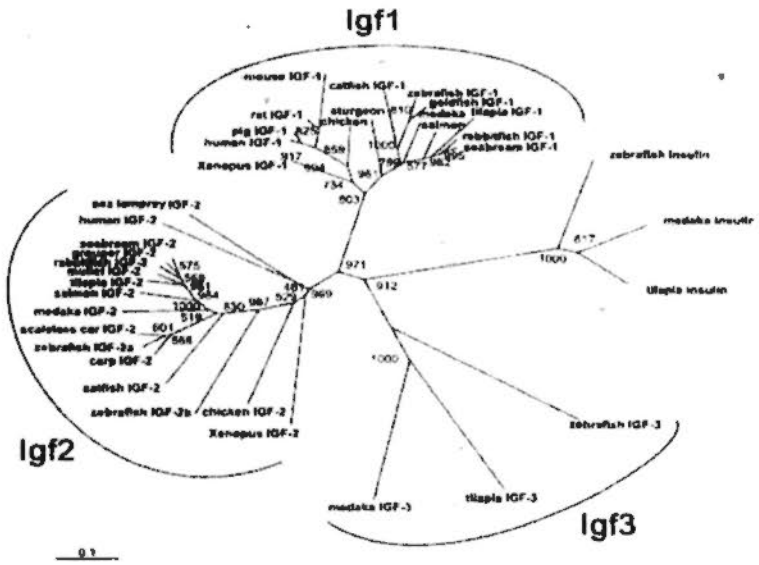

Fig. 1-5 Phylogenetic analysis of protein sequences of Igfs from different vertebrates

	B	C	A	D	E
Igf1	29	12	21	8	47
	(100)	(100)	(100)	(100)	(100)
Igf2a	32	11	21	6	97
	(79.3)	(54.5)	(71.4)	(50.0)	(14.9)
Igf2b	27	10	21	6	93
	(77.8)	(30.0)	(71.4)	(50.0)	(14.9)
Igf3	28	12	21	6	78
	(64.3)	(33.3)	(61.9)	(33.3)	(14.9)

Fig. 1-6 Comparison of domains of the four Igf subtypes in zebrafish.

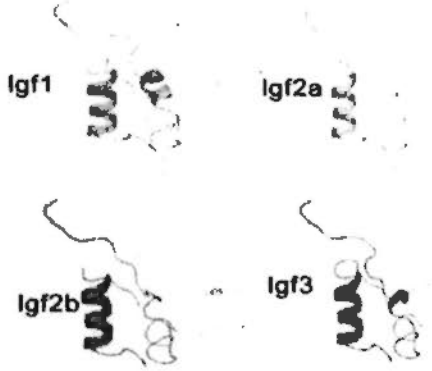

Fig. 1-7 Predicted protein structures of the four Igf subtypes in zebrafish.

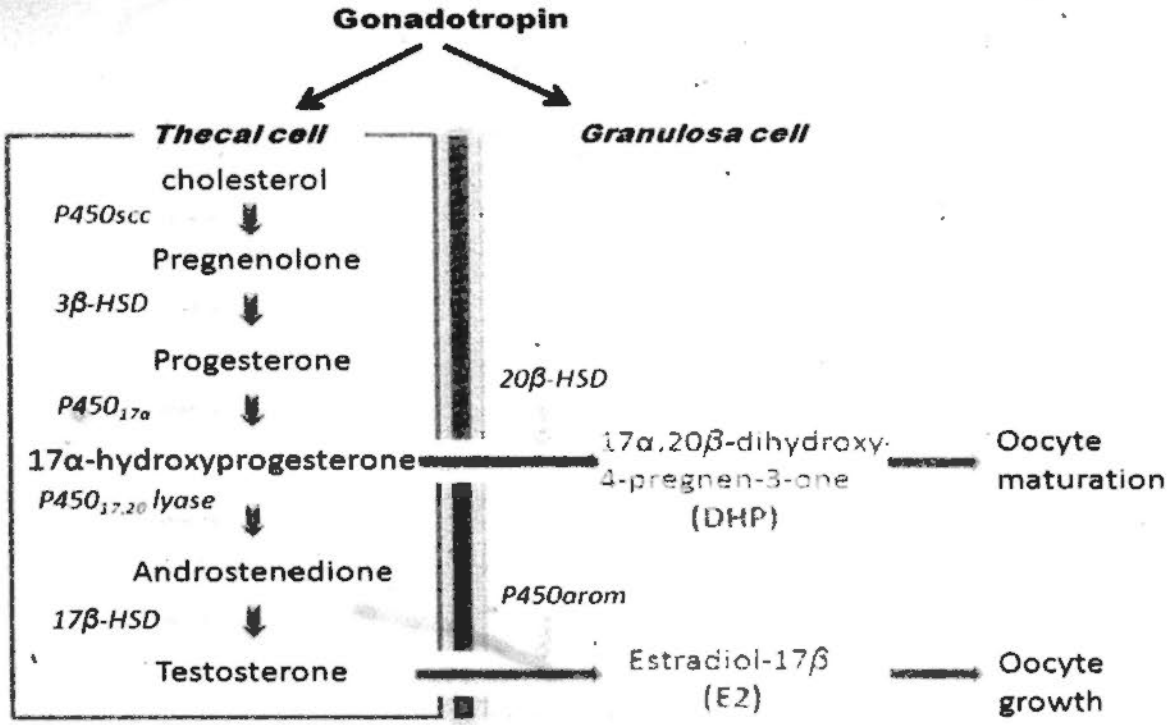

Fig. 1-8 Two-cell type model for the production of estradiol and DHP during fish oocyte growth and maturation.

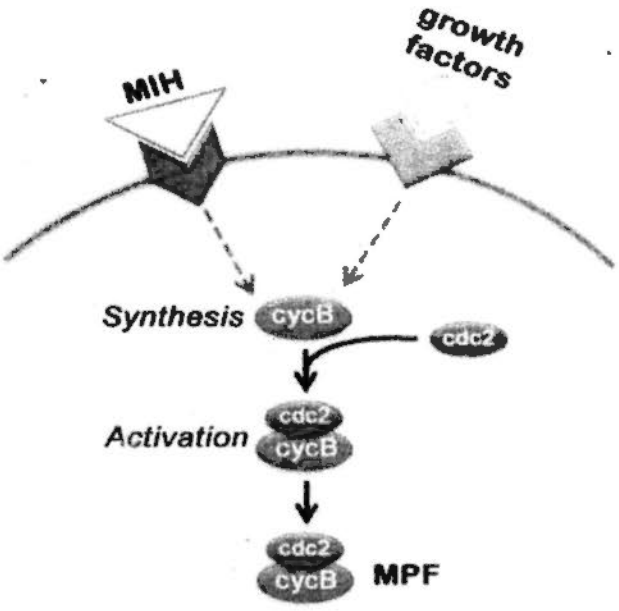

Fig. 1-9 The formation of MPF in fish oocyte.

Chapter 2 Insulin-like growth factor 3 is involved in oocyte maturation of zebrafish

2.1 Introduction

The insulin-like growth factor (Igf) system regulates a variety of cellular processes including growth, proliferation, survival, migration, and differentiation (Wood et al., 2005). Since the identification of two Igf peptides (Igf1 and Igf2) (Rinderknecht and Humbel, 1978a; Rinderknecht and Humbel, 1978b), increasing attention has been paid to their functions on the ovary. Igf1 and Igf2 have been found to localize in the ovary of a variety of vertebrate species (el-Roeiy et al., 1993; Kagawa et al., 1995; Oliver et al., 1989; Perks et al., 1995; Quesnel, 1999). In addition, the expression of Igf binding proteins and Igf receptors has also been demonstrated in the ovary (Armstrong et al., 2002; Wandji et al., 1998; Zhou et al., 1991). Previous studies on the Igf system in the ovary of different species suggested that the regulation of ovarian function by the Igf system is conserved throughout animal evolution (Chang et al., 2002; Lorenzo et al., 1994; Lorenzo et al., 1996; Sakaguchi et al., 2000; Sirotkin et al., 2000; Xia et al., 1994; Yoshimura et al., 1996a).

Previous reports have demonstrated the expression of the Igf system including the Igf peptides, Igf receptors, and Igf binding proteins in fish gonads. Igf1 mRNA and protein have been demonstrated to be present in the ovary of several fish species including seabream (Kagawa et al., 1995), tilapia (Berishvili et al., 2006) and sturgeon (Wuertz et al., 2007). Functional analysis revealed that Igf1 can induce meiotic resumption in common carp (Mukhejee et al., 2006) and striped bass (Weber and Sullivan, 2000), and can also induce oocyte maturation competence in white bass (Weber and Sullivan, 2000). Igf2 mRNA and protein have also been studied in the ovary of tilapia (Schmid et al., 1999b) and seabream (Radaelli et al., 2003). Igf2 can

induce oocyte maturation in striped bass (Weber and Sullivan, 2000). These results indicate the important role of the Igf system in fish ovarian functions. In view of the discovery of a gonad-specific Igf subtype (Igf3) in fish (Wang et al., 2008) and the fact that Igf1 and Igf2 are ubiquitously expressed in most tissues, it is envisaged that Igf3 might play a more important role in fish reproduction.

Apart from its gonad-specific expression (Berishvili et al., 2010; Wang et al., 2008), a recent study also reported the presence of Igf3 in the zebrafish embryo (Zou et al., 2009). The importance of this novel Igf in teleost reproduction and development warrants detailed study on its gene expression and regulation. In addition, it is not known how Igf3 exerts it action in the gonad. The elucidation of the physiological functions of Igf3 would lead to a fuller understanding of the role of the Igf system in fish reproduction.

The present study was aimed to study the temporal and spatial gene expression patterns of Igf3 on the mRNA and protein level in ovary. In addition, functional studies including regulation of gene expression by gonadotropin and actions on oocyte maturation by this gene were also investigated.

2.2 Materials and Methods

2.2.1 Animals

Zebrafish (*Danio rerio*) were purchased locally. Fish were maintained under 14-h light/10-h dark cycles, in circulating freshwater aquaria at 26-28°C. Fish were fed twice daily with newly hatched brine shrimp (Brine Shrimp Direct, USA). Fish experiments were conducted in accordance to the regulations of the Animal Experimentation Ethics Committee of The Chinese University of Hong Kong. The animals were anesthetized in tricaine before handling.

2.2.2 Chemicals

AR grade chemicals and human chorionic gonadotropin (hCG) were obtained from Sigma-Aldrich (USA), culture media from Gibco (USA), and enzymes from Promega (USA) unless otherwise stated.

2.2.3 Cell culture and transient transfection

The human embryonic kidney 293 cell line (HEK 293, ATCC number: CRL-1573) and mouse spermatogonial cell line (GC-1, ATCC number: CRL-2053) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/ml) and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere containing 5% CO_2. The zebrafish liver cell line (ZFL, ATCC number: CRL-2643) was maintained at 28°C with complete growth medium as recommended by ATCC. Transient transfection was carried out using Lipofectamine transfection reagent (Invitrogen, USA).

2.2.4 Rapid amplification of cDNA ends analysis

Total RNAs from embryos of 48 h post-fertilization (hpf) and adult ovary of zebrafish were prepared by the RNeasy Mini Kit (Qiagen, USA) according to the manufacturer's instructions. All the RNAs were digested by Rnase-free DNase I and purified. SMART cDNAs were reversely transcribed from the RNAs using the SMART cDNA Synthesis Kit (Clontech, USA). 5'RACE and 3'RACE were performed using specific primers (Table 1) and the amplification products were cloned into the T/A cloning vector pGEM-T Easy (Promega, USA) for direct sequencing.

2.2.5 RNA isolation and RT-PCR

Total RNA samples were isolated from the tissues, ovarian follicles, embryos and

fries of zebrafish, using TRIzol Reagent (Invitrogen, USA). The amount and purity of the RNA were determined by spectrophotometry. RT-PCR was performed as previously described (Wang et al., 2008). All primers used in this study are listed in Table 2-1. Annealing temperature for PCR ranges from 55 to 65°C, depending on the primer set used. For real-time PCR, the standards for *igf3* and *gapdh* (glyceraldehyde-3-phosphate dehydrogenase) were prepared by amplification of cDNA fragments with the specific primers (Table 1). The amplicons were purified by the PCR Purification Kit (Qiagen, USA) and cloned into pCR-4 vector. These plasmids were used to construct the standard curves in the real-time PCR assays. Real-time PCR was carried out on an ABI Real-Time PCR Fast System (ABI, USA) using the SYBR Green PCR Master Mix Kit (ABI, USA). For semiquantitative RT-PCR analysis, PCR was carried out on a Thermal Cycler 9600 (Eppendorf, GER). The number of cycles used were: 32 for *igf3*; 35 for *gdf9* (growth differentiation factor 9); 35 for *lhcgr* (luteinizing hormone/choriogonadotropin receptor); and 28 for *gapdh*.

2.2.6 *In situ* hybridization

Intact ovaries from adult female zebrafish were carefully dissected and fixed in 4% buffered paraformaldehyde overnight at 4°C, and then embedded in tissue freezing medium (Leica, GER) at −25°C. The fixed tissues were cut on an 8 μm Leica CM 1850 microtome, and were mounted onto superfrost glass slides (Menzel, GER). A cDNA fragment of *igf3* was amplified by RT-PCR with specific primers (Table 1) and cloned into pGEM-T easy transcription vector (Promega, USA). Sense and antisense *igf3* riboprobes were synthesized from the pGEM-T easy transcription vector construct containing *igf3* and were linearized with *Nde*I or *Nco*I endonuclease (NEB, UK). The RNA probes were labeled using the DIG RNA Labeling Kit (Roche, USA). Tissue sections were first prehybridized for 30 min, and then a total of 250 μl

of hybridization buffer containing 150 ng of DIG-labeled sense or antisense *igf3* riboprobe was added to each slide and incubated in a humidified box at 42°C for 16 h. After hybridization, sections were washed twice in 2× saline-sodium citrate (SSC) (1× SSC = 0.15 M NaCl and 15 mM sodium citrate) at room temperature for 15 min and then in 1× SSC and 0.1× SSC at 55°C for 1 h sequentially. The hybridization signals were detected using anti-DIG conjugated with alkaline phosphatase and visualized with the nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate substrate solution (Roche, USA). Lastly, the sections were dehydrated through graded ethanol and xylene, then mounted and photographed.

2.2.7 Antibody production, Western blot analysis and immunohistochemistry

Two peptides (EGARARCGRELVDD and RSGGPRSRGKGIVDQC) of zebrafish Igf3 were selected as antigens for the production of polyclonal antibodies (Abgent, China). The synthetic peptides were used to immunize New Zealand White rabbits for antibody production. For confirmation of antibody specificity, the zebrafish *igf3* cDNA fragment corresponding to the prepropeptide was amplified by RT-PCR with specific primers (Table 2-1) and cloned into the pEGFP-N1 vector. For Western blot analysis, the cell or tissue lysates were firstly separated by 10% or 15% SDS-PAGE gels, or a 16% Tris-tricine SDS-PAGE gel. The separated proteins were transferred onto PVDF membranes and immunoblotted with the primary antibodies against Igf3. The protein bands were visualized by a Western blotting kit (Millipore, USA) after incubation with secondary antibody conjugated with horseradish peroxidase. For immunohistochemistry, the following procedures were conducted at room temperature. Sections were postfixed for 10 min in 4% paraformaldehyde in PBS, washed twice in PBS, blocked for 60 min in 5% normal goat serum in PBS, and incubated with either the Igf3 antiserum or preimmune serum overnight at 4°C. For diaminobenzidine tetrahydrochloride/hydrogen peroxide (DAB) staining, the sections

were washed and probed with donkey anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (Amersham, UK). After a 2-h incubation period, the sections were washed in PBS, color developed with DAB, dehydrated through graded ethanol and xylene, then mounted and photographed. For fluorescence staining, the sections were washed and probed with goat anti-rabbit Alexa 594 (Invitrogen, USA), washed in PBS, cell nuclei visualized with 4'-6-diamidino-2-phenylindole (DAPI) (Invitrogen, USA) counterstaining, then mounted and photographed.

2.2.8 Expression, purification and bioactivity evaluation of recombinant Igf3

The zebrafish *igf3* cDNA fragment corresponding to the predicted mature peptide was amplified by RT-PCR with specific primers (Table 2-1) and cloned into the pProEX-HTb vector. One liter of recombinant *E. coli* harboring this plasmid was grown in LB medium at 37°C until the absorbance value reached 0.5-0.8 at 600 nm. Cells were collected three hours after induction by IPTG and the collected cells were resuspended in 1xPBS. After sonication, the total lysate was divided into the soluble fraction and insoluble fraction by centrifugation. SDS-PAGE was carried out to check the expression of Igf3. The insoluble fraction was kept and dissolved in 1xPBS with 8M urea. Recombinant Igf3 was affinity purified with Ni-NTA agarose (Qiagen, USA). The purified protein was dissolved in 1xPBS with 8M urea, pH4.5, and concentrated in a concentrator (Millipore, USA). The concentrated protein was dialyzed in a tubing of a molecular weight cut-off of 3500 Da (Pierce, USA) as follows: 1xPBS with 4M urea at room temperature for 24 h; 1xPBS with 2M urea at 4°C for 24 h; 1xPBS with 1M urea and 0.4M L-arginine at 4°C for 24 h; 1xPBS with 0.4M L-arginine at 4°C for 24 h. The refolded Igf3 was collected and concentrated. The concentration was determined by the BCA Protein Assay Kit (Pierce, USA). The bioactivity of the recombinant protein was evaluated by the alamar blue assay on two

cell lines. GC-1 cells and ZFL cells were seeded onto 96-well plates at a density of 1×10^3 and incubated for 24 h at 37°C with 1% FBS. The cells were then incubated in FBS-free medium in the presence of the recombinant zebrafish Igf3. After 48 h of incubation, the medium was replaced with 10% alamar blue reagent (Biosource, USA) and incubated for 1 h in the dark. The yielded color was measured on an automated fluorescent plate reader (TECAN, USA) at an excitation wavelength of 485 nm and emission wavelength of 595 nm.

2.2.9 Preparation of ovarian fragments

After anesthetization and decapitation, the two ovaries from each fish (8–10 fish for each experiment) were carefully dissected out and placed in a dish containing 60% Leibovitz L-15 medium. Each ovary was halved in the middle, and each half was transferred into a well of a 12-well plate where it was briefly dispersed into small fragments before drug treatment.

2.2.10 Isolation of ovarian follicles

The ovaries were dissected out from 15–20 female zebrafish after anesthetization and decapitation, and placed in a 100-mm culture dish containing 60% Leibovitz L-15 medium. The follicles of different stages were manually isolated and grouped according as the following stages: primary growth follicles (PG, stage I; below 0.1 mm in diameter), previtellogenic (PV, stage II or cortical alveolus stage; about 0.30 mm in diameter), early vitellogenic (EV; about 0.40 mm in diameter), midvitellogenic (MV; about 0.50 mm in diameter), and full grown but immature (FG; about 0.65 mm in diameter). This staging system that we have adopted is based on the original definition of Selman (Selman et al., 1994) as modified by Ge (Wang and Ge, 2004b) and Thomas (Pang and Thomas, 2010). The isolation process normally lasted for 4–6 h at room temperature before incubation and drug treatment at 28°C

for different periods of time.

2.2.11 Separation of the follicular cell layer from the follicles

It has recently been reported that cold-shock treatment of the follicles makes it easier to mechanically separate the two follicle compartments, viz. the denuded oocyte and the follicular cell layer (Liu and Ge, 2007). In the present study, we pretreated FG follicles at low temperature (4°C) for about 30 min. The follicular cell layer was then carefully peeled off from the follicle with fine forceps without damaging the oocyte inside. The isolated follicular cell layers and the denuded but intact oocytes from 5–10 follicles were pooled and subject to RNA extraction with TRIzol Reagent separately. The denuded oocytes were also stained by propidium iodide (PI) to demonstrate complete removal of the follicular cell layer.

2.2.12 Follicle incubations

Zebrafish were sacrificed and ovaries excised as described above. Follicles of different stages were separated and incubated (30-40 follicles/well) in 24-well culture plates at 28°C. After treatment, follicles that underwent germinal vesicle breakdown (GVBD) were identified by their ooplasmic clearing (due to proteolytic cleavage of vitellogenin) (Selman et al., 1994). Each group had four replicate wells and each experiment was repeated three times. Data were analyzed by one-way ANOVA followed by the Student-Newman-Keuls post test using the GraphPad Instat Software (GraphPad Software, USA).

2.3 Results

2.3.1 Identification and characterization of two spliced forms of *igf3* in zebrafish

Using 5'RACE and 3'RACE, two alternative splicing events on the zebrafish

igf3 gene was identified. The two transcripts, *igf3, transcript variant 1* (*igf3 tv1*, NCBI GenBank database accession no. HQ241070) and *igf3, transcript variant 2* (*igf3_tv2*, NCBI GenBank database accession no. HQ241071) are different from each other in their 5' UTR and signal peptide regions (Fig. 2-1A). By employing gene specific primers designed from the cloned sequences, the expression patterns of the two *igf3* splice variants in different adult zebrafish tissues, during early development and during larvae development were studied (Fig. 2-1B, C and D). Strong expression of *igf3_tv1* was observed in adult ovary and testis (Fig. 2-1B) and also during larvae development (Fig. 2-1D). Interestingly, no expression of *igf3 tv1* was detected in all the other adult tissues examined (Fig. 2-1B). Some weak expression of *igf3 tv1* was detected during early development (Fig. 2-1C). In contrast, *igf3 tv2* is only expressed during early development (Fig. 2-1C). No expression of *igf3 tv2* was detected after 6 dpf (Fig. 2-1D) and also none in all adult tissues examined including the gonads (Fig. 2-1B). The differential expression of the two splice variants in the gonads is very conspicuous, with high expression of *igf3_tv1* but no expression of *igf3 tv2* at all (Fig. 2-1B).

2.3.2 Establishement of real-time PCR for zebrafish *igf3* and *gapdh*

Specific DNA fragaments were amplified from the ovarian cDNA, the PCR products of igf3 and gapdh were purified and cloned into pCR-4 vector. The plasmids were diluted and used as the standards in real-time assays. The log copy number of the standard templates exhibited a linear relationship with Ct value (Fig. 2-2A and B). The specificity of PCR amplification was further confirmed by agarose gel electrophoresis (Fig. 2-2C).

2.3.3 Expression of Igf3 mRNA and protein in adult zebrafish gonads

Two specific polyclonal peptide antibodies were developed against zebrafish Igf3,

one of them (generated from the peptide EGARARCCGRELVDD) can recognize the zebrafish Igf3 prepropeptide and the other (generated from the peptide RSGGPRSRGKGIVDQC) can recognize the mature peptide. The specificity of the antibody which can recoginze the zebrafish Igf3 prepropeptide was confirmed in the following way. Using Western blot analysis, a specific band at 49 kDa corresponding to the calculated molecular weight of the zebrafish Igf3 prepropeptide-GFP fusion protein was observed in HEK293 cells transfected with pEGFP-N1-Igf3, while no immunoreactivity was observed in HEK293 cells transfected with the empty vector (Fig. 2-3A). The specificity of the other antibody which can recognize the mature peptide was confirmed in the bacterial system where the expression of recombinant Igf3 mature peptide can be detected by this antibody (Fig. 2-9B).

One specific band at ~24 kDa was observed in the ovary and testis by Western blotting using the zebrafish Igf3 antibody which can recognize the prepropeptide. An intense band of Igf3 prepropeptide was found in the ovary, but the level in the testis is much lower with no signal detected at all in the other tissues (Fig. 2-3B). The expression of Igf3 mature peptide can be detected in the ovary only (Fig. 2-3C).

The expression of *igf3* mRNA level in adult ovary and testis using real-time PCR was also investigated. The results show that the expression of *igf3* in the ovary is much higher than that in the testis (Fig. 2-3D).

2.3.4 Expression of Igf3 in zebrafish ovarian follicles

Both real-time PCR and semiquantitative RT-PCR were performed to assess the relative levels of *igf3* expression in the ovarian follicles of different stages from PG to FG. Follicles of different stages from zebrafish were separated (Fig. 2-4A). A significant gradual increase in *igf3* expression was observed during follicle development. Although it could be detected in all the stages by RT-PCR (Fig. 2-4B),

the level of *igf3* mRNA is low in follicles of the early stages. The level increases in the MV stage, and reaches the highest in the FG stage (Fig. 2-4C). Consistent with this result, *in situ* hybridization also showed that the follicles at the FG stage exhibited the strongest staining and the signal is weak at the early stages (Fig. 2-4D).

Immunohistochemistry was also performed to detect the protein expression in the zebrafish ovary. The expression in follicles of different stages was visualized by both fluorescence and DAB staining. Strong immunoreactivity was observed in the follicular cell layers surrounding the oocytes after the EV stage (Fig. 2-5A, B). Using DAPI staining, the signal was found in both the thecal and granulosal cells of the FG stage (Fig. 2-5A). Weak cytoplasmic immunoreactivity was also observed in the oocytes. No signal was detected in control sections incubated with the preimmune serum (data not shown).

The expression of *igf3* mRNA in ovarian follicles was further examined using semiquantitative RT-PCR. This was done by separating the somatic follicular cell layer from the full grown follicles and analyzing the expression of *igf3* in the two compartments. Clean separation was confirmed by PI staining of the denuded oocytes revealing the absence of the follicular cells (Fig. 2-6A). Furthermore, two marker genes viz. *gdf9* and *lhcgr* were used as controls, the former being oocyte specific and the other being follicular cell specific (Liu and Ge, 2007). Different from the *lhcgr* and *gdf9*, *igf3* was expressed in both the oocyte and the follicular cell layer (Fig. 2-6C). The real-time PCR results show that the expression of *igf3* in the follicular cell layer is higher than in the oocyte (Fig. 2-6B).

2.3.5 Gonadotropin regulates expression of *igf3* in zebrafish ovary via a cAMP-dependent pathway

As demonstrated above, zebrafish *igf3* mRNA expression exhibits a significant trend of increase during follicle development, with the highest level detected in the

FG stage. This suggests the potential roles of endocrine agents, particularly gonadotropins from the pituitary, in regulating *igf3* expression, especially in view of the fact that luteinizing hormone receptor shows sequential and progressive increases during zebrafish follicle growth (Kwok et al., 2005).

Two approaches were adopted to address this issue, one using ovarian fragments and the other using isolated follicles. When tested on zebrafish ovarian fragments, hCG increased *igf3* expression in a time-dependent manner, with the maximal effect reached after 2 h of treatment (Fig. 2-7A). In addition, treatment of zebrafish ovarian fragments or follicles of FG stage with different concentrations of hCG for 2 h consistently increased the expression of *igf3* in a dose-dependent manner (Fig. 2-7B and C). Interestingly, the effect of hCG was dependent on the stage of follicles used. When tested on follicles of different stages, hCG caused significant increase of *igf3* expression in the FG follicles, but with little effects on the follicles of EV stage (Fig. 2-7D).

Since cAMP is the major second messenger involved in gonadotropin signaling , we have also studied the effect of increasing intracellular cAMP level on the expression of zebrafish *igf3*. Both the membrane diffusible cAMP analog (8-Br-cAMP) and the phosphodiesterase inhibitor IBMX (which can increase intracellular cAMP level) were used in the experiments. Treatment of zebrafish different stages follicles with both agents increased the expression of *igf3* in a dose- time- and stage- dependent manner. Desensitization of the activation was observed at higher concentrations of the drugs used, as was previously reported in a similar system (Wang and Ge, 2003a; Wang and Ge, 2004a) (Fig.2-8).

2.3.6 Expression, purification and bioactivity evaluation of recombinant zebrafish Igf3

In order to analyze the biological function of Igf3, recombinant zebrafish Igf3

was prepared from a bacterial system. The amino acid sequence of mature Igf3 is shown in Fig. 2-9A. The mature zebrafish Igf3 peptide HIS tag fusion protein was expressed in *E. coli* (Fig. 2-9B). Results of Western blot analyses using the antibody which can recognize the zebrafish Igf3 mature peptide and an anti-HIS antibody (Santa Cruz, USA) both confirmed the expression of the recombinant protein (Fig. 2-9C). After purification, the purified recombinant protein was analyzed by SDS-PAGE with commasie blue staining (Fig. 2-9D). Since the Igf signaling pathway has been demonstrated to enhance cell proliferation, the biological activity of this recombinant protein was evaluated on GC-1 cells and ZFL cells using the alamar blue assay. The recombinant zebrafish Igf3 was demonstrated to stimulate the proliferation of both cell lines in a dose-dependent manner (Fig. 2-9E and F).

2.3.7 Effects of Igf3 on zebrafish oocyte maturation

To assess the effect of Igf3 on oocyte maturation, the full grown zebrafish follicles were incubated in the absence or presence of the recombinant zebrafish Igf3 at different concentrations, and GVBD of the oocytes was scored after different times of treatment. The recombinant zebrafish Igf3 could significantly enhance zebrafish oocyte maturation in clearly dose- and time-dependent manners (Fig. 2-10A). Some proportion of the early stage follicles including MV (0.48-0.56mm in diameter) and EV (0.38-0.47mm in diameter) follicles also underwent maturation after treatment with the recombinant Igf3(Fig. 2-10B).

2.4 Discussion

The presence of multiple spliced forms of Igfs including Igf3 in zebrafish has been reported (Zou et al., 2009). In this study, the two *igf3* transcripts in zebrafish were characterized. These two transcripts, namely *igf3 tv1* and *igf3 tv2*, have different 5'-UTR and translation initiation sites, but producing the same mature peptide. In

addition, we have provided evidence showing that the two *igf3* transcript variants have distinct temporal expression patterns in zebrafish. During early development, *igf3 tv2* is highly expressed after the pre-mid-blastula transition. This is in big contrast to the expression *igf3 tv1* which is low throughout embryogenesis. In adult zebrafish, *igf3 tv1* is exclusively expressed in the gonads only, while *igf3 tv2* cannot be detected in all the adult zebrafish tissues examined. The different expression patterns of the two *igf3* transcripts in zebrafish suggest that *igf3 tv1* functions exclusively in the adult gonads while *igf3 tv2* functions mainly during early development. It is highly likely that these two *igf3* transcripts possess different promoters which are subject to the regulation of the different transcription factors prevalent at different developmental stages of zebrafish as well as in the different adult tissues. Further studies on the differential regulation of expression of these two transcripts of *igf3* are highly warranted.

Both Igf3 prepropeptide and mRNA are significantly higher in the ovary than in the testis. Furthermore, the Igf3 mature peptide can only be detected in the ovary of adult zebrafish. These results indicate the major biological action of Igf3 is the regulation of ovarian functions.

To provide clues on the functional roles of *igf3* in the ovary of zebrafish, we have analyzed its temporal expression pattern in follicles of different stages using real-time PCR. Interestingly, the expression of *igf3* in the follicles was found to increase progressively during the development of the follicles, with the highest level detected at the FG stage. This result was also confirmed by *in situ* hybridization, with the strongest signal detected in follicles of the FG stage. This expression profile is very different from that of *igf1* and *igf2* in zebrafish ovary where *igf1* expression was found to increase from PG to EV stage but decreased at the FG stage, while the expression of *igf2* was maintained at a constant level during folliculogenesis (Yu and Ge, 2008). The different expression patterns of the *igf*s indicate their different roles

on ovarian functions. The expression profile of *igf3* is similar to that of the luteinizing hormone receptor which also exhibits progressive increases during follicle growth in zebrafish (Kwok et al., 2005). The increasing trend of *igf3* expression during follicle growth suggests its potential roles on vitellogenic growth, and its high expression at the FG stage indicates the possible role of *igf3* on oocyte maturation.

Using immunohistochemistry and RT-PCR, the Igf3 protein and mRNA is mainly found in the follicular cell layer. These results are consistent with our previous study in tilapia indicating that the expression of *igf3* mRNA is also confined to the follicular cell layer (Wang et al., 2008).

The increasing expression of *igf3* during zebrafish follicle development and the high expression of Igf3 in the somatic follicular cell layer has prompted us to speculate that this growth factor is subject to endocrine regulation. Gonadotropin is the major regulator of ovarian development and functions in many organisms. Evidence also shows that the actions of gonadotropin are mediated or modulated by the intra-ovarian paracrine/autocrine growth factors (Ge, 2005). Consistent with the report from a recent study that expression of *igf3* can be up-regulated by hCG (Nelson and Van Der Kraak, 2010a), results from the present study showed that *igf3* in the zebrafish ovary is strongly stimulated by hCG treatment. This has been demonstrated in the present study not only on the ovarian fragments in time- and dose- dependent manners, but also on the isolated follicles as well in dose- and stage-dependent manners. The action of hCG on the follicles is particularly prominent on follicles of the FG stage. Gonadotropin is known to regulate target gene expression primarily via activation of the cAMP-PKA pathway (Leung and Steele, 1992). Our results showed that both 8-Br-cAMP and IBMX could mimic the effects of hCG on follicles of the FG stage, suggesting the cAMP is likely a second messenger involved in the action of hCG on the follicles in stimulating *igf3*

expression. A putative cAMP response element (CRE) can actually be found on the promoter of the *igf3* gene in zebrafish. All these results suggest that Igf3 in zebrafish follicles serves as a downstream factor of gonadotropin to transmit its action from one cell layer to another in a paracrine manner. The regulation of expression of *igf3* in relation to the functional characterization of its promoter warrants further studies.

In the present study, we have demonstrated for the first time that Igf3 exerts a potent action in stimulating oocyte maturation in zebrafish. Bioactive recombinant zebrafish Igf3 was expressed and prepared from a bacterial system successfully. Treatment of zebrafish follicles with the recombinant Igf3 significantly induced oocyte maturation in time- and dose-dependent manners. Interestingly, the early stage follicles including EV and MV also exhibited response to Igf3 treatment. It should be noted that in these stages, the follicles can hardly undergo oocyte maturation by treatment with hCG or with 17α, 20β-dihydroxyprogesterone (DHP) alone (Pang and Ge, 2002a). We have also made some attempts to address whether Igf3 exerts its action on the follicular cell layer or on the oocyte. However, it was found that most of the oocytes underwent GVBD spontaneously in the absence of any hormones or growth factors after removal of the follicular cell layer. A recent paper also reported the same phenomenon that about 75% of the full-grown denuded oocytes in zebrafish underwent GVBD after removing the follicular cells without any additional hormones or growth factors (Pang and Thomas, 2010). It was also demonstrated in that study that estrogen in the follicular cells could partially inhibit this oocyte maturation process through GPER. On the other hand, it has been reported previously that oocyte maturation could be stimulated by Igf1 in denuded oocytes of *Cyprinus carpio* (Paul et al., 2009), suggesting the direct action of Igf1 on the oocyte. It is therefore possible that Igf3 might also exert its action on the oocyte directly. A definitive proof or disproof of this point would await solving the technical problem of preventing spontaneous GVBD of denuded oocytes in zebrafish. There

are several reports demonstrating that Igf1 and Igf2 could induce oocyte maturation in several fish species including zebrafish (Kagawa et al., 1994; Negatu et al., 1998; Nelson and Van Der Kraak, 2010b; Weber and Sullivan, 2000), but all these studies have employed recombinant human Igf1 or Igf2. This is far from ideal not only because of the heterologous nature of the mammalian growth factors used, but also because of the fact that Igf1 and Igf2 are not specific to the fish ovary. The gonad-specific expression patterns of Igf3 and the regulation of *igf3* expression by gondatropin in the zebrafish ovary strongly support the important functional role of Igf3 on the ovary, especially in oocyte maturation.

Although the mechanisms underlying the action of Igf3 on oocyte maturation is unclear, we speculate that Igf3 might exert its effect by activating the phosphoinositide 3 (PI3) kinase signal transduction cascade to promote oocyte maturation. It has been shown that in many species such as in star fish (Sadler and Ruderman, 1998), *Xenopus* (Muslin et al., 1993) and pig (Shimada and Terada, 2001) that activation of PI3 kinase is sufficient to induce oocyte maturation. In fish, Weber and Sullivan (Weber and Sullivan, 2001) reported the involvement of PI3 kinase in mediating Igf1 signaling during oocyte maturation in striped bass. More recent studies also indicated that PI3 kinase is an initial component of the signal transduction pathway that precedes maturation-promoting factor activation during Igf1 induced oocyte maturation in *Cyprinus carpio* (Paul et al., 2009). So far, there is no information on the cognate receptor for Igf3. However, the structure of Igf3 is similar to other Igfs despite the low level of sequence homology among them (Wang et al., 2008). Zou *et al.* (Zou et al., 2009) have shown that Igf3 can trigger the Akt pathway which is a major downstream effector of the Igf1 receptor in mammals and zebrafish. We also provided evidence in this study that Igf3 can activate cell proliferation. It is therefore possible that Igf3 exerts its effects through the Igf type I receptor (Igf1r) to trigger the downstream pathways such as PI3k signaling in

zebrafish ovary. Moreover, the expression of Igf1r in the ovary has been demonstrated in several fish species. The ovarian *igf1r* mRNA increased at the onset of vitellogenesis in female sterlet (Wuertz et al., 2007). The Igf1r was found in the granulosa and theca cells of gilthead seabream and coho salmon (Maestro et al., 1997; Perrot et al., 2000). In zebrafish, two types of *igf1r*s have been identified (Maures et al., 2002), and are found to be expressed in both the follicular cells and oocyte (Yu and Ge, 2008). Our preliminary results showed Igf3 in the follicles can rapidly activate the phosphorylation of p42/44 MAPK which is the major downstream factor of PI3 kinase (data not shown). The mechanisms of Igf3 actions on oocyte maturation are currently under investigation.

In summary, the present study has identified two spliced forms of *igf3* in zebrafish, one which is exclusively expressed in adult gonads and the other expressed during early development. The protein level of Igf3 in the adult zebrafish ovary is significantly higher than that in the testis, suggesting the major role of this growth factor on ovarian functions. In the zebrafish ovary, the expression of *igf3* is stage-dependent and the highest level is seen in the FG stage follicles. The Igf3 protein is mainly detected in the follicular cell layer of the follicles. The expression of *igf3* in the zebrafish ovary was strongly stimulated by gonadotropin and cAMP. Recombinant Igf3 could enhance oocyte maturation in follicles of EV, MV and FG stages. All these evidence strongly supports a functional role of Igf3 in the ovary of zebrafish, by acting as a downstream mediator of gonadotropin action in the ovary.

Gene	Sequence(5' to 3' direction)	Strand	Application	Size (bp)
igf3_tv1	AAAAAGACATGCCATCAGACGC	S	*in situ* hybridization	574
	ATGAATGTTGGCGTCTCAGTTG	AS		
	GCCAAACGCCTTCAGATAATGC	S	Real-time PCR	203
	GCTGCTCCAGGTTTGCCTATGT	AS		
	ACAAGCTTACCATGCCATCAGACGCAATGCC	S	Construction of pEGFP-N1-Igf3	579
	ACGGATCCCGCCGCACTTTCTTGGATTTGG	AS		
	ACTGGATCCGCCAGAGCACGCTGCGGACGA	S	Construction of pProEX-HTb-Igf3	207
	ACTCCTCGAGTCAATCACGCCGCACTTTCTTGGA	AS		
	GGCGTCTCAGTTGTCGAACTGAGG	AS	5`RACE	
	GGAGGTCACATCCACGCACAC	AS	5` nested RACE	
	GCCAGAGCACGCTGCGGAC	S	3'RACE	
	GACTCCGGAGGACCAGTTCTGG	S	3`nested RACE	
igf3_tv2	CGCATAATTCAATCAAAGTCCG	S	RT-PCR	383
	GCTGCTCCAGGTTTGCCTATGT	AS		
gdf9	GAGTCTGTTGAACCCGACG	S	RT-PCR	544
	GCAGGTGGATGTCCTTCTTA	AS		
lhcgr	AAGGACGAGTCGCTGAAAC	S	RT-PCR	663
	GATTCATTGTGGCGTATTCA	AS		
18s	CCTGAGAAACGGCTACCACATCC	S	RT-PCR	220
	AGCAACTTTAGTATACGCTATTGGAG	AS		
gapdh	CGACCTCACCTGCCGCCTTACA	S	Real-time PCR	187
	GTCATTGAGGGAGATGCCAGCG	AS		
β-actin	CGAGCAGGAGATGGGAACC	S	RT-PCR	101
	CAACGGAAACGCTCATTGC	AS		

Table. 2-1 Primers used in Chapter 2.

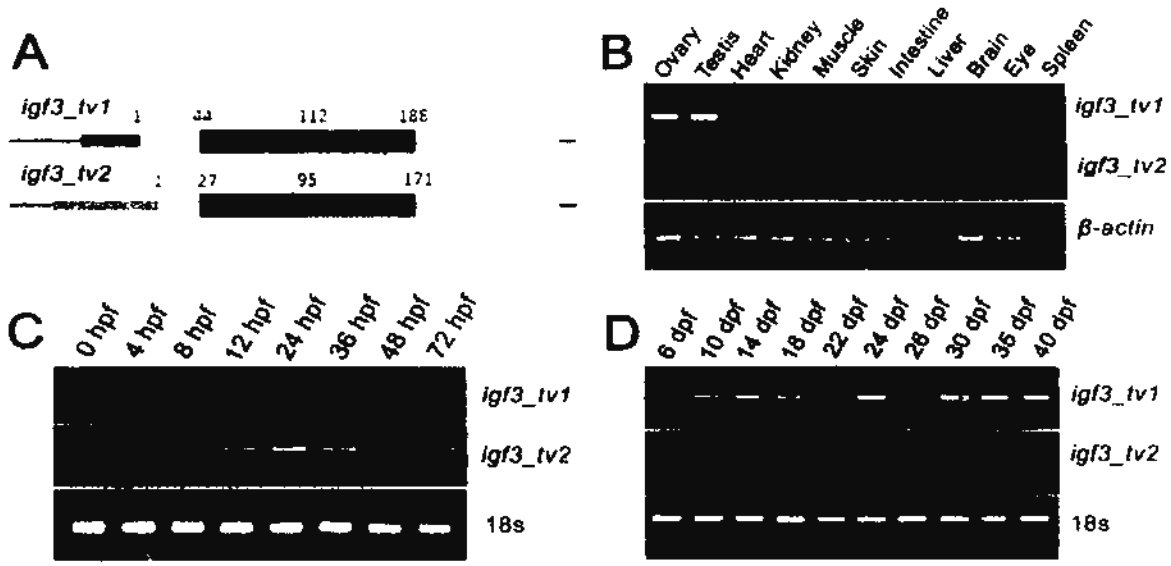

Fig. 2-1 Two spliced transcripts of the zebrafish *igf3* gene.
(A) Zebrafish *igf3* is spliced into two kinds of transcripts: *igf3_tv1* and *igf3_tv2*. The alternatively spliced regions at the 5′ end are shown in different colors. Black and red indicate the 5′ UTR regions of the two transcripts. Green, blue, violet and yellow indicate the signal peptide, mature peptide, E domain and 3′UTR of the two transcripts respectively. Expression of *igf3_tv1* and *igf3_tv2* was studied by RT-PCR in different adult tissues (B), during early development (C) and during gonad development (D), using 18s ribosomal RNA or *β-actin* as internal control. hpf, hours post fertilization; dpf, days post fertilization.

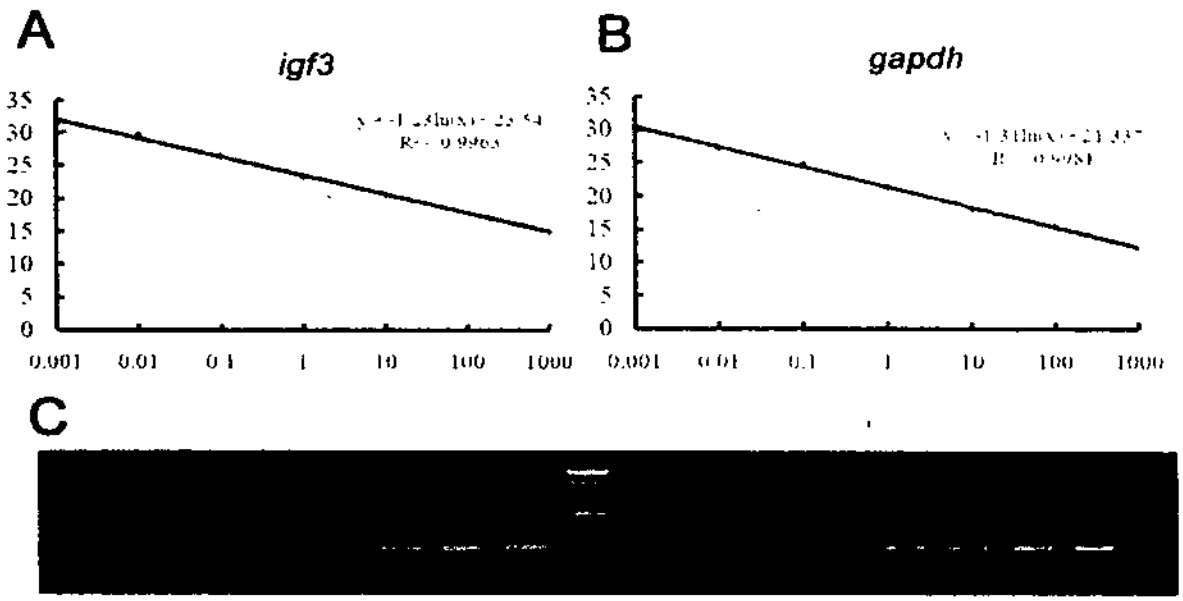

Fig. 2-2. Validation of real-time PCR assay for zebrafish *igf3* and *gapdh* expression. A, Standard curve generated by serially-diluted *igf3* plasmid; B, Standard curve generated by serially-diluted *gapdh* plasmid: C, Electrophoresis of PCR product of *igf3* and *gapdh*.

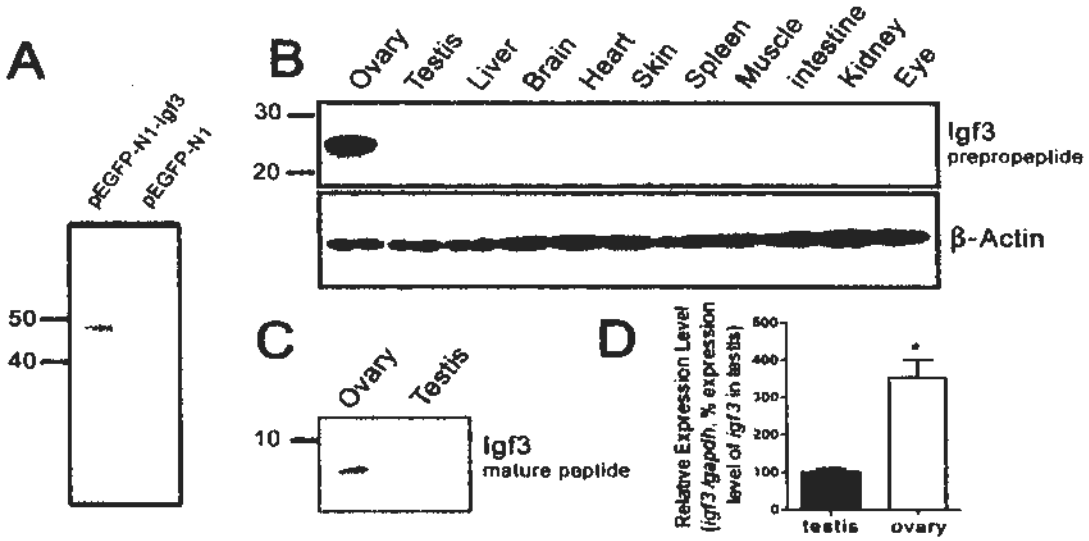

Fig. 2-3 Expression of Igf3 in the adult zebrafish gonads.
(A) Using the antibody which can recognize the zebrafish Igf3 prepropeptide, Western blot analysis was performed on HEK-293 cells transiently transfected with the vector pEGFP-N1-Igf3 harboring the zebrafish Igf3 coding region (which includes the signal peptide, mature peptide and E domain) insert or the empty vector (pEGFP-N1) without the zebrafish *igf3* insert. (B) Using the antibody which can recognize the zebrafish Igf3 prepropeptide, Western blot analysis was performed on adult zebrafish ovary and testis showing predominant expression in the ovary. Equal loading was confirmed by Western blot probed with an anti-β-actin antibody. (C) Using the antibody which can recognize the zebrafish Igf3 mature peptide, the expression of Igf3 mature peptide was detected by Western blot in adult zebrafish ovary only. (D) Real-time PCR results showing higher expression of *igf3* mRNA in ovary than in testis. Each value represents the mean ± SEM (n=4) (*P<0.05).

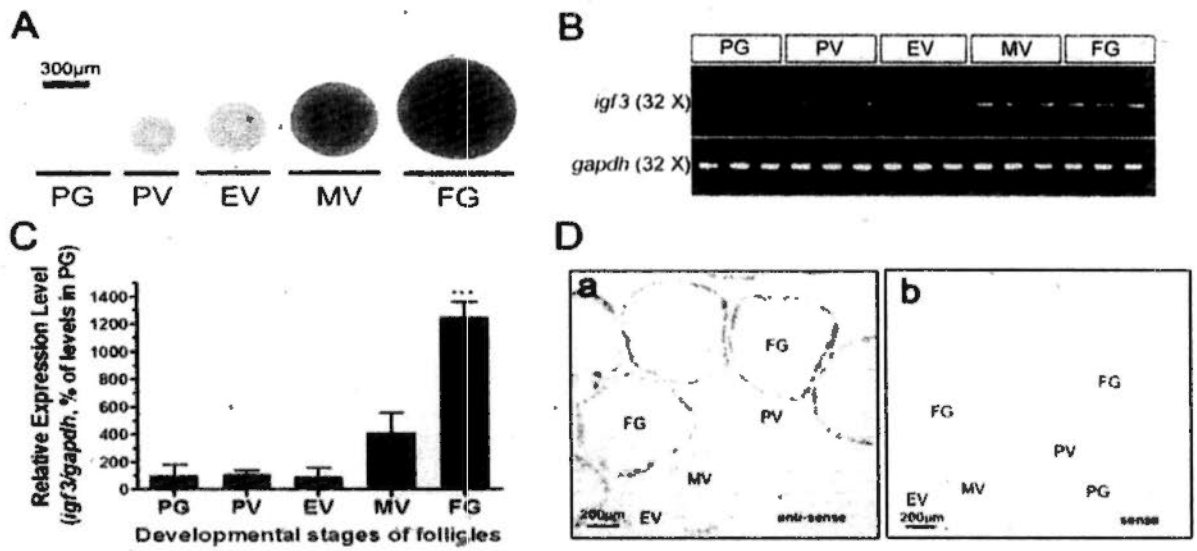

Fig. 2-4. Stage-dependent expression of *igf3* in developing zebrafish follicles.
(A) The morphology of zebrafish follicles at different stages. PG, primary growth (stage I); PV, previtellogenic stage (stage II); EV, early vitellogenic stage (early stage III); MV, midvitellogenic stage (mid stage III); FG, full grown stage (late stage III). (B) RT-PCR results showing the expression of *igf3* and *gapdh* in zebrafish follicles of different stages. (C) Real-time PCR results of *igf3* expression in zebrafish follicles of different stages. Each value represents the mean ± SEM (n=4) (***P<0.001). (D) Detection of *igf3* mRNA in the zebrafish ovary by *in situ* hybridization with antisense (left panel) and sense (right panel) probes. Scale bars, 100 μm.

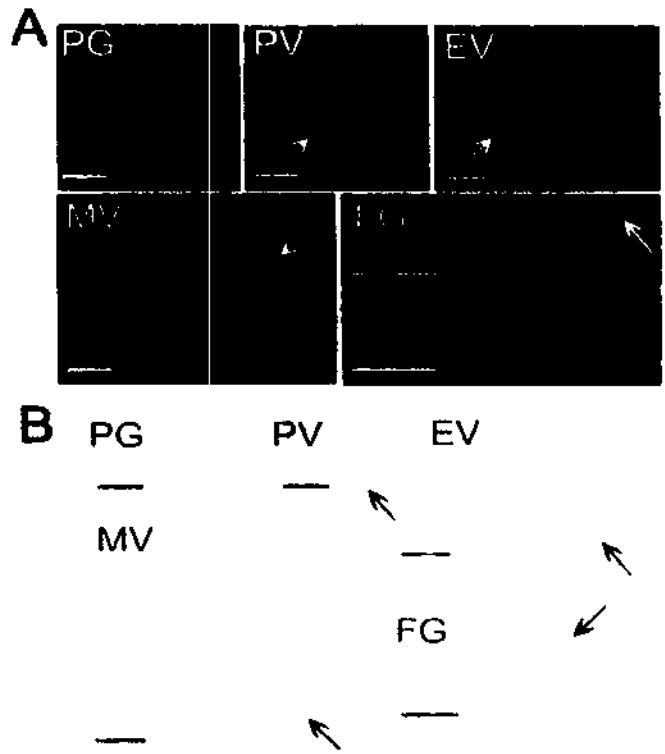

Fig. 2-5 Spatial expression of Igf3 protein in adult zebrafish ovary.
Sections of zebrafish ovary were probed with the anti-Igf3 antibody against the prepropeptide, and the secondary antibodies used were Alexa Fluor 488 anti-mouse for fluorescent labeling, followed by DAPI staining (blue color) (A), and horseradish peroxidase-conjugated for DAB staining (B). Positive staining was observed mainly in the follicular cells. Scale bars, 100 μm.

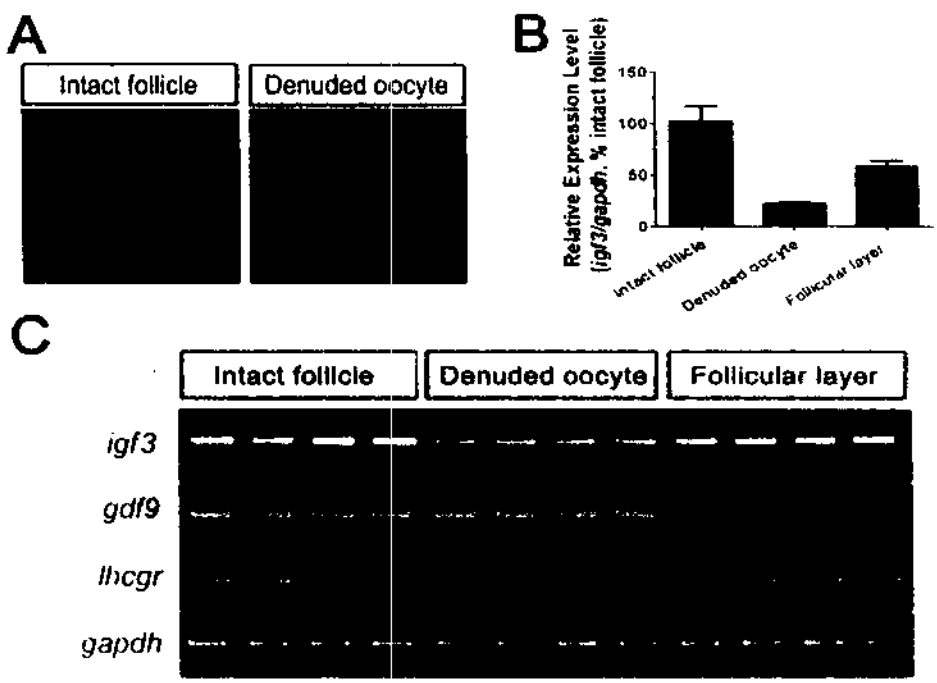

Fig. 2-6 Spatial distribution of *igf3* expression in full grown zebrafish follicles.
(A) Propidium iodide staining of intact oocyte with follicular cells (left panel) and oocytes without follicular cells (right panel). (B) Real-time PCR results of *igf3* expression in the intact follicles, denuded oocytes and follicular cell layer. (C) RT-PCR results for the expression of *igf3*, *gdf9*, *lhcgr*, and *gapdh* in intact follicles, denuded oocytes, and isolated follicular cell layers.

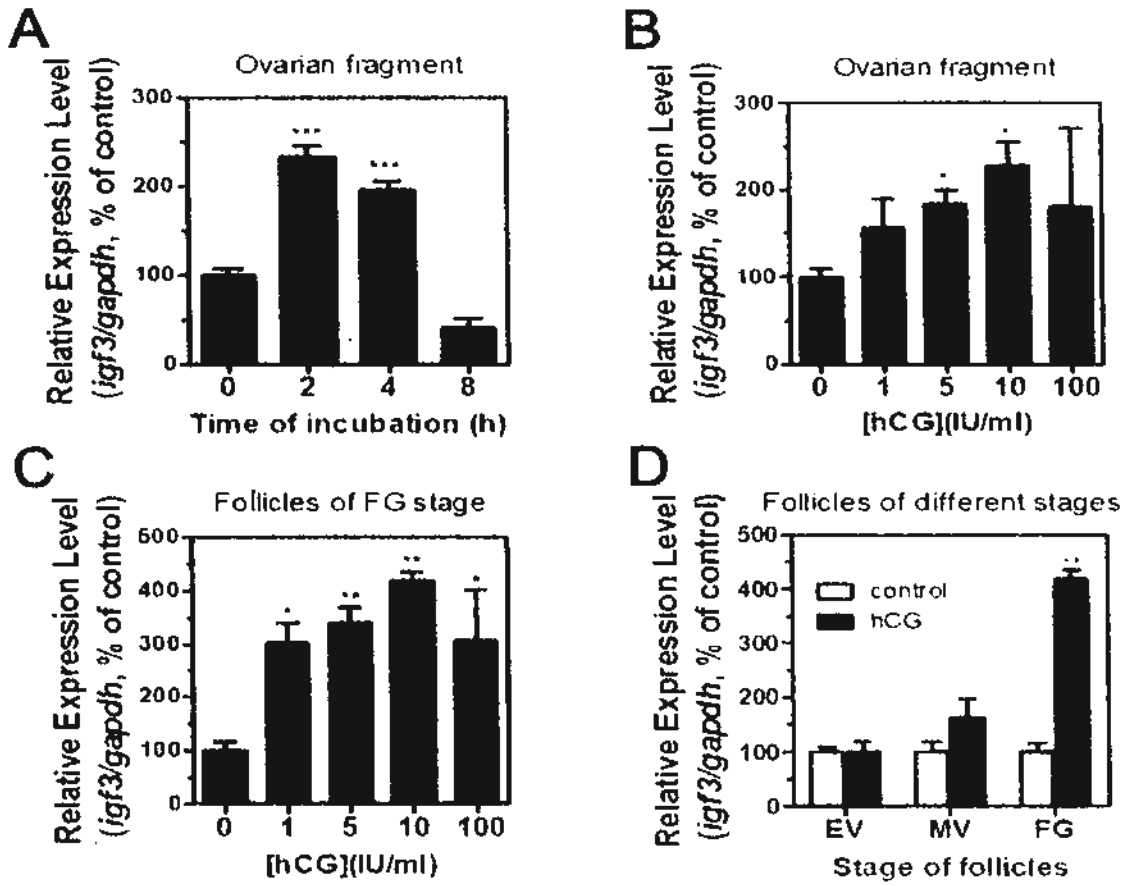

Fig. 2-7 Regulation of *igf3* expression in zebrafish ovary and follicles by hCG.
(A) Time course of hCG (10 IU/ml) action on *igf3* expression in zebrafish ovarian fragments. (B) Dose-response of hCG action (2 h incubation) on *igf3* expression in zebrafish ovarian fragments. (C) Dose-response of hCG action (2 h incubation) on *igf3* expression in zebrafish FG follicles. (D) Stage dependence of hCG action (10 IU/ml, 2 h incubation) on *igf3* expression in zebrafish follicles of different stages. Each value represents the mean + SEM of three independent experiments each performed in quadruplicates (*P<0.05; **P<0.01; ***P<0.001 vs control).

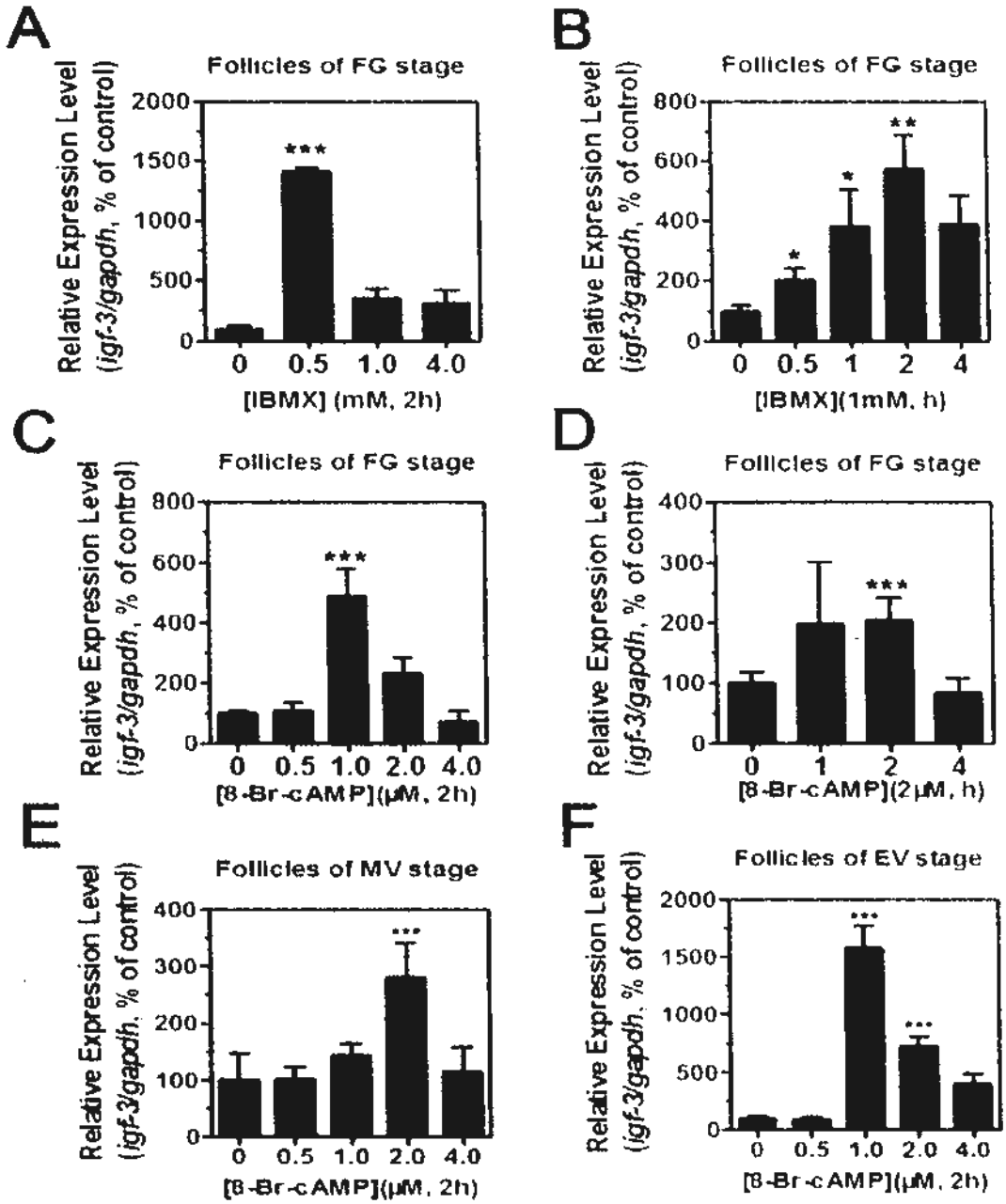

Fig. 2-8 Regulation of *igf3* expression in zebrafish follicles by cAMP.

(A) Dose-response of IBMX action (2 h incubation) on *igf3* expression in zebrafish FG follicles. (B) Time-response of IBMX action (1mM) on *igf3* expression in zebrafish FG follicles. (C) Dose-response of 8-Br-cAMP action (2 h incubation) on *igf3* expression in zebrafish FG follicles. (D) Time-response of 8-Br-cAMP action (1uM) on igf3 expression in zebrafish FG follicles. (E) Dose-response of 8-Br-cAMP action (2 h incubation) on *igf3* expression in zebrafish MV follicles. (F) Dose-response of 8-Br-cAMP action (2 h incubation) on *igf3* expression in zebrafish EV follicles. Each value represents the mean + SEM three independent experiments each performed in quadruplicates (*P<0.05; **P<0.01; ***P<0.001 vs control).

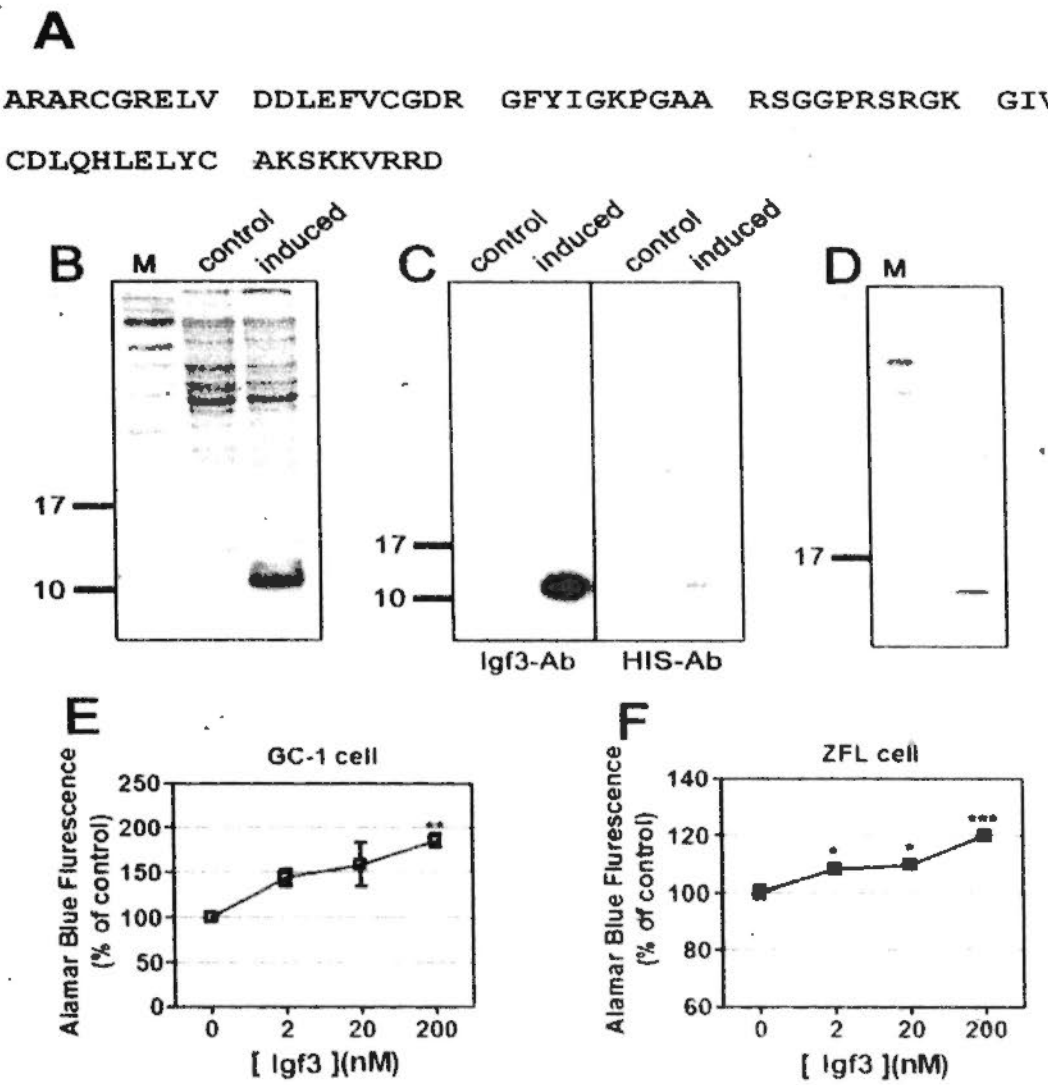

Fig. 2-9 Purification, characterization and bioactivity evaluation of recombinant zebrafish Igf3 protein.

(A) Amino acid sequence of mature Igfs; (B) Igf3 protein was expressed in *E. coli* and analyzed by SDS-PAGE, followed by commasie blue staining. A strong band can be seen in the IPTG induced group. (C) Western blot results with both the antibody which can recognize Igf3 mature peptide and an anti-His antibody. A specific band can be detected using both antibodies. (D) The purified Igf3 protein was analyzed by SDS-PAGE followed by commasie blue staining. A specific band can be detected. (E and F) Bioactivity of the recombinant Igf3 was evaluated on cultured GC-1 and ZFL cells. Both cell lines were treated with different concentrations of recombinant Igf3 for 48 h. Cell proliferation was then assessed using the alamar blue assay. Each value represents the mean ± SEM of three independent experiments each performed in quintuplicates (*P<0.05; **P<0.01; ***P<0.001 vs control).

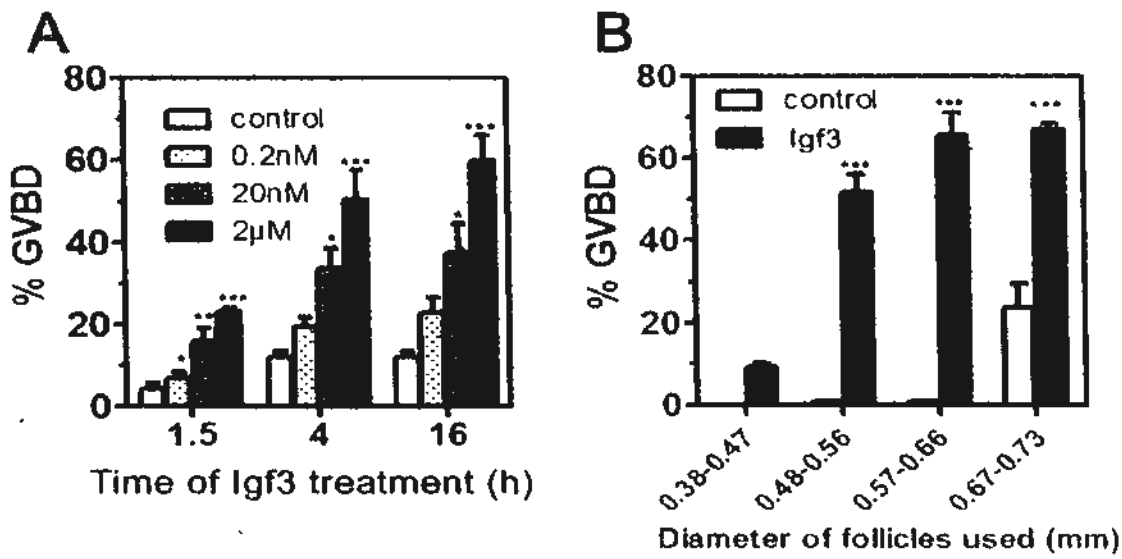

Fig. 2-10 Action of recombinant Igf3 on zebrafish oocyte maturation.
(A) Time- and dose-dependent actions of recombinant zebrafish Igf3 on oocyte maturation. (B) Stage-dependent responsiveness of zebrafish oocytes to recombinant Igf3 (2 μM). GVBD was scored after treatment. Each value represents the mean ± SEM of three independent experiments each performed in quadruplicates (*P<0.05; **P<0.01; ***P<0.001 vs control).

Chapter 3 The potential downstream signaling pathway involved in Igf3-induced oocyte maturation in zebrafish

3.1 Introduction

Female fertility requires precise regulation of oocyte meiosis. Fish oocytes, like those of other vertebrates, begin meiosis but subsequently become arrested at prophase until gonadotropin (luteinizing hormone (LH)) released from the pituitary causes the meiotic cell cycle to resume. In response to the LH surge, the oocytes proceed from prophase to second metaphase. The prophase-to-metaphase transition is characterized by germinal vesicle breakdown (GVBD), which is indicative of oocyte maturation. So far, much is still unknown about the ovarian signaling events in the ovary that regulate this process (Nagahama and Yamashita, 2008).

It has been demonstrated in several vertebrates that LH acts via an interaction with its cognate LH receptor, a member of the large superfamily of G protein coupled receptors (GPCRs) (Ascoli et al., 2002; Richards, 2001). Under the regulation of LH, maturation-inducing hormones (MIHs) acting as steroid mediators are synthesized in the follicular layer of the ovary to initiate maturationa events. Progesterone was considered an effective MIH in amphibians, but some studies have also suggested that androgens are MIHs in *Xenopus* oocytes. In most fish, 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) is considered an MIH (Nagahama and Yamashita, 2008). It has been demonstrated in both fish and amphibians that oocyte maturation is stimulated by MIH, which can bind to GPCRs and activate various signal transduction pathways. This signaling event ultimately induces the activation of maturation-promoting factor (MPF), a complex of two proteins, cdc2 and cyclin B. The action of MPF is universal among species; it catalyzes the entry into the M phase of meiosis I and meiosis II. However, different signaling pathways lead to MPF activation in different species (Kishimoto, 2003).

In recent years, evidence has suggested that local paracrine factors are also

involved in the regulation of meiotic reinitiation in oocytes (Ge, 2007), and Igf system is one of these local factors. The Igf system is composed of ligands, receptors, and Igf binding proteins (Igfbps). Since the identification of Igf1 and Igf2, the role of this system in the ovary has attracted considerable attention. *In vitro* studies in many species of vertebrates and gene knockout studies in mice have revealed that Igfs are key intraovarian regulators of many aspects of ovarian function including oocyte maturation (Yoshimura, 2003).

It has been established in the past several decades that Igf1 and Igf2 induce oocyte maturation through binding and stimulation of Igf type I receptors in *Xenopus* and rabbit (Janicot et al., 1991; Taghon and Sadler, 1994; Yoshimura et al., 1996a; Zhu et al., 1998), and that the action of Igf1 on oocyte maturation occurs via a steroid-independent pathway in striped bass and *Fundulus* (Negatu et al., 1998; Weber and Sullivan, 2000). Ligand occupancy of Igf1r leads to tyrosine phosphorylation of its intracellular domain and subsequent activation of signaling cascades. The downstream pathway originates with the interaction of the Igf1r with one of its major substrates, insulin receptor substrate 1 (IRS-1) (Myers et al., 1994). IRS-1 associates with and activates phosphatidylinositol 3-kinase (PI3-k), which subsequently recruits signaling proteins such as Akt/protein kinase B (PKB) (Cantley, 2002; Kulik et al., 1997). It has been demonstrated in star fish (Okumura et al., 2002), *Xenopus* (Andersen et al., 1998) and mouse (Hoshino et al., 2004) that the activation of PI3-k and Akt is sufficient to induce oocyte maturation. In common carp, the effect of Igf1 on oocyte maturation can be blocked by inhibition of PI3-k (Paul et al., 2009). Evidence from *Xenopus* has shown that one potential downstream target of PI3-k/Akt after induction by Igf1 is PDE3, which could degrade cAMP in oocytes (Andersen et al., 1998).

Igf3 is a newly characterized Igf ligand in teleosts and was first reported by our group (Wang et al., 2008). The gonad-specific expression of Igf3 suggests an

important role in the reproductive function of fish. Previously, using zebrafish as model, we have found the unique expression pattern of Igf3 correlates with that of the LH receptor in the ovary. The expression of Igf3 could be regulated by LH via a cAMP-dependent pathway, and a role for Igf3 in oocyte maturation was also demonstrated (Li et al., 2011). However, the molecular mechanism on how Igf3 stimulate oocyte maturation is still unknown.

In the present study, we aimed to investigate the potential downstream signaling pathway involved in Igf3-induced oocyte maturation in zebrafish. Three questions were addressed in this study. Is the steroidogenesis required for Igf3-induced oocyte maturation? Does Igf3 stimulate oocyte maturation through Igf1r? Are the major downstream factors of Igf pathway including PI3-K, PDE3 and MAPK are necessary for Igf3-induced oocyte maturation?

3.2 Materials and Methods

3.2.1 Animals

See Section 2.2.1.

3.2.2 Chemicals

AR grade chemicals and human chorionic gonadotropin (hCG) were obtained from Sigma-Aldrich (USA), culture media from Gibco (USA), and enzymes from Promega (USA) unless otherwise stated.

3.2.3 RNA isolation and RT-PCR

Total RNA samples were isolated from ovarian follicles of zebrafish using TRIzol Reagent (Invitrogen, USA). The amount and purity of the RNA were determined by NanoDrop 2000C Spectrophotometer (Thermo, USA). For real-time PCR, the standards for target genes and *gapdh* (glyceraldehyde-3-phosphate dehydrogenase) were prepared by amplification of cDNA fragments with the specific

primers (Table 3-1). The amplicons were purified by the PCR Purification Kit (Qiagen, USA). These purified PCR products were used to construct the standard curves in the real-time PCR assays. Real-time PCR was carried out as described in Section 2.2.5.

Gene	Sequence(5' to 3' direction)	Strand	Application	Size (bp)
p450c17a1	ACAGTCCTCCGCACATCTTCTTT	S	Real-time PCR	224
	TGTGGAACTGTAGTCAGCAAACG	AS		
p450c17a2	CTGGTGTTCAGCTCCTCCTATCA	S	Real-time PCR	254
	TTAGCCTCTTCAGGTCCTTGTTG	AS		
cyp19a	GTTGTCTCCTACTGTCGGTTCAT	S	Real-time PCR	192
	CAGACCCAGTTTACTTCCAAAGC	AS		
17b-HSD1	GGTTTGCCATTCAACGAGGTCTAC	S	Real-time PCR	180
	CTCCAGCTCCTTGTCTCCAGTCT	AS		
gapdh	CGACCTCACCTGCCGCCTTACA	S	Real-time PCR	187
	GTCATTGAGGGAGATGCCAGCG	AS		

Table 3-1 Primers used in Chapter 3.

3.2.4 Western blot analysis

Follicles lysates were firstly separated by 10% or 15% SDS-PAGE gels. The separated proteins were transferred onto PVDF membranes and immunoblotted with the primary antibodies. The protein bands were visualized by a Western blotting kit (Millipore, USA) after incubation with secondary antibody conjugated with horseradish peroxidase.

3.2.5 Isolation of ovarian follicles

See Section 2.2.10.

3.2.6 Follicle incubations

See Section 2.2.12.

3.3 Results

3.3.1 Effects of transcriptional and translational inhibitors on Igf3-induced oocyte maturation

To understand the role of steroidogenesis in Igf3-induced oocyte maturation in zebrafish, we tested whether the action of Igf3 in inducing oocyte maturation involves transcriptional and/or translational events. Experiments were performed to examine the actions of actinomycin D (an inhibitor of transcription) and of cycloheximide (an inhibitor of translation) on Igf3-induced oocyte maturation. The results show that the action of Igf3 on oocyte maturation was dramatically suppressed in a dose-dependent manner by a 4-h incubation with cycloheximide (Fig. 3-1A). However, actinomycin D did not cause any significant reduction on the action of Igf3 on oocyte maturation at different doses (Fig. 3-1B).

3.3.2 Effects of a steroidogenesis inhibitor on Igf3-induced oocyte maturation and effects of Igf3 on the expression of steroidogenesis enzymes

We then tested whether the action of Igf3 in inducing oocyte maturation involves steroidogenesis. Experiments were performed to examine the actions of trilostane (a 3β-HSD inhibitor) on Igf3-induced oocyte maturation. The results show that the action of Igf3 on oocyte maturation was not affected at any of several concentrations of trilostane with several incubation times (Fig. 3-2A).

The expression of some important steroid metabolizing enzymes including *cyp19a*, *p450c17a1*, *p450c17a2* and *17b-HSD1* was measured by real-time PCR after treatment with recombinant Igf3 protein. The results show that the expressions of these enzymes was not altered after treatment with Igf3 (Fig. 3-2B).

3.3.3 Effects of Igf1rs inhibitors on Igf3-induced oocyte maturation and effects of Igf3 on the activation of Igf1rs

To investigate whether Igf3 activates oocyte maturation through Igf1rs in zebrafish, we employed two Igf1rs inhibitors (NVP-AEW541 and NVP-AEW742) to address this issue. After treatment with NVP-AEW541, the action of Igf3 on zebrafish oocyte maturation was abolished in a clear dose-dependent manner (Fig. 3-3A). This result could not be explained by the toxicity of the inhibitors on the follicles, as the actions of DHP on oocyte maturation were not affected by (Fig. 3-3B). Similar results were obtained for another inhibitor NVP-AEW742 (Fig. 3-3C). The effects of DHP on oocyte maturation were not significantly influenced by this inhibitor either (Fig. 3-3D). Furthermore, the action of Igf3 on the phosphorylation of Igf1rs was also tested by Western blot analysis. The results show that the phosphorylation of Igf3 was stimulated after treatment with recombinant zebrafish Igf3 protein in a time-dependent manner. The translation of cyclin B was also stimulated by Igf3 in a time-dependent manner, with β-Actin was used as the loading control (Fig. 3-3E).

3.3.4 Effects of PI3 kinase inhibitors on Igf3-induced oocyte maturation

PI3 kinase is one of the major downstream factors of the Igf1r signaling pathway. To investigate whether PI3 kinase activation is involved in Igf3-induced oocyte maturation, two common PI3K inhibitors (wortamannin and LY294002) were used. Incubation of zebrafish FG-stage follicles with different concentrations of either wortamannin or LY294002, significantly reduced the effect of Igf3 on oocyte maturation in a dose-dependent manner (Fig. 3-4).

3.3.5 Effects of a PDE3 inhibitor on Igf3-induced oocyte maturation

Cyclic nucleotide phosphodiesterases (PDEs) are enzymes that degrade and

inactivate cAMP, an event associated with the resumption of meiosis. Type 3 phosphodiesterase (PDE3) is an oocyte-specific PDE that is required for the effect of PI3 kinase/Akt on oocyte maturation (Conti et al., 2002). The role of PDE3 in Igf3-induced oocyte maturation was tested with cilostamide, a specific inhibitor of PDE3. The induction of oocyte maturation by Igf3 was prevented in a dose dependent manner by incubating the follicles with cilostamide (Fig. 3-5).

3.3.6 Effects of Igf3 on MAP kinase activation and effects of MAP kinase inhibitors on Igf3-induced oocyte maturation

To further investigate the early signal transduction components involved in Igf3-induced oocyte maturation, the possible role of mitogen-activated protein kinase (MAPK) was examined. The level of phosphorylated ERK1/2 increased after 30 min in response to Igf3 (Fig. 3-6A). This stimulatory action of Igf3 could not be attributed to altered ERK protein levels, as the amount of total ERK1/2 protein was not changed by the Igf3 treatment. Additionally, a specific inhibitor of MAP kinase kinase (MEK), PD98059, was tested. FG-stage follicles were pre-incubated for 1 h with increasing doses (1, 5 and 25 μM) of PD98059 followed by incubation with Igf3 (2 μM). Oocyte maturation was examined at different time points. PD98059 significantly inhibited Igf3-induced oocyte maturation in a dose-dependent manner (Fig. 3-6B).

3.4 Discussion

In our previous study, we demonstrated a role for Igf3 in oocyte maturation in zebrafish. In the present study, we extended those results by investigating how Igf3 exerts its effects on oocyte maturation. We investigated whether the steroidogenesis is involved in Igf3-induced oocyte maturation. The action of Igf3 was suppressed by cycloheximide but not by actinomycin D suggesting that Igf3 acts by regulating protein synthesis and may not act via the activation of steroidogenesis ezyme

expression during oocyte maturation. Trilostane is an inhibitor of 3β-HSD that is able to attenuate DHP production, and we found that the effect of Igf3 on oocyte maturation was not significantly influenced by this inhibitor. Our results are consistent with other studies of Igf1 that reported the failure of inhibitors of 3β-HSD to block Igf1-induced oocyte maturation in fish ovarian follicles (Kagawa et al., 1994; Kagawa et al., 1995; Negatu et al., 1998; Weber and Sullivan, 2000). The action of Igf3 on the induction of GVBD via a steroid-independent pathway is further supported by the inability of Igf3 to regulate the expression of important steroid metabolizing enzymes including *cyp19a*, *p450c17a1*, *p450c17a2* and *17b-HSD1*. After treatment with recombinant Igf3 protein, the results of real-time PCR showed that the expression of these enzymes was not altered. This finding suggests that Igf3 exerts no significant effects on the estrogen and DHP synthesis pathways in zebrafish follicles.

To study the downstream signaling pathway involved in Igf3-induced oocyte maturation in zebrafish, we investigated whether Igf3 bind to and activate Igf1rs. There are two types of Igf receptors including Igf type 1 receptor (Igf1r) and Igf type 2 receptor (Igf2r). The latter is also called mannose 6-receptor and has a high affinity for Igf2 in mammals. Igf2r binds and targets Igf2 for lysosomal degradation without inducing an intracellular response (Kornfeld, 1992). Available information indicates that the C domain of human Igf1 is responsible for its high affinity for Igf1r but not for Igf2r, and diversity exists between the amino acid sequences of Igf3 and other Igfs in the C domain. Previous research has also indicated that this diversity in the C domain suggests the presence of a currently unknown receptor for Igf3. Alternatively, the A and B domains of Igf3 are highly conserved, and are essential for receptor binding and the subsequent biological actions of all Igfs. The cysteine residues in the B domain are conserved, and the predicted tertiary structures of the four Igfs in zebrafish are similar. All of these features indicate the possibility that Igf3 binds to

Igf1r. To substantiate this hypothesis, we employed two specific Igf1rs inhibitors that have been demonstrated to successfully the phosphyorylation of Igf1rs in zebrafish (Chablais and Jazwinska, 2010). The results from the present study showed that the effects of Igf3 on oocyte maturation were diminished by two specific Igf1rs inhibitors in a dose-dependent manner. Furthermore, we also demonstrated that Igf1rs can be activated by Igf3 in a time dependent manner. In comparison with FG stage follicles, the strongly activated Igf1rs was detected in naturally matured oocytes, which implies an important role for the Igf pathway in the process of oocyte maturation. All of this evidence suggests that Igf3 stimulates oocyte maturation through Igf1rs in zebrafish.

In the present study, two PI3 kinase inhibitors with different mechanisms of action, wortamannin and LY294002, blocked the effect of Igf3 on oocyte maturation in a dose-dependent manner. These results are consistent with similar studies on Igf1 in other species including *Xenopus* (Schmitt and Nebreda, 2002), striped bass (Weber and Sullivan, 2001) and *Cyprinus carpio* (Paul et al., 2009). The requirement of PI3 kinase activation for steroid-induced oocyte maturation has also been reported in star fish (Sadler and Ruderman, 1998), Atlantic croaker (Pace and Thomas, 2005), *Rana dybowskii* (Ju et al., 2002) and white bass (Weber and Sullivan, 2005). All of these findings suggest that the early signaling pathways activated by Igfs and MIH during oocyte maturation may be similar in zebrafish.

It has been found that PDE3 is the predominant PDE isoform in mammalian oocytes (Tsafriri et al., 1996), and the activity of a PDE3 is required for the effects of Pi3-k/Akt action on oocyte maturation (Andersen et al., 1998). The current finding that the inhibition of PDE3 by cilostamide significantly reduced Igf3-mediated effects on oocyte maturation in zebrafish supports the importance of PDE3 in Igf-induced oocyte maturation.

Results from the present study also demonstrated that MAP kinase is rapidly and

strongly activated in Igf3-stimulated oocytes. This activation is also specific to the induction of GVBD and takes place about 30 min before cyclin B becomes activated. Activated ERK was also detected in natural matured oocytes. MAP kinase activation is universal during oocyte maturation, but its requirement for GVBD induction is uncertain. Earlier studies have shown that MOS/MAPK pathway plays a major role in progesterone-induced meiotic resumption in Xenopus oocytes (Sagata et al., 1989). However, available information from starfish, goldfish, Atlantic croaker and mouse oocytes have shown that MAP kinase activation is not essential for oocyte maturation (Ferrell, 1999; Liang et al., 2007). In this study, the effects of Igf3 on oocyte maturation were blocked by an MAPK inhibitor, PD98059, in a dose dependent manner. Therefore, these evidence obtained from our study strongly suggests that the activation of MAP kinase serves as an important component of the Igf3-induced signal transduction cascade leading to the stimulation of GVBD.

Taken together, the present study has indicated that Igf3 stimulate the oocyte maturation via a steroid-independent manner and that Igf3 induces oocyte maturation through Igf1rs. PI3 kinase, PDE3 and MAP kinase are necessary for Igf3-mediated oocyte maturation in zebrafish. This evidence not only helps us understand the molecular mechanism of the effects of Igf3 on oocyte maturation but also supplies valuable information for the study of the signaling pathway involved in meiotic resumption in fish.

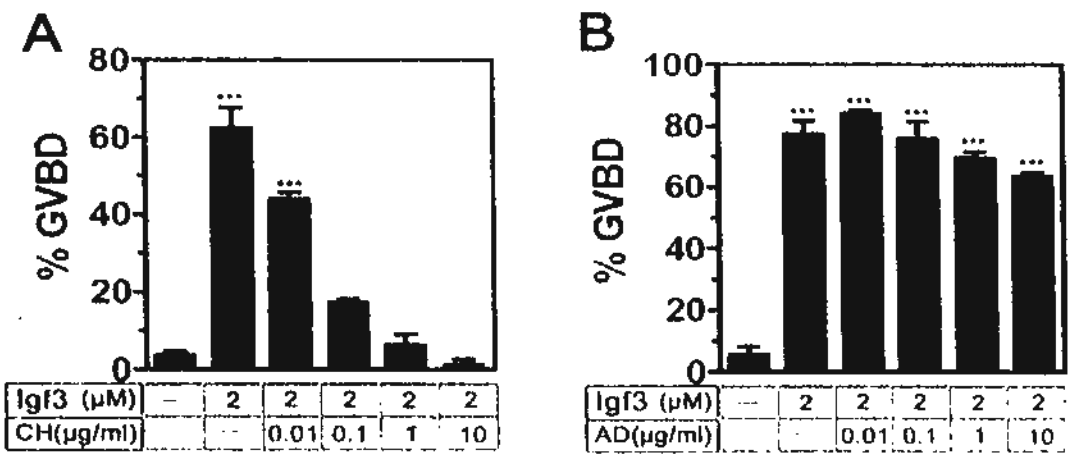

Fig. 3-1 Effects of cycloheximide (CH) and actinomycin D (AD) on Igf3-induced zebrafish oocyte maturation.

(A) Dose-dependent inhibition of Igf3-induced zebrafish oocyte maturation by CH. (B) The effect of AD on Igf3-induced oocyte maturation. Full grown follicles were treated with recombinant Igf3 (2 μM) in the absence or presence of AD or CH, and GVBD was scored after treatment. Each value represents the mean ± SEM of three independent experiments each performed in quadruplicate (***P<0.001 vs control).

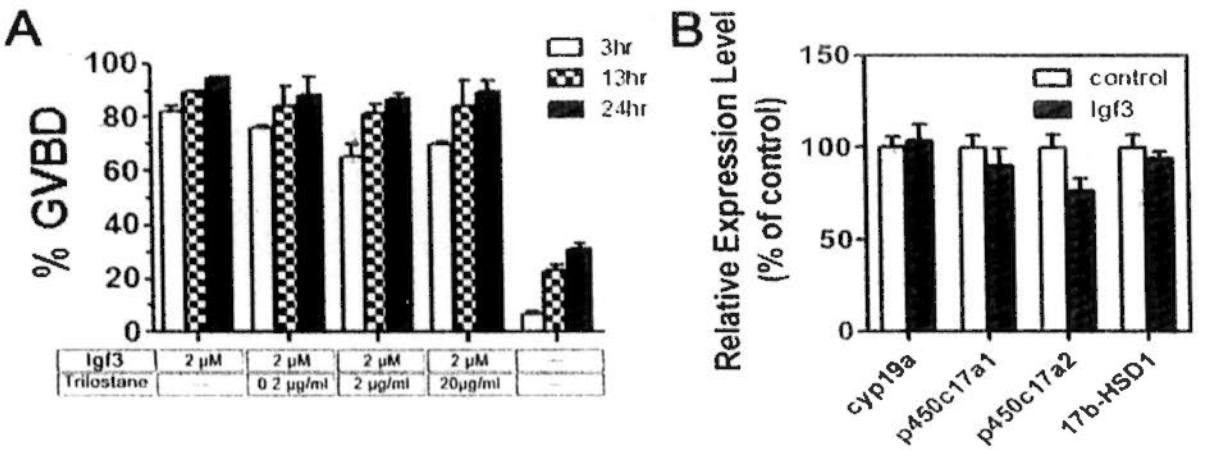

Fig. 3-2 Effects of steroidogenesis inhibitor on Igf3-induced oocyte maturation and effect of Igf3 on expression of steroidogenesis enzymes

(A) The lack of action of trilostane (a 3β-HSD inhibitor) on Igf3-induced oocyte maturation in different concentrations (0.2, 2 and 20 μg/ml). Full-grown follicles were treated with recombinant Igf3 (2 μM) in the absence or presence of trilostane, and GVBD was scored after treatment. (B) Relative mRNA level of *cyp19a*, *p450c17a1*, *p450c17a2* and *17b-HSD1* in MV follicles treated with zebrafish Igf3 protein for 1 h. White bar, control; Black bar, Igf3 treatment. Each value represents the mean ± SEM of three independent experiments each performed in quadruplicates.

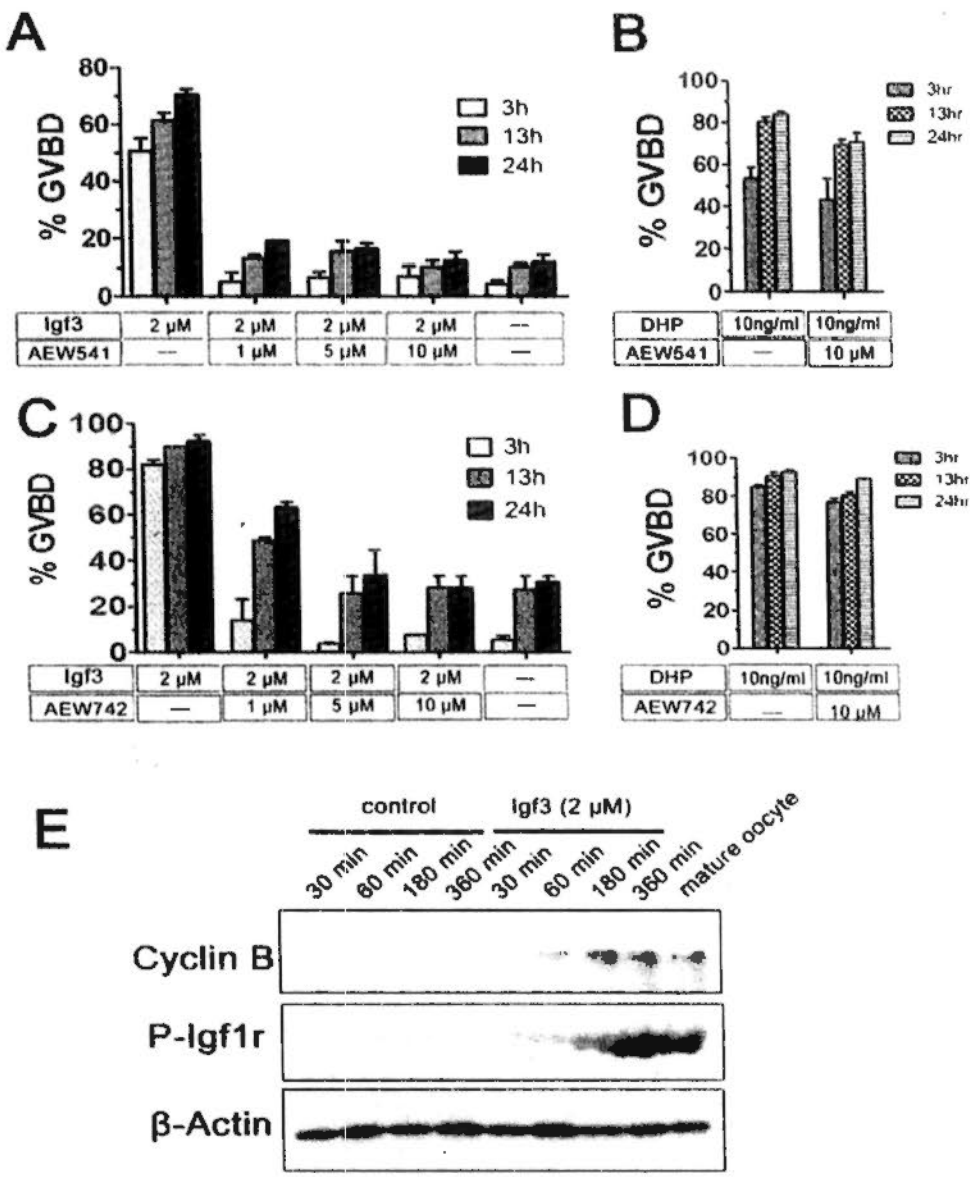

Fig. 3-3 Igf3 stimulates oocyte maturation through Igf1r in zebrafish.
(A) Inhibition of Igf3-induced oocyte maturation by different doses of NVP-AEW541; (B) DHP-induced oocyte maturation was not significantly blocked by different doses of NVP-AEW541; (C) Inhibition of Igf3-induced oocyte maturation by different doses of NVP-AEW742; (D) DHP-induced oocyte maturation was not significantly blocked by different dose of NVP-AEW742; (E) Western blot results regarding the effect of Igf3 on the phosphorylation of Igf1rs in zebrafish FG-stage follicles.

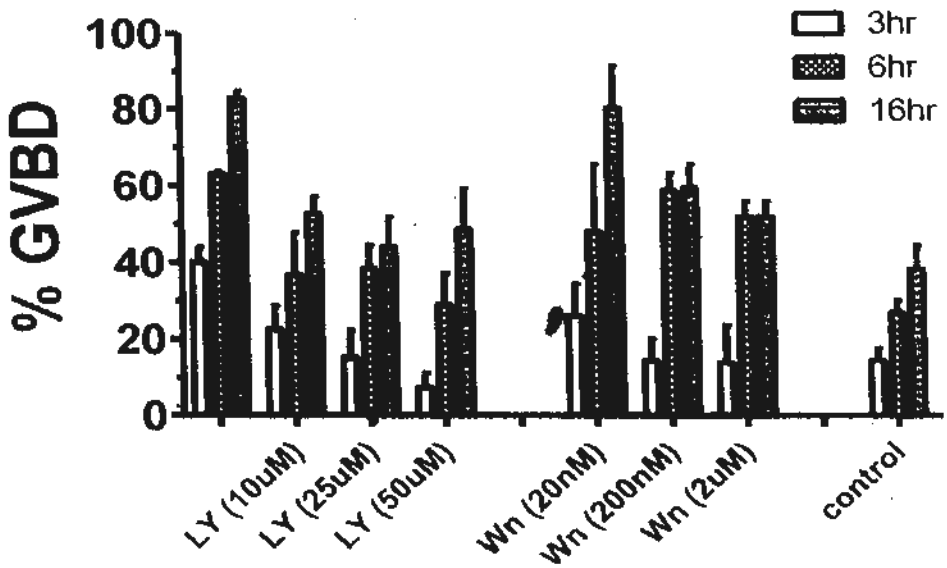

Fig. 3-4 Effect of PI3-k inhibitors on Igf3-induced oocyte maturation.
The percentage of zebrafish follicles showing GVBD after incubation with Igf3 (2 μM), in the presence of graded doses of either PI3-k inhibitor, LY294002 (LY) or Wortmannin (Wn), at different incubation time points. Full-grown zebrafish follicles were pre-incubated for 1 h in the presence of each inhibitor. Data are representative of three independent experiments.

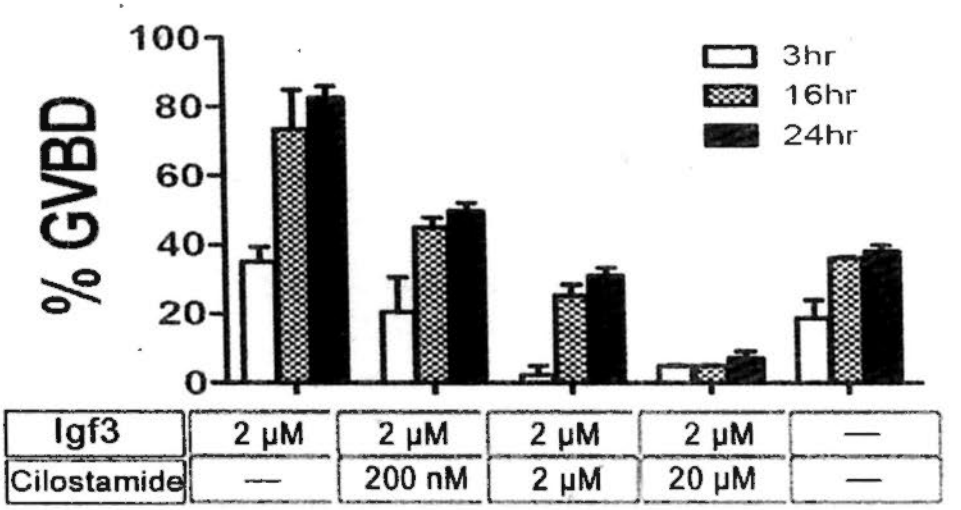

Fig. 3-5 Effects of a PDE3 inhibitor on Igf3-induced oocyte maturation.
The percentage of zebrafish foolicles showing GVBD after incubation with Igf3 (2 μM), in the presence of graded doses of the PDE3 inhibitor, cilostamide at different incubation time points. Full-grown zebrafish follicles were pre-incubated for 1 h in the presence of the inhibitor. Data are representative of three independent experiments.

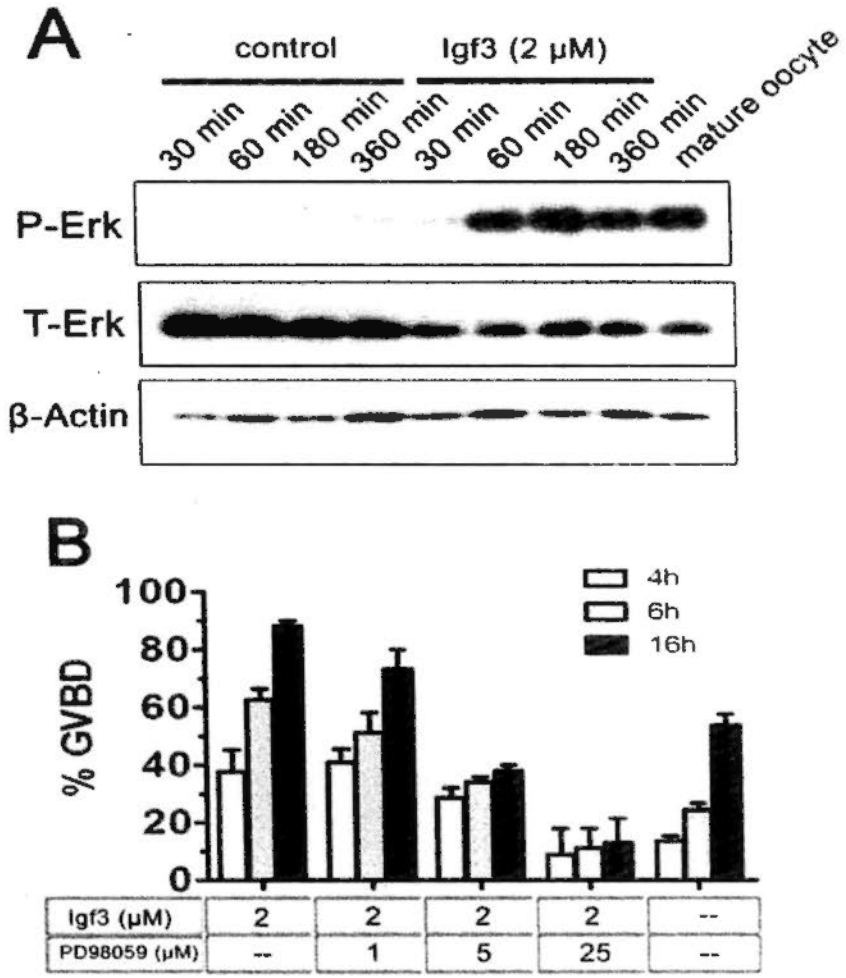

Fig. 3-6 MAP kinase is required for Igf3-induced oocyte maturation in zebrafish. (A) Western blot analyses of phosphorylated Erk (P-Erk1/2) and total Erk (T-Erk1/2) incubated with or without Igf3 (2 μM) for 30-360 min; (B) The percentage of zebrafish follicles showing GVBD after incubation with Igf3 (2 μM) in the presence of graded doses of the MEK inhibitor, PD98059 (PD) at different incubation time points. Full-grown zebrafish follicles were pre-incubated for 1 h in the presence of the inhibitors.

Chapter 4 Insulin-like growth factors in the zebrafish ovary: differential expression and regulation by gonadotropin

4.1 Introduction

It has been conclusively that gonadatropins secreted from the pituitary control many aspects of gonadal development and function in vertebrates (Hillier, 2001). This concept of classical endocrine control of reproductive function is now extended to a more complex regulatory system, including paracrine and autocrine modulating mechanisms (Ge, 2005; Hillier, 2001; Matzuk et al., 2002). As the significance of putative intraovarian regulators became increasingly recognized, much of the attention has centered on insulin-like growth factors (Igfs). Igfs are low molecular weight peptide hormones with structures related to insulin and relaxin in vertebrates and insulin-like peptides (ILPs) in invertebrates. Activation of the Igf signaling pathway occurs when Igfs bind their cognate receptor tyrosine kinases, which leads to the activation of a number of downstream signaling cascades, including pathways involving mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)-Akt (Wood et al., 2005).

In mammals, the expression of the genes encoding Igf1 and Igf2 in the ovary has now been clearly established in several species, including the rat (Adashi et al., 1997), sheep (Perks et al., 1995; Teissier et al., 1994), cow (Perks et al., 1999), pig (Yuan et al., 1996) and human (Zhou and Bondy, 1993). However, there is some heterogeneity in the localization of Igf1 and Igf2 in the ovary among different species. For example, in the rat and mouse, the expression of *igf1* mRNA was detected in the granulosa cells of developing preantral follicles and antral follicles. However, the expression level of *igf1* mRNA in the ovary of cow and sheep is extremely low (Perks et al., 1995; Perks et al., 1999), whereas *igf2* expression is detectable in the thecal cells of both species, with high expression in early growing follicles (Armstrong and Webb, 1997). In humans, Igf2 expression is high in

granulosa and thecal compartments, and its expression increases in granulosa cells as follicle size increases. In contrast, Igf1 is only weakly detectable, suggesting that the importance of Igf2 in the human ovarian Igf system overweighs that of Igf1 (el-Roeiy et al., 1993; Yoshimura, 2003; Zhou and Bondy, 1993).

Igf1 knockout mice do not contain any antral follicles and are unable to ovulate, even after treatment with exogenous gonadotropins, which directly indicates the essential role of the Igf system in the ovary (Baker et al., 1996). Both Igf1 and Igf2 have been demonstrated to stimulate the growth of secondary follicles, promote the proliferation of granulosa cell, inhibit apoptosis and promote oocyte maturation (Sirotkin, 2010).

There are a few reports on the regulation of *igfs* by gonadatropin in the ovary. The available information on the regulation of Igf1 by gonadotropin is controversial in the mouse ovary. Either LH or FSH can significantly increase the secretion of Igf1 in granulosa cells (Hsu and Hammond, 1987). However, Zhou and colleagues found that FSH does not regulate granulosa cell Igf1 gene expression, as the latter appears normal in FSH knockout ovaries and is unperturbed by hypophysectomy in normal animals (Zhou et al., 1997). In humans, several lines of evidence support the conclusion that the LH and FSH regulate *igf2* mRNA expression and secretion in granulosa cells (Ramasharma and Li, 1987; Voutilainen and Miller, 1987).

Despite several reports demonstrating that human Igf1 and Igf2 induce oocyte maturation in several fish species including zebrafish (Kagawa et al., 1994; Negatu et al., 1998; Nelson and Van Der Kraak, 2010b; Weber and Sullivan, 2000), information on the regulation and bioactivity of ovarian paracrine/autocrine Igf action is still largely unknown. Recently, a gonad-specific Igf (Igf3) was identified in our lab (Wang et al., 2008). We also demonstrated the regulation of *igf3* by gonadotropin through the cAMP pathway and the role of Igf3 in oocyte maturation in zebrafish (Li et al., 2011). Considering that four Igfs have been identified in

zebrafish, the present study was undertaken to study the expression and regulation of these Igfs in the zebrafish ovary. After analyzing the temporal expression profiles of all four Igfs during ovarian folliculogenesis; the regulation of the four Igfs by gonadotropin and cAMP was investigated in primary follicle cells and fully grown follicles. Evidence from the present study suggests functional convergence and differentiation of the four Igfs in zebrafish ovary.

4.2 Materials and Methods

4.2.1 Chemicals

All chemicals were obtained from Sigma (St. Louis, MO), and restriction enzymes were from Promega (Madison, WI) unless otherwise stated. Human CG (hCG) and 3-iso-butyl-1-methylxanthine (IBMX) were purchased from Sigma. hCG was dissolved in water and diluted to the final concentrations with the medium, whereas IBMX was first dissolved in ethanol and then diluted to the desired concentrations with the medium before use.

4.2.2 Animal

See Section 2.2.1.

4.2.3 RNA isolation and RT-PCR

Total RNA samples were isolated from the tissues, ovarian follicles, embryos and fries of zebrafish, using TRIzol Reagent (Invitrogen, USA). The quality and quantity of the RNA were determined by NanoDrop 2000C Spectrophotometer (Thermo, USA). RT-PCR was performed as Section 2.2.5. All primers used in this study are listed in Table 4-1. For real-time PCR, the standards for *igf1*, *igf2a*, *igf2b*, *igf3* and *gapdh* (glyceraldehyde-3-phosphate dehydrogenase) were prepared by amplification of cDNA fragments with the specific primers (Table 4-1). The amplicons were purified by the PCR Purification Kit (Qiagen, USA). These PCR products were used

to construct the standard curves in the real-time PCR assays. Real-time PCR was carried out as described in Section 2.2.5.

4.2.4 Isolation of ovarian follicles

See section 2.2.10.

4.2.3 Primary culture of the ovarian follicle cells

The primary culture of zebrafish ovarian follicle cells was performed according to the protocol of Ge's lab (Pang and Ge, 2002b). Briefly, the follicles around vetellogenic stage from 15 to 25 females were carefully selected and cultured in 25 cm^2 flask for 6 days in the medium of M199+10% fetal bovine serum (FBS) under the condition of 28 °C and 5% CO_2. The medium was changed on the third day of the incubation. After the 6-day incubation, the cells were then sub-cultured in 24-well plates at the density of 100 000 cells/1 ml per well for 24 h in M199+10% FBS before hormone and drug treatment.

4.3 Results

4.3.1 Differential gene expression of four *igfs* during zebrafish folliculogenesis

Real-time PCR assays to detect the *igfs* and *gapdh* were established. Specific *igfs* or *gapdh* DNA fragments were amplified from the ovarian cDNA, and these PCR products were purified and used as standards in the assays. Standard curves were generated according to the cycle threshold value (Fig. 4-1). RT-PCR was first performed to examine the gene expression of the four *igfs* in the zebrafish ovary. All four *igfs* transcripts were detected in the ovary (Fig. 4-2A). Using real-time PCR, the expression levels of the four *igf* transcripts in the ovary were compared. The mRNA level of *igf1* was extremely compared with that of the other *igfs*, and the expression of *igf3* was highestof the four *igfs* (Fig. 4-2B).

The expression profiles of all *igfs* during folliculogenesis were further assessed

using real-time PCR. The level of *igf1* slightly increased from the primary growth (PG) stage through the early vitellogenic (EV) stage and decreased thereafter (Fig. 4-3A). On the other hand, *igf2a* and *igf2b* steadily increased during folliculogenesis (Fig. 4-3B and C). The expression profile of *igf3* during folliculogenesis was the most drastic among the four *igf* ligands. The expression of *igf3* was low in the early stages of the follicles development but dramatically increased in the full grown (FG) stage (Fig. 4-3D).

4.3.2 Effects of gonadotropin (hCG) on the expression of the four *igfs* in the FG-stage follicles

To examine whether gonadotropin regulates the expression of all *igfs* in the zebrafish follicles, intact FG-stage follicles were first treated. Treatment of cultured zebrafish ovarian follicles with hCG for 2 hours at different concentrations caused a slight change in *igf1* expression (Fig. 4-4A) but a significant change in the expression of *igf2a* (Fig. 4-4B), *igf2b* (Fig. 4-4C) and *igf3* (Fig. 4-4D). The expression of *igf2a* was decreased by hCG in a dose-dependent manner. Although the differences were not statistically significant, increased levels of *igf2b* were observed at different concentrations of hCG, reaching a peak with treatment with 1 IU/ml hCG treatment, hCG stimulated the level of *igf3* at a low concentration, but a high dosage of hCG had a strong desensitizing effect on the responsiveness of the cells. At 100 IU/ml, the stimulation of receptor expression declined significantly compared with the stimulation observed at 10 IU/ml.

4.3.3 Effects of gonadotropin (hCG) on the expression of four *igfs* in the cultured zebrafish follicle cells

To further study the regulation of Igfs by gonadotropin, a primary culture of zebrafish ovarian follicle cells was established (Fig. 4-5).

The regulation of *igfs* by gonadotropin was examined in the zebrafish ovarian follicle cells. Treatment of the cultured zebrafish ovarian follicle cells with hCG for 2 hours at different concentrations caused a significant change in the expression of *igf2a*, *igf2b* and *igf3*. Consistent with the results of treatment of the FG stage follicles, the expression of *igf1* was not changed after treatment with different concentrations of hCG (Fig. 4-6A), whereas hCG decreased the expression of *igf2a* (Fig. 4-6B) and increased the expression of *igf2b* (Fig. 4-6C) and *igf3* (Fig. 4-6D).

4.3.4 Effects of IBMX on the expression of four *igfs* in the cultured zebrafish follicle cells

Because cAMP is considered to be the principal second messenger involved in gonadotropin signaling, we also studied the effects of increased intracellular cAMP levels on the expression of *igfs* to demonstrate whether cAMP is involved in the gonadotropin regulation of *igfs*. Different concentrations of IBMX (an inhibitor of phosphodiesterase) were used to treat the cultured zebrafish follicle cells for 2 h. Similar to the effect of hCG, *igf1* expression was not changed by IBMX (Fig. 4-7A), but IBMX suppressed the expression of *igf2a* (Fig. 4-7B) and increased the expression of *igf2b* (Fig. 4-7C) and *igf3* (Fig. 4-7D).

4.4 Discussion

In the present study, we first tracked the expression of the *igf1*, *igf2a*, *igf2b* and *igf3* genes in the adult zebrafish ovary. The results showed that the expression of all four *igfs* expression can be detected in the ovary by RT-PCR. However, the expression levels of these *igfs* were different. The level of *igf1* mRNA was extremely low in the ovary, but its expression could still be detected by both RT-PCR and real-time PCR. The mRNA expression levels of other *igfs* including *igf2a*, *igf2b* and *igf3*, were similar in the ovary, with *igf3* showing the highest expression level. Detection of *igfs* expression in the zebrafish ovary is consistent with the results of a

recent study, although the expression of *igf1* was not detected in that study (Nelson and Van Der Kraak, 2010a). In mammals, there are only two Igf ligands known as Igf1 and Igf2. The expression of both Igf1 and Igf2 in the ovary has been extensively studied in many species and great diversity in Igfs expression exists in these species (Mazerbourg et al., 2003). In rodents, Igf1 is the predominant subtype of Igfs in the ovary, whereas in primate, the expression of Igf2 is significantly higher than Igf1 in ovary indicating the importance of Igf2 in the primate ovarian Igf system (Yoshimura, 2003). The present study suggests that Igf2a, Igf2b and Igf3 may have a more important paracrine/autocrine role in the zebrafish ovary compared to Igf1.

In zebrafish, both Igf1 and Igf2 promote the development of oocyte maturation competence and stimulate final oocyte maturation, although Igf1 and Igf2 recombinant proteins from a human source were used in the experiments drawing those conclusions (Nelson and Van Der Kraak, 2010b); moreover, the involvement of Igf3 in final oocyte maturation was also demonstrated by our lab (Li et al., 2011). Our previous study showed that the expression of *igf3* was up-regulated by hCG in the ovarian fragments and separated follicles; however, the regulation of other *igfs* including *igf1*, *igf2a* and *igf2b* remains largely unknown. This study demonstrated that the expression of *igf2b* and *igf3* was up-regulated in a dose-dependent manner in FG-stage follicles. These results are consistent with our previous results regarding *igf3* and a recent study of the regulation of *igf2b* by Nelson et al. (Nelson and Van Der Kraak, 2010a). In contrast to its stimulation of *igf2b* and *igf3*, treatment with hCG had a inhibitory effect on the expression of *igf2a*. No effect on the *igf1* expression was observed under the same conditions. To determine whether gonadotropin regulates the expression of *igfs* in the follicle cells, a primary culture of ovarian follicle cells was prepared from zebrafish vitellogenic stage follicles. This stage was chosen because both gonadotropin (hCG) and Igfs have powerful effects on follicles at this stage (Li et al., 2011; Nelson and Van Der Kraak, 2010b; Pang and

Ge, 2002a). All four *igfs* were expressed in the cultured follicle cells, as demonstrated by RT-PCR (data not shown). Similar to the results with FG-stage follicles, hCG also exhibited the similar effects on the levels of mRNA levels of all four *igfs*, confirming the actions of hCG on the four *igf* ligands. These results also indicate that the regulation of *igf2a*, *igf2b* and *igf3* in follicle cells can be regulated by hCG. Although information on the regulation of *igfs* in the ovaries of other model animal is limited, the up-regulation of zebrafish *igf2b* by gonadotropin agrees well with reports that LH and FSH regulate *igf2* mRNA expression and secretion in human granulosa cells (Ramasharma and Li, 1987; Voutilainen and Miller, 1987). *igf1* is the major form of *igf* in the mouse ovary of mouse and could be up-regulated either luteinizing hormone (LH) or follicle-stimulating hormone (FSH) (Hsu and Hammond, 1987). Moreover, evidence from research on the testis also supports the notion that gonadotropins modulate Igf expression in mammals and amphibians, for example, *igf1* mRNA levels were increased by FSH in newt testicular organ culture (Yamamoto et al., 2001). In rodent and procine Sertoli cells, Igf1 secretion was stimulated by FSH and LH (Cailleau et al., 1990; Naville et al., 1990). In addition, opposing effects of hCG on the expression of *igf2a* and *igf2b* were observed in the present study. Although the mechanisms by which these two subtypes of Igf are differentially controlled in zebrafish are not yet clear, but the inverse effects of hCG on their expression suggest that pituitary gonadotropins are likely to be involved. It is generally accepted that gonadotropins regulate target gene expression primarily via activation of the cAMP-PKA pathway. To further investigate the effects of cAMP on the expression of *igfs* in follicles cells, we carried out experiments to examine the response of the four *igfs* to IBMX, which increases intracellular cAMP levels. The results showed that IBMX mimicked the differential regulation of *igf2a*, *igf2b* and *igf3* by hCG, suggesting that cAMP is likely to be the major second messenger involved in the differential effects of gonadotropin on the expression of these *igfs*

expression in follicle cells. The up-regulation of *igf3* expression by cAMP in follicle cells is consistent with our previous study of ovarian fragments and different stage of follicles (Li et al., 2011). *igf2b* expression was stimulated by cAMP in zebrafish follicle cells, similar to studies in humans, in which cAMP stimulated the expression of *igf2* in cultured granulosa cells (el-Roeiy et al., 1993).

Taken together, our results show that all four *igfs* are expressed in the ovary of zebrafish and exhibit differential expression profiles during folliculogenesis. The present study not only demonstrated that hCG stimulated *igf2b* and *igf3* expression and suppressed *igf2a* expression, but also provided evidence for the first time that the effect of gonadotropin can be mimicked by IBMX, which increases the intracellular levels of cAMP. This finding suggests the possibility of the involvement of cAMP in the gonadotropin-mediated regulation of the differential expression of *igf2a*, *igf2b* and *igf3*. Ongoing studies are addressing the precise mechanisms of gonadotropin regulation on Igf signaling and the role of Igfs in oocyte maturation.

Gene	Sequence(5' to 3' direction)	Strand	Application	Size (bp)
igf1	AGGTCACACAACCGTGGCATC	S	Real-time PCR	235
	TAGTTTCTGCCCCCTGTGTTTCC	AS		
igf2a	GGTCTTCCCAGTGTCACAGGCTC	S	Real-time PCR	167
	TGCTCCTCATCTTGGATTTTCTC	AS		
igf2b	CATCATTCTGTTTGCCATACCTG	S	Real-time PCR	177
	ACACAAACTGTAGAGCGTCCACC	AS		
igf3	GCCAAACGCCTTCAGATAATGC	S	Real-time PCR	203
	GCTGCTCCAGGTTTGCCTATGT	AS		
18s	CCTGAGAAACGGCTACCACATCC	S	RT-PCR	220
	AGCAACTTTAGTATACGCTATTGGAG	AS		
gapdh	CGACCTCACCTGCCGCCTTACA	S	Real-time PCR	187
	GTCATTGAGGGAGATGCCAGCG	AS		

Table. 4-1 Primers used in Chapter 4.

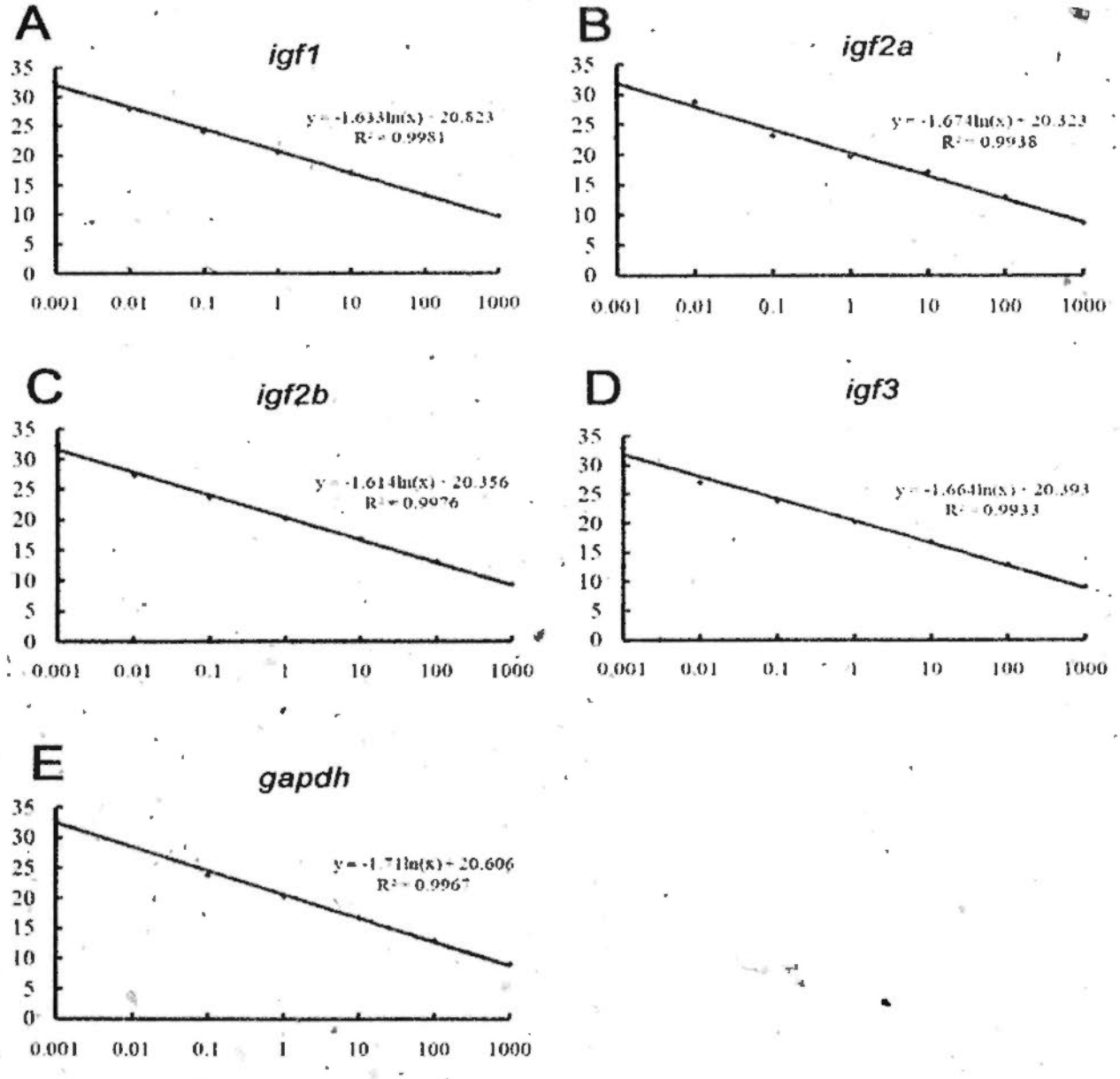

Fig. 4-1. Validation of real-time PCR assay for the expression of zebrafish *igf1 igf2a*, *igf2b*, *igf3* and *gapdh*.
Standard curve generated by serially diluted purified products of *igf1* (A); *igf2a* (B); *igf2b* (C); *igf3* (D) and *gapdh* (E).

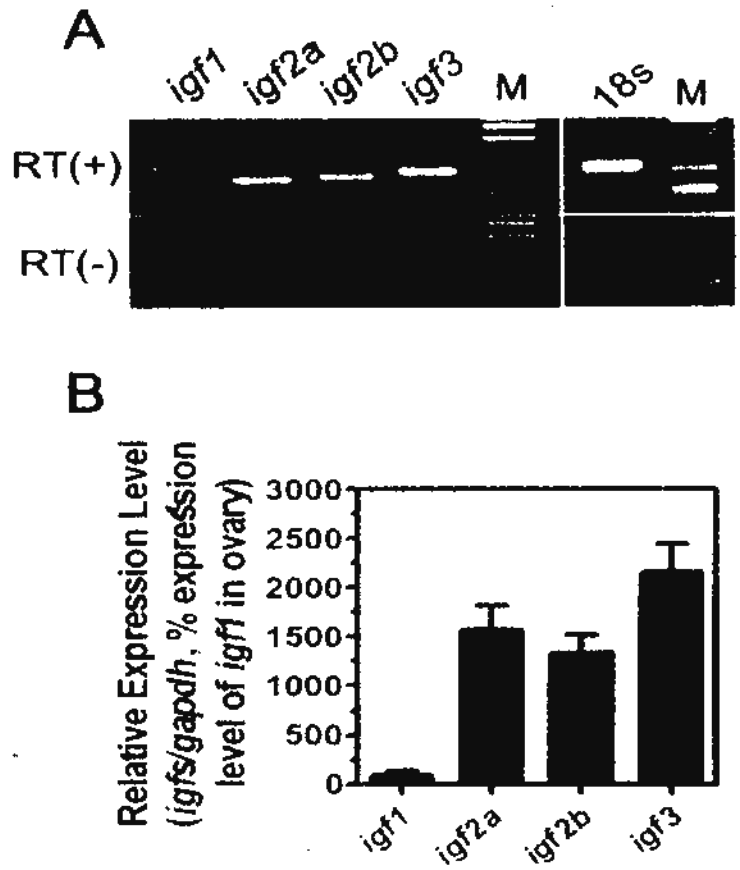

Fig. 4-2 The expression of *igf1*, *igf2a*, *igf2b* and *igf3* in the zebrafish ovary.
(A) RT-PCR detection of *igf1*, *igf2a*, *igf2b* and *igf3* expression in the ovary. RT(+): with reverse transcription; RT(-): RNA without reverse transcription; M: marker. (B) Real-time PCR detection of *igf1*, *igf2a*, *igf2b* and *igf3* expression in the ovary.

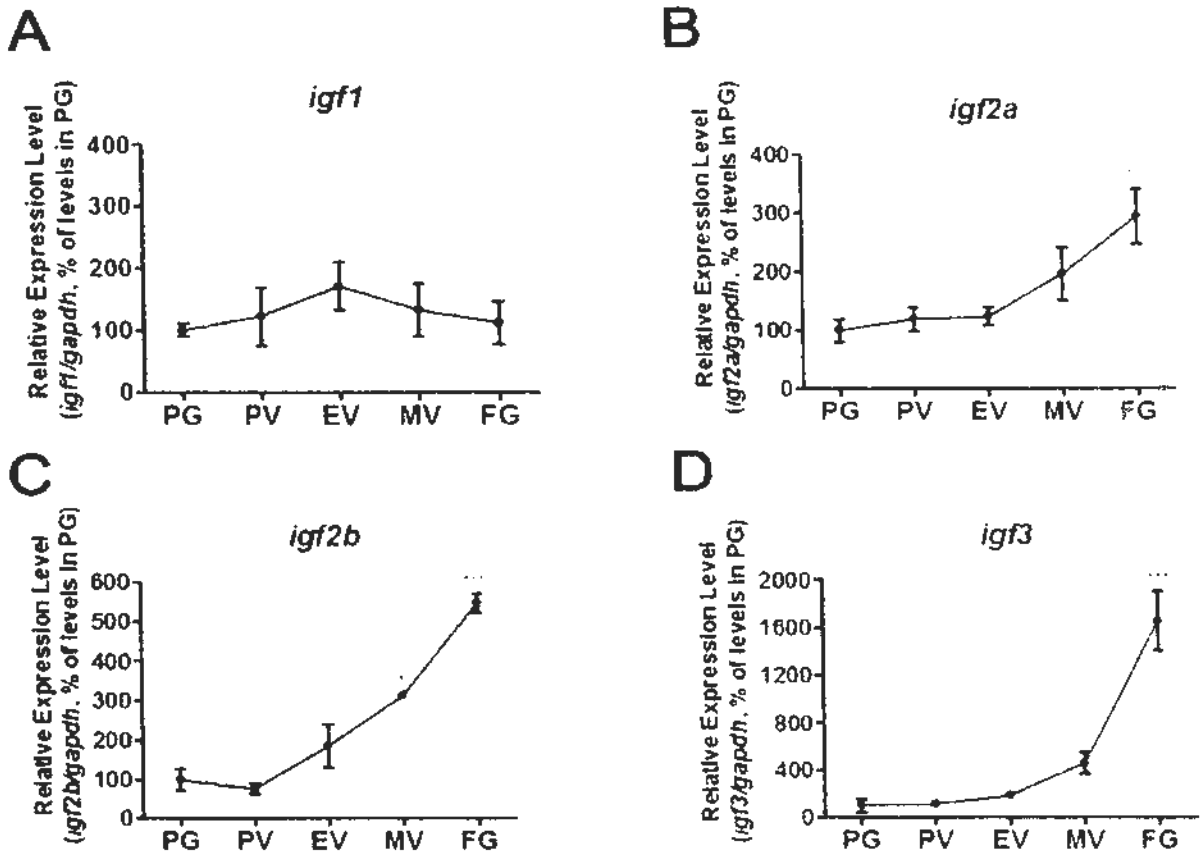

Fig. 4-3 Temporal expression of *igf1*, *igf2a*, *igf2b* and *igf3* during folliculogenesis. Relative levels of *igf1* (A), *igf2a* (B), *igf2b* (C) and *igf3* (D) mRNA in the follicles at the different stages; each value represents the mean ± SEM of three independent experiments, each performed in quadruplicate (*P<0.05; **P<0.01; ***P<0.001 vs. control).

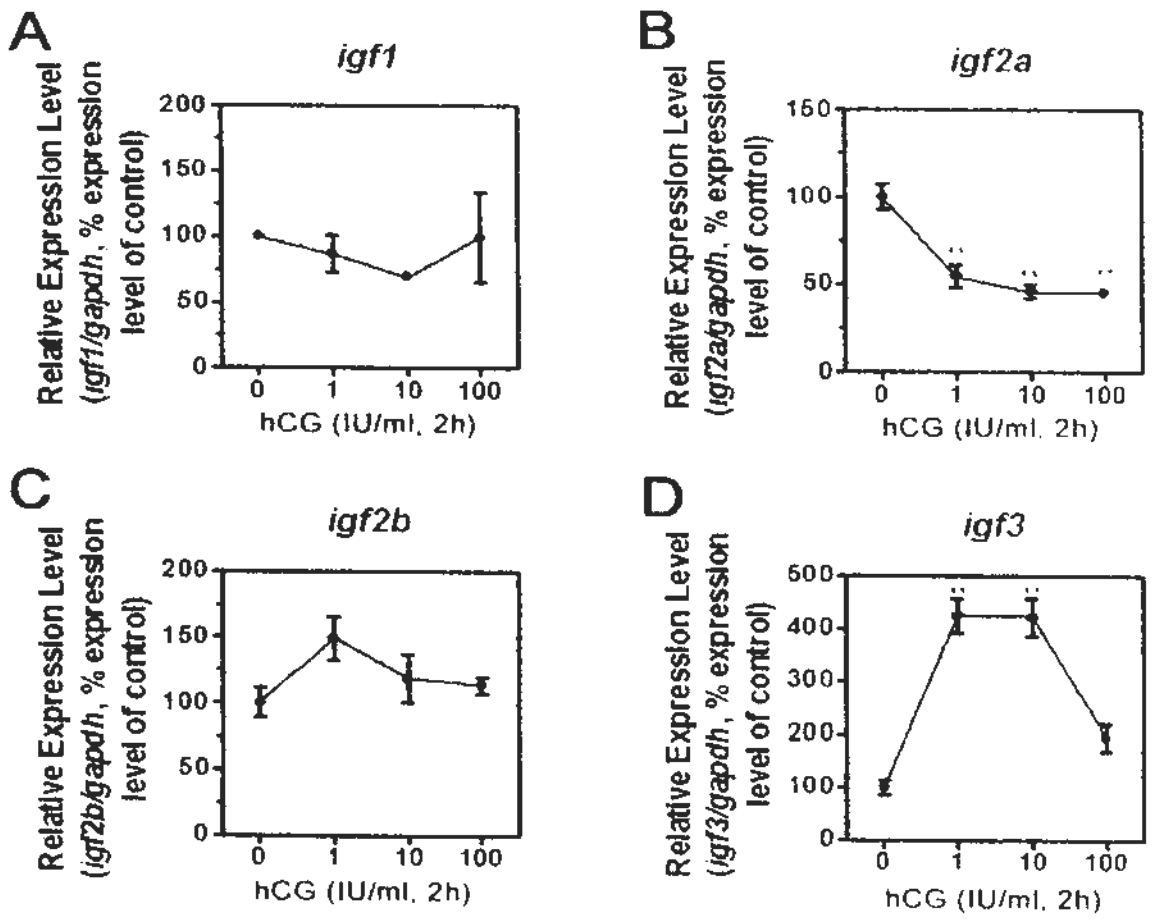

Fig. 4-4 Dose response of gonadotropin (hCG) effects on the expression of *igf1*, *igf2a*, *igf2b* and *igf3* in FG-stage follicles.

Expression levels were normalized to gapdh, and expressed as a percentage of the control. Regulation of *igf1* (A), *igf2a* (B), *igf2b* (C) and *igf3* (D) expression by different concentrations of hCG. Each value represents the mean ± SEM of three independent experiments each performed in triplicate (*P<0.05; **P<0.01; ***P<0.001 vs. control).

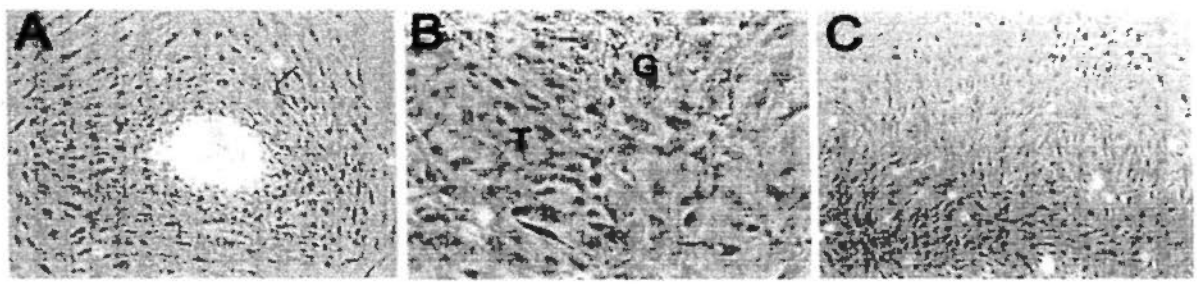

Fig. 4-5 Primary culture of the zebrafish ovarian follicle cells.
(A) A primary culture of follicle cells after 6 days of incubation. (B) The two types of epithelial cells present in the culture with distinct morphologies. G, granulosa cells; T, theca cells. (C) Subcultured follicle cells in a 24-well plate before treatment.

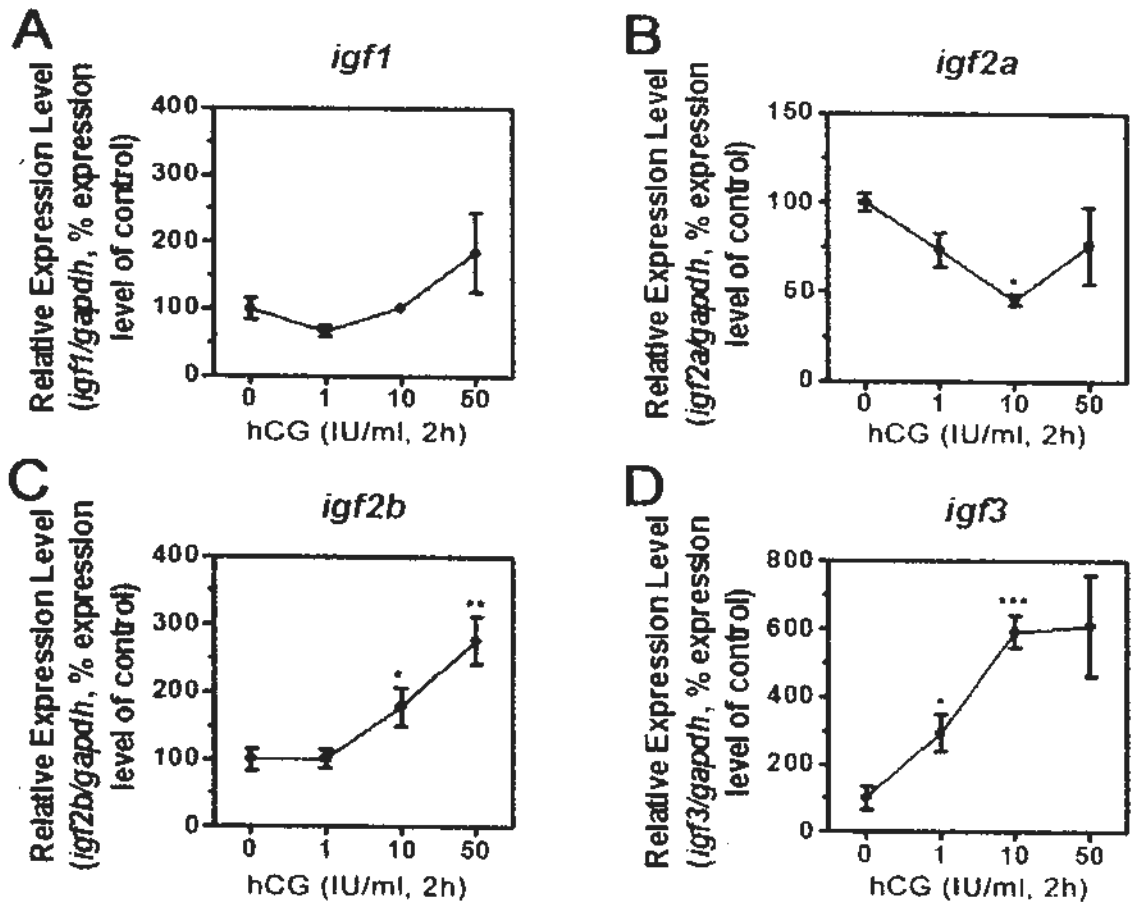

Fig. 4-6 Dose response of gonadotropin (hCG) effects on the expression of *igf1*, *igf2a*, *igf2b* and *igf3* in primary cultured follicle cells.
Expression levels were normalized to *gapdh* and expressed as a percentage of the control. Regulation of *igf1* (A), *igf2a* (B), *igf2b* (C) and *igf3* (D) expression by different concentrations of hCG. Each value represents the mean ± SEM of three independent experiments each performed in triplicate (*P<0.05; **P<0.01; ***P<0.001 vs. control).

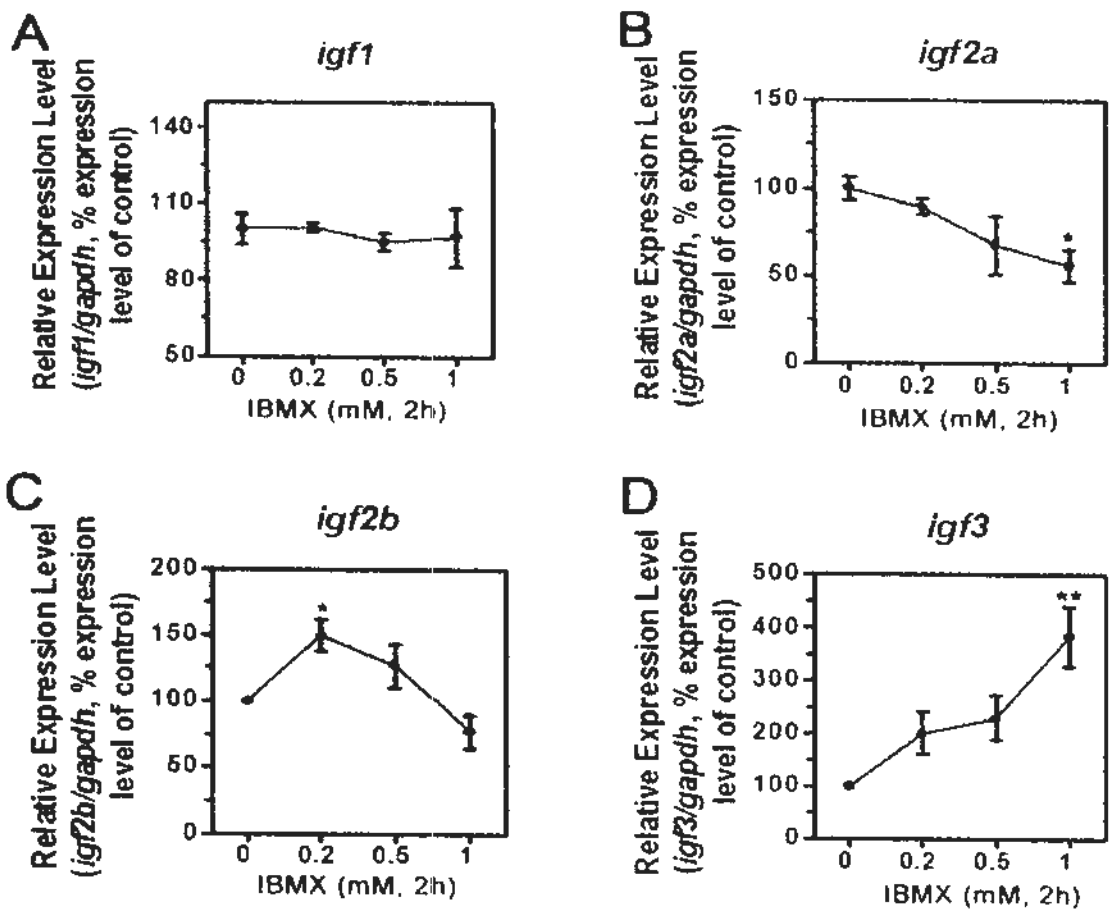

Fig. 4-7 Dose response of IBMX effects on the expression of *igf1*, *igf2a*, *igf2b* and *igf3* in primary cultured follicle cells.

Expression levels were normalized to *gapdh* and expressed as a percentage of the control. Regulation of *igf1* (A), *igf2a* (B), *igf2b* (C) and *igf3* (D) expression by different concentrations of IBMX. Each value represents the mean ± SEM of three independent experiments each performed in triplicate (*P<0.05; **P<0.01 vs. control).

Chapter 5 Developmental expression of the insulin-like growth factor system in zebrafish embryos

5.1 Introduction

The essential role of the insulin-like growth factor (Igf) system in normal organism growth and development is well known. At the cellular level, this system is involved in many aspects of cellular responses, including growth, proliferation, survival, migration, and differentiation (Wood et al., 2005). In mammals, the Igf system is comprised of two ligands (Igf1 and Igf2), six Igf binding proteins (Igfbp1 through Igfbp6), and two types of receptors that mediate the actions of the ligands, Igf type 1 receptor (Igf1r) and Igf type 2 receptor, also known as mannose-6-phosphate receptor. Both Igf1 and Igf2 stimulate cellular responses mainly through Igf1r; in contrast, the mannose-6-phosphate receptor is primarily responsible for clearing released Igf2 (Le Roith et al., 2001).

In the past few decades, the central importance of Igf signaling in the growth and development of vertebrates has been established, and direct evidence from knockout animals has demonstrated that Igf1, Igf2 and Igf1r are required for normal development in the mouse embryo (Baker et al., 1993; Liu et al., 1993). However, the specific roles for each individual member of the Igf system (Igfs, Igf1r and Igfbps) in early development are still poorly defined. Attempts to elucidate the cellular actions of the members of the Igf system have not been easy partly due to the inaccessibility of the mammalian fetus which is enclosed in the uterus. Zebrafish embryos provide an experimental platform that is appropriate for the study of the developmental functions of the members of Igf system, making them well suited for discerning the developmental roles of Igfs, Igf1r, and Igfbps (Maures et al., 2002). In recent years, there has been a rapid accumulation of knowledge about Igf biology in zebrafish.

In contrast to mammals, which have only one Igf1 gene and one Igf2 gene, recent

studies have indicated the existence of four Igf genes in zebrafish. The zebrafish *igf1* gene was first cloned by Chen et al. (Chen et al., 2001), and *igf2* (*igf2a*) was first cloned by Maures et al. (Maures et al., 2002). Subsequently, a duplicated zebrafish *igf2* gene (*igf2b*) was identified by Lien et al. (Lien et al., 2006). Most recently, the fourth Igf, Igf3, was cloned and characterized in zebrafish by our lab (Wang et al., 2008). Moreover, a single Igf1r is found in mammals, whereas two Igf1rs (Igf1ra and Igf1rb) are present in zebrafish (Ayaso et al., 2002; Maures et al., 2002). The functions of Igf signaling in early development have been studied in zebrafish for years. Eivers and colleagues first reported that Igf signaling is important for early neural development and embryonic growth by overexpressing *igf1* mRNA and microinjection of dominant-negative *igf1r* mRNA in zebrafish embryo (Eivers et al., 2004). The extensive work on zebrafish by Schlueter and colleagues has revealed that both Igf1ra and Igf1rb are essential for early development. Igf1r inhibition caused neuronal apoptosis, primordial germ cells migration and significant impairment of eye, inner ear, and heart development, (Schlueter et al., 2007a; Schlueter et al., 2006; Schlueter et al., 2007b). By overexpressing specific *igf* mRNAs in zebrafish embryos, Zou and colleagues showed that all four Igf genes are functional in early development and have roles in the development of the notochord (Zou et al., 2009).

Activation of the Igf signaling pathway depends on the binding of Igf ligands, but the specific role of these ligands in the early development of zebrafish is still poorly understood. It was believed that the gene expression of zebrafish *igf1* and *igf2* (*igf2a*) were ubiquitous during the early development of zebrafish (Maures et al., 2002). Recently, Sang and colleagues first reported the distinct, specific localization of *igf1*, *igf2a* and *igf2b* transcripts in zebrafish embryos at the 18S and prim-5 stages (Sang et al., 2008). They provided further evidence that the differential roles of *igf2a* and *igf2b* are required for localized organ development through events including

primordial germ cell migration, dorsal midline development, and pronephros and ventral forebrain formation (Sang et al., 2008; White et al., 2009).

To provide additional clues about the role of the Igf system in early development, the present study aimed to reveal the temporal and spatial gene expression patterns of all four *igf* ligands including (*igf1*, *igf2a*, *igf2b* and *igf3*) and two *igf1rs* (*igf1ra* and *igf1rb*) in the early development of zebrafish. This information not only provides the direct information to the study of Igf signaling in zebrafish but also yields valuable insights into the function of the Igf system in developmental regulation and the evolution of growth regulatory mechanisms in vertebrates.

5.2 Materials and Methods

5.2.1 Animals

See. Section 2.2.1. The stage of zebrafish embryos were according to Kimmel et al. (Kimmel et al., 1995).

5.2.2 RNA isolation and real time PCR

Total RNA samples were isolated from embryos of zebrafish, using TRIzol Reagent (Invitrogen, USA). The quality and quantity of the RNA were determined by NanoDrop 2000C Spectrophotometer (Thermo, USA). RT-PCR was performed as Section 2.2.5. For real-time PCR, the standards for *igf1*, *igf2a*, *igf2b*, *igf3* and 18S ribosome RNA were prepared by amplification of cDNA fragments with the specific primers (Table 5-1). The amplicons were purified by the PCR Purification Kit (Qiagen, USA) and these PCR products were used to construct the standard curves in the real-time PCR assays. Real-time PCR was carried out as Section 2.2.5.

5.2.3 Whole mount *in situ* hybridization

A cDNA fragment of *igf1*, *igf2a*, *igf2b*, *igf3*, *igf1ra* and *igf1rb* was amplified by RT-PCR with specific primers (Table 5-1), in order to confirm the signals by *in situ*

hybridization, two different fragments correspoding to the specific genes were amplified. The specific PCR products were purified by the PCR Purification Kit (Qiagen, USA) and cloned into pGEM-T vector (Promega, USA). The plasmid DNA of zebrafish *igf1*, *igf2a*, *igf2b*, *igf3*, *igf1ra* and *igf1rb* was linearized by restriction enzyme digestion, followed by *in vitro* transcription reactions with either T7 or Sp6 RNA polymerase to generate the antisense or sense RNA riboprobes using the DIG RNA Labeling Kit (Roche, USA). The chorions of zebrafish embryos before 48 hpf were removed manually with Dumont Watchmaker's Forceps no. 5. The embryos were then fixed in 4% paraformaldehyde (PFA) in 1×PBS buffer (137 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2HPO4 and 1.4 mM KH_2PO_4, pH at 7.4) overnight at 4°C. The embryos were washed with PBS several times and transferred gradually into 100% methanol (MeOH) and stored at -20°C until use. In WISH, the embryos were rehydrated in graded concentrations of MeOH (75%, 50% and 25%) to 1×PBS buffer. After pre-hybridization with Hyb buffer (50% formamide, 5×SSC (sodium chloride sodium citrate), 50 µg/ml Heparin, 5 mM EDTA, 0.5 mg/ml ribosomal RNA (Sigma R7125), 0.46 ml 1M citric acid (pH 6.0) and 0.1% Tween-20 to a final volume of 50 ml), the embryos were mixed with the RNA probes dissolved in the Hyb buffer at 65 °C overnight. Following hybridization with the RNA probes, embryos were washed sequentially in Hyb buffer, 75% Hyb buffer, 50% Hyb buffer, 25% Hyb buffer (in 2×SSC buffer), 2×SSC (0.3 M NaCl and 0.03 M Na citrate, pH at 7.0) at 65°C for 15 min each and then 0.2×SSC for 30 min twice at room temperature. The embryos were further washed sequentially by 75% 0.2×SSC, 50% 0.2×SSC, 25% 0.2×SSC (in PBT buffer) and PBT (1×PBS with 1% Tween-20) for 5 min at room temperature. After washing, the embryos were incubated with anti-DIG antibody (1:5000) in PBT buffer containing 2% lamb serum at 4 °C overnight. The embryos were then washed by PBT for 30 min three times and AP buffer (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris pH 9.5 and 0.1% Tween-20) for 5 min three times. The embryos were then

incubated with staining solution (1% NBT in AP buffer) at room temperature in the dark. The staining reaction was monitored by color examination using stereo microscope. The embryos were then destained in 100% EtOH, 75% EtOH (in H_2O), 50% EtOH (in PBT), 25% EtOH (in PBT) and PBT for 5 min at room temperature. The embryos were stored in PBT buffer at 4°C and pictured on a Leica camera DFC420.

5.3 Results and discussions

The expression level of *igf1* from 0 hours post fertilization (hpf) to 72 hpf was determined by real-time PCR. The *igf1* transcript was barely detected before 6 hpf, around the gastrulation stage, but the expression then slightly increased during development and reached its highest levels 72 hpf (Fig. 5-1A). The distribution of *igf1* transcript in the embryo was then investigated by whole-mount *in situ* hybridization. *igf1* transcript was not detected before 6 hpf (at the shield stage) (Fig. 5-1B to E). After the somitogenesis stage, the hybridization signal was restricted to the trigeminal ganglia and the eye at 18 hpf (the 18 somite stage) (Fig. 5-1F and G). At 24 hpf (Fig. 5-1H and I) and 48 hpf (Fig. 5-1J to L), the expression of *igf1* was still only detected in the trigeminal ganglia, but the signal in the eyes was centered in the lens. At 72 hpf, a strong signal could be detected in the otic vesicle, and weak expression of *igf1* could be observed in the trigeminal ganglia (Fig. 5-1M to O). The results from real-time PCR assays are consistent with the studies by Sang et al. (Sang et al., 2008) and Zou et al. (Zou et al., 2009), which also showed a very low expression of *igf1* before 12 hpf in early development. However, our results are in contrast to those reported by Maures et al. (Maures et al., 2002). Studies in mice have shown that the expression of *igf1* is not detected in preimplantation-stage embryos (Heyner et al., 1989). In teleosts, including seabream (Duguay et al., 1996) and rainbow trout (Ayson et al., 2002; Gabillard et al., 2003), *igf1* mRNA levels are relatively low throughout the embryonic period in comparison with adulthood. In

chickens, *igf1* transcripts are first detected at the gastrulation stage (Allan et al., 2003). This evidence suggests that *igf1* is a zygotic gene conserved across different species of vertebrates. Zou et al. (Zou et al., 2009) and Eivers et al. (Eivers et al., 2004) found defects in dorsal structure when overexpressing *igf1*. However, they studied gene function by transcriptionally overexpressing *igf1* in embryos. This approach is far from ideal due to the activation of Igf1r in a ubiquitous or ectopic manner throughout the embryo. Therefore, the precise role of Igf1 in specific organs during early development is still largely unknown. *igf1* transcripts were found to localize to zebrafish trigeminal ganglia between 12 hpf to 72hpf; these ganglia are the primary sensory neurons that mediate touch sensitivity (Metcalfe et al., 1990). Therefore, locally produced *igf1* may have a previously unrecognized paracrine/autocrine role in the differentiation or proliferation of the neuronal cells.

Real-time PCR indicated that *igf2a* transcript is present from the 0 hpf to 72 hpf. The expression level dramatically increased after 4 hpf and reached its peak at 8 hpf, followed by gradual decrease during development (Fig. 5-2A). Using whole-mount *in situ* hybridization, we found that *igf2a* transcripts were ubiquitous in early cleavage, gastrula-stage embryos (data not shown) and in the early somitogenesis stage (Fig. 5-2B and C). At 14 hpf, weak *igf2a* expression was detected in the notochord (Fig. 5-2D and E). In embryos at 16 hpf embryos, *igf2a* transcript was readily detected in the notochord (Fig. 5-2F and G). The expression of *igf2a* was still restricted to the notochord at 18 hpf (Fig. 5-2H and I). At 24 hpf, the signal was weak in the notochord, and a strong signal appeared in the area of the brain, including the middle brain, dorsal middle brain and otic vesicle (Fig. 5-2J). A strong signal could be detected in the pharyngeal arch region, middle brain and otic vesicle at 48 hpf (Fig. 5-2K). At 72 hpf, the signal in the brain became weaker and a signal appeared in the liver (Fig. 5-2L).

According to results of real-time PCR, the expression of *igf2b* was very low in

early development, and then dramatically increased after 24 hpf and remained high (Fig. 5-3A). Using whole-mount *in situ* hybridization, the expression of *igf2b* was barely detected before 6 hpf (the shield stage) (Fig. 5-3B and C). A weak *igf2b* transcripts signal was first detected at 12 hpf, and the expression was restricted to the floor plate region (Fig. 5-3D and E). Signals could also be detected in the floor plate at 14 hpf (Fig. 5-3F and G). At 18 hpf, the signals were restricted to the hypochord and floor plate (Fig. 5-3H and I). At 24 hpf, the expression in the hypochord and floor plate became weaker, and a strong signal appeared in the area of the brain, including the middle brain, dorsal middle brain and otic vesicle (Fig. 5-3J). At 48 hpf, a signal was detected in the middle brain and the pharyngeal arch region (Fig. 5-3K and L). A signal was only observed in the heart and liver at 72 hpf (Fig. 5-3M). Results from the present studies demonstrate that the spatially distinct expression of *igf2a* and *igf2b* in 18S and prim-5 stage of zebrafish embryos (Sang et al., 2008).

Based on sequence analysis, it is clearly that both *igf2a* and *igf2b* are the orthologous to human *igf2* (Wang et al., 2008; Zou et al., 2009). Published reports have shown the distribution of both *igf2a* and *igf2b* in the 18-somite and prim-5 stages of zebrafish embryos (Sang et al., 2008; White et al., 2009), and those analyses have been investigated further in the present study on more specific stages of embryo. A distinct expression pattern of both *igf2a* and *igf2b* in the midline at the 18-somite stage was detected in our study, in accordance with the previous study. Differential expression of *igf2a* and *igf2b* was also found in later developmental stages, for example, at 48 hpf, *igf2a* transcript was detected in the pharyngeal arch region, midbrain and otic vesicle, in contrast to *igf2b* signals, which were located in the fin and otic vesicle. These results strongly suggest distinct and overlapping function for both Igf2a and Igf2b in specific developmental stages.

Our previous study identified two *igf3* splice forms (termed as *igf3_tv1* and *igf3_tv2*) in zebrafish (Li et al., 2011). We demonstrated that *igf3_tv2* is the major

form found during early zebrafish development, whereas *igf3 tv1* is highly expressed in the larval and adult zebrafish. Here, the expression of *igf3_tv2* was analyzed during the early development of zebrafish. Real-time PCR showed that the expression of *igf3 tv2* could be detected after 4 hpf (the sphere stage) and kept increasing until 24 hpf, at which point its expression decreased. The whole-mount *in situ* hybridization signal was ubiquitous at 6 hpf, and a strong signal was observed in the pharyngeal arch region along with a weak signal in the brain region at 10 hpf, 18 hpf and 24 hpf. Expression was restricted to the hypobranchial muscle (sternohyoideus) at 48 hpf and 72 hpf, with weak expression in the brain at 48 hpf. Interestingly, the localization of *igf3* mRNA is similar to the localization of *igbp1* transcripts, which is restricted to the region of the pharyngeal pouch at 48 hpf in zebrafish (Maures and Duan, 2002). This correlation raises the possibility of the regulation of *igf3* by *igfbp1* in this region. The specific expression of *igf3* implies its involvement in the formation of the pharyngeal arch. Considering the importance of Igf signaling in early development, *igf3* may be involved in areas of zebrafish embryo where it is expressed.

We also examined the expression of both *igf1ra* and *igf1rb* during zebrafish embryogenesis (Fig. 5-5). By *in situ* hybridization, expression of *igf1ra* could be easily detected after 4 hpf (the sphere stage) (Fig. 5-5A), and *igf1ra* was ubiquitously expressed at 8 hpf (75% epiboly stage), 10 hpf (bud stage) and 24 hpf (prim-5 stage) (Fig. 5-5C-E). At 72 hpf (protruding-mouth stage) (Fig. 5-5F), the high expression of *igf1ra* was detected primarily in the brain. Similarly, *igf1rb* was widely expressed at 4 hpf (sphere stage), 8 hpf (75% epiboly stage) and 10 hpf (bud stage) (Fig. 5-5G-I), However, *igf1rb* transcripts were highly expressed in the brain region at 24 hpf (prim-5 stage) (Fig. 5-5J), and at 72 hpf (protruding-mouth stage) (Fig. 5-5L), a strong *igf1rb* signal was detected in the brain, liver and heart regions. Our results reveal distinct expression profiles of both *igf1ra* and *igf1rb* after 24 hpf for the first

time. These results are not consistent with a previous study by Maures et al. (Maures et al., 2002). In their study, both receptors were found to be ubiquitously expressed throughout embryogenesis. Schlueter and his colleagues have already investigated the divergent and overlapping functions of Igf1ra and Igf1rb in growth, muscle differentiation, and inner ear, skeletal muscle, and heart development before 48 hpf (Schlueter et al., 2006). Our results not only provide direct evidence to support their conclusions, but also further reveal the possibility of differential functions of both *igf1rs* in the later stages of zebrafish embryonic development.

Taken together, the present study has revealed the spatial and temporal expression patterns of four *igf* ligands including *igf1*, *igf2a*, *igf2b* and *igf3*, as well as two *igf* receptors, *igf1ra* and *igf1rb*, in the early development of zebrafish. All of these genes exhibited the distinct and dynamic expression profiles during zebrafish embryogenesis. Further investigations into the functions of these Igf ligands in zebrafish will provide valuable insight into the diversity and evolution of Igf signaling in vertebrates.

Gene	Sequence(5' to 3' direction)	Strand	Application	Size (bp)
igf1	AGGTCAGACAACCGTGGCATC	S	Real-time PCR	236
	TAGTTTCTGCCCCCTGTGTTTCC	AS		
	TAGAGGACAGCGGGAGGAATGA	S	*in situ* hybridization	566
	GGTCCATGATCTCATTGCGAAT	AS		
	ACGGATCCACCATGTCTAGCGGTCATTTC	S		486
	ACCTCGAGCTACATGCGATAGTTTCTG	AS		
igf2a	GGTCTTCCCAGTGTCACAGGCTC	S	Real-time PCR	168
	TGCTCCTCATCTTGGATTTTCTC	AS		
	CATGAATCATACCACTCGCCCG	S	*in situ* hybridization	680
	TGTCCTCAGTTTCCTGCCTTCC	AS		
	ACCTCGAGCTACATGCGATAGTTTCTG	S		594
	ACCTCGAGTCATTTTCGGGATGTGCTGATC	AS		
igf2b	CATCATTCTGTTTGCCATACCTG	S	Real-time PCR	178
	ACACAAACTGTAGAGCGTCCACC	AS		
	CCCTTCTTCCTACCCCACCAAA	S	*in situ* hybridization	759
	TTGTGCAGCCACGCTTGAGTTC	AS		
	ACGGATCCACCATGGACAGTTTCGTAAATAAGGTC	S		639
	ACCTCGAGTCACTTGTGGCTAACGTAGTT	AS		
igf3	CGCATAATTCAATCAAAGTCCG	S	Real-time PCR	258
	GCTGCTCCAGGTTTGCCTATGT	AS		
	GACAACAGCTACTCGCATAATTC	S	*in situ* hybridization	232
	CATCTGAACGTTGCAGTAGAAAT	AS		
	AAAAAGACATGCCATCAGACGC	S		574
	ATGAATGTTGGCGTCTCAGTTG	AS		
igf1ra	CAGACCTCCAGACAGGAAGCGT	S	*in situ* hybridization	634
	CGTGGCCATCATTATCCCTGTT	AS		
	TCGATCCACATTGGCCCCCTCT	S		343
	AGACAAAGGGAGGAGGGAAATG	AS		
igf1rb	CCCAGACCTCCAGACAGGCGAC	S	*in situ* hybridization	705
	TCCAAGCCTGTCATTGTTTCGG	AS		
	GTCTTCCAGCCTGCAGCCCCTT	S		486
	AGTCAGTGATCCTGTCTGGCGG	AS		
18s	CCTGAGAAACGGCTACCACATCC	S	Real-time PCR	220
	AGCAACTTTAGTATACGCTATTGGAG	AS		

Table 5-1 The primers used in Chapter 5.

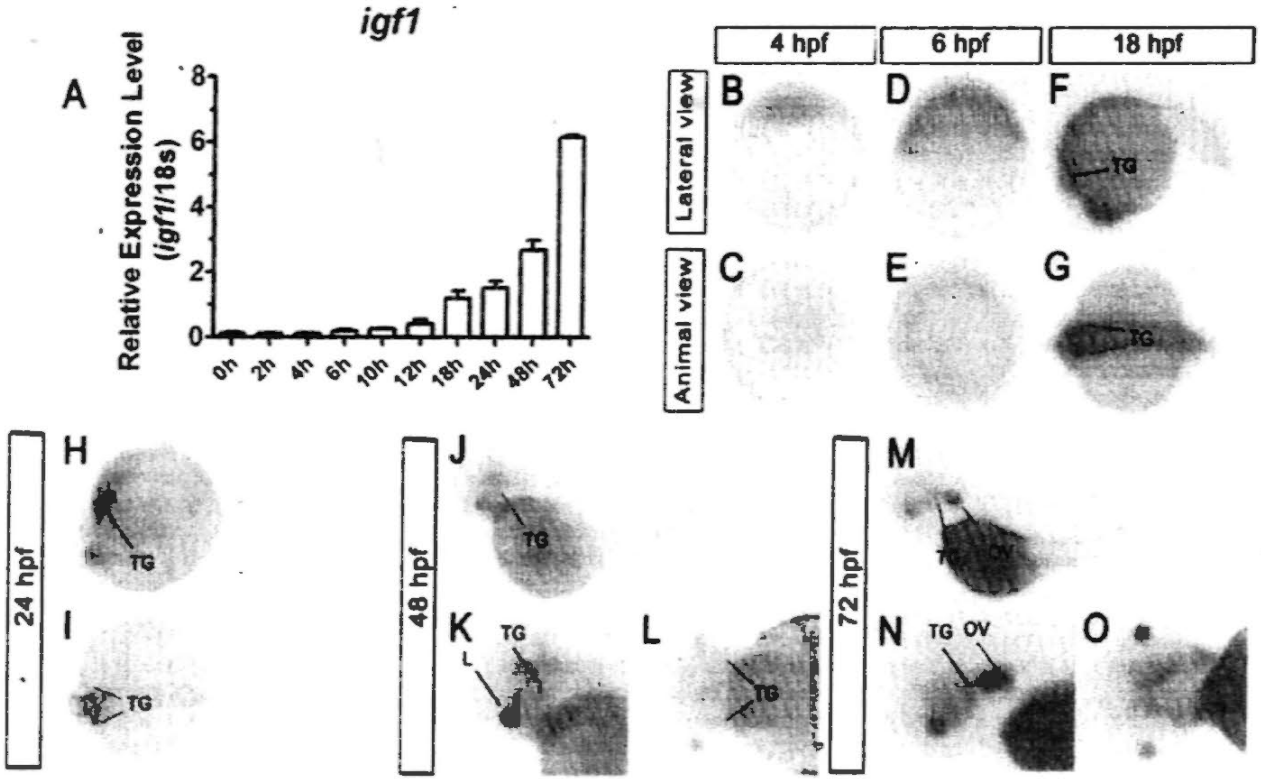

Fig. 5-1 The expression of *igf1* during early development of zebrafish. (A) The real-time PCR result showing the temporal expression of *igf1* relative to a housekeeping gene (*18s*) in zebrafish embryos during the first 72 hpf; (B-O) The results of whole mount in situ hybridization showing the spatial expression of *igf1* during embryogenesis. B and C: 4 hpf embryo (sphere stage), lateral and animal view; D and E: 6 hpf (shield stage), lateral and animal view; F and G: 18 hpf (18-somite stage), lateral and animal view; H and I: 24 hpf (prim-5 stage), lateral and dorsal view; J,K,L: 48 hpf (long-pec stage), lateral and dorsal view; M,N and O: 72 hpf (protruding-mouth stage), lateral and ventral view. TG, trigeminal ganglia. TG, trigeminal ganglia; OV, otic vesicle; L, lens.

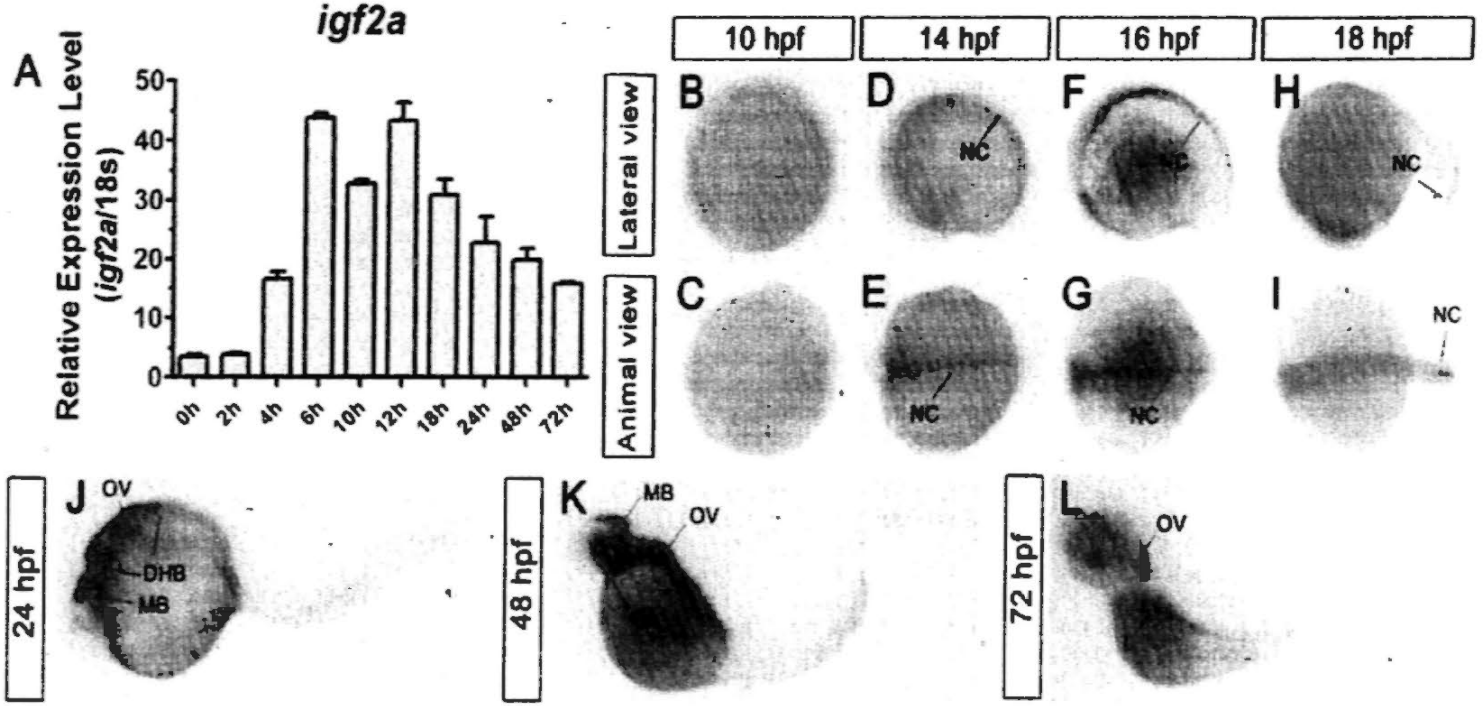

Fig. 5-2 The expression of *igf2a* during early development of zebrafish. (A) The real-time PCR result showing the temporal expression of *igf2a* relative to a housekeeping gene (*18s*) in zebrafish embryos during the first 72 hpf; (B-L) The results of whole mount in situ hybridization showing the spatial expression of *igf2a* during embryogenesis. B and C: 10 hpf (bud stage), lateral and animal view; D and E: 14 hpf (10-somite stage), lateral and animal view; F and G: 16 hpf (14-somite stage), lateral and animal view; H and I: 18 hpf (18-somite stage), lateral and animal view; J: 24 hpf (prim-5 stage), lateral view; K: 48 hpf (long-pec stage), lateral view; L: 72 hpf (protruding-mouth stage). MB, midbrain; PA, pharyngeal arch region; OT, otic vesicle; L, liver; DHB, dorsal middle brain; NC, notochord.

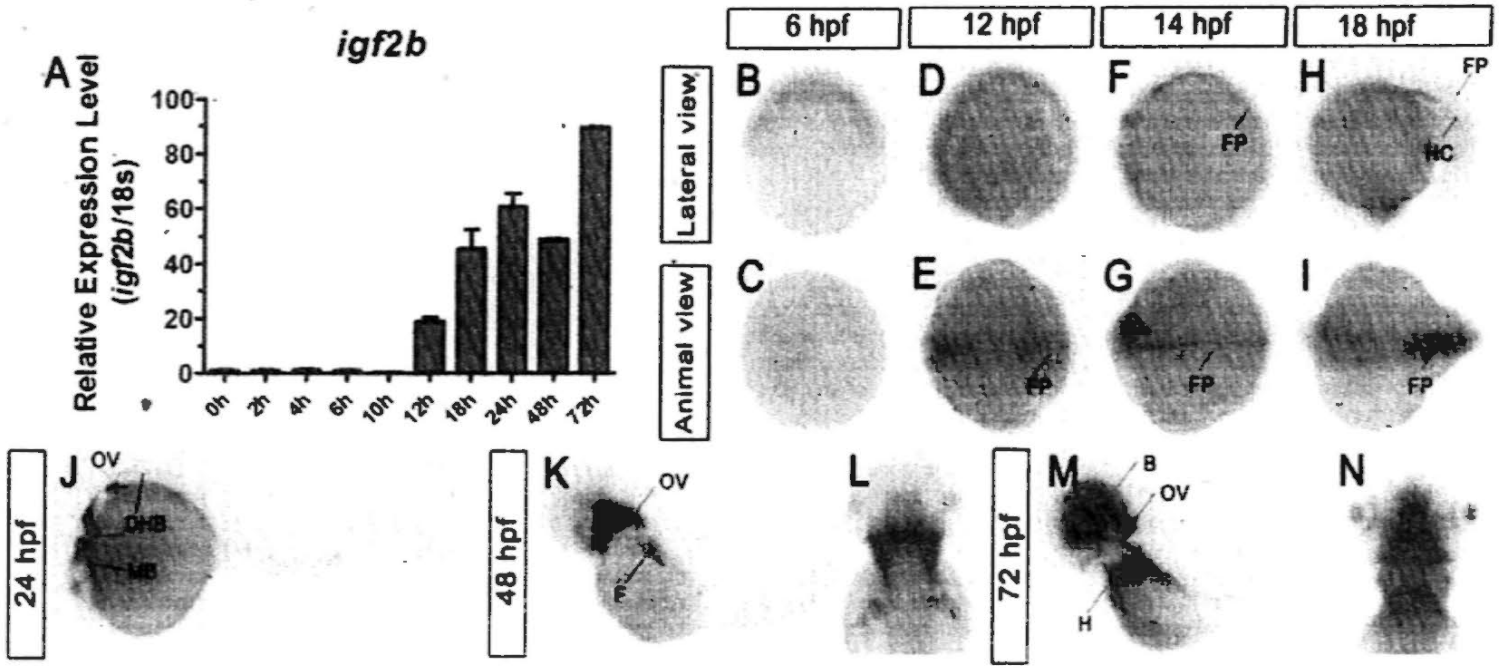

Fig. 5-3 The expression of *igf2b* during early development of zebrafish. (A) The real-time PCR result showing the temporal expression of *igf2b* relative to a housekeeping gene (*18s*) in zebrafish embryos during the first 72 hpf; (B-L) The results of whole mount *in situ* hybridization showing the spatial expression of *igf2b* during embryogenesis. B and C: 6 hpf (shield stage), lateral and animal view; D and E: 12 hpf (5-somite stage), lateral and animal view; F and G: 14 hpf (10-somite stage), lateral and animal view; H and I: 18 hpf (18-somite stage), lateral and animal view; J: 24 hpf (prim-5 stage), lateral view; K and L: 48 hpf (long-pec stage), lateral and vetral view. L: 72 hpf (protruding-mouth stage), lateral and dorsal view. MB, midbrain; PA, pharyngeal arch region; OT, otic vesicle; L, liver; DHB, dorsal middle brain; NC, notochord; F, fin.

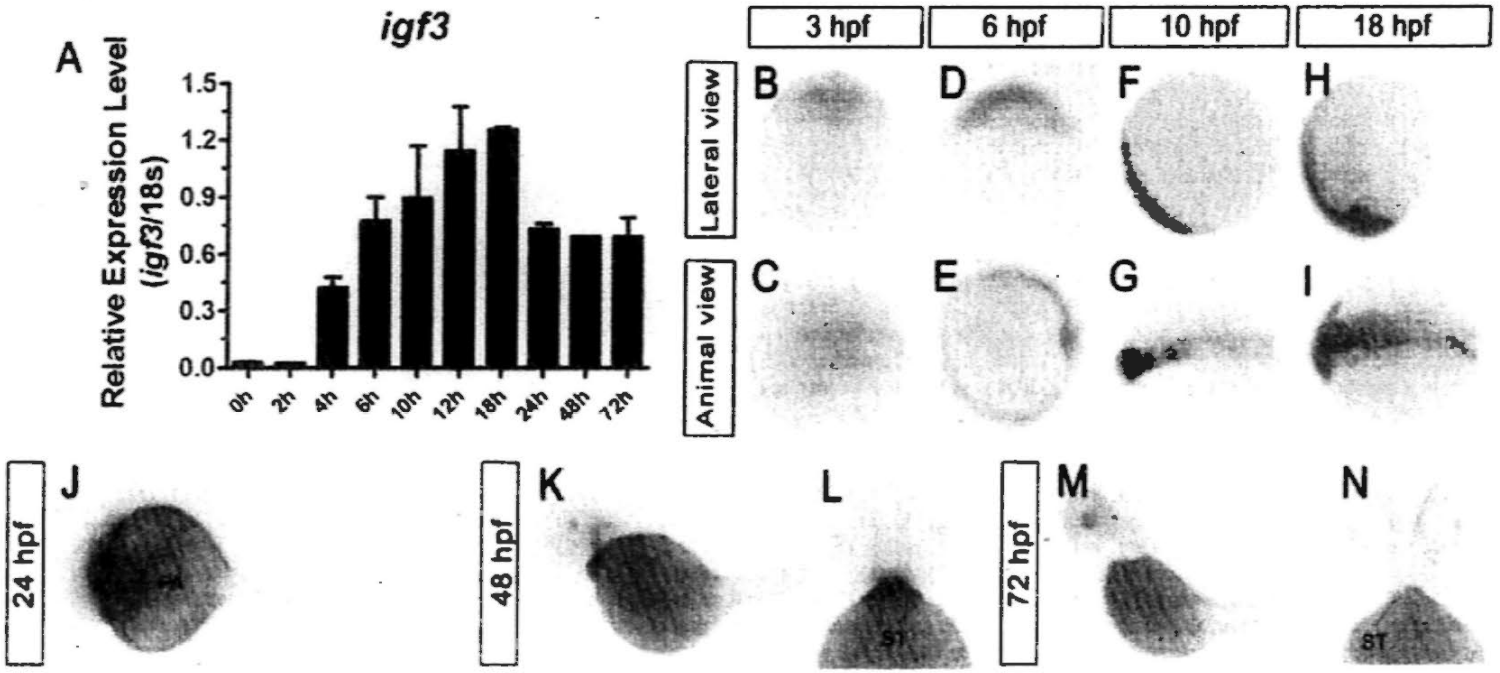

Fig. 5-4 The expression of *igf3* during early development of zebrafish. (A) The real-time PCR result showing the temporal expression of *igf3* relative to a housekeeping gene (*18s*) in zebrafish embryos during the first 72 hpf; (B-L) The results of whole mount *in situ* hybridization showing the spatial expression of *igf3* during embryogenesis. B and C: 3 hpf (1k-cell stage), lateral and animal view; D and E: 6 hpf (shield stage), lateral and animal view; F and G: 10 hpf (bud stage), lateral and animal view; H and I: 18 hpf (18-somite stage), lateral and animal view; J: 24 hpf (prim-5 stage), lateral view; K and L: 48 hpf (long-pec stage), lateral and dorsal view. L: 72 hpf (protruding-mouth stage), lateral and dorsal view. MB, midbrain; PA, pharyngeal arch region; ST, sternohyodieus (hypobranchial muscle).

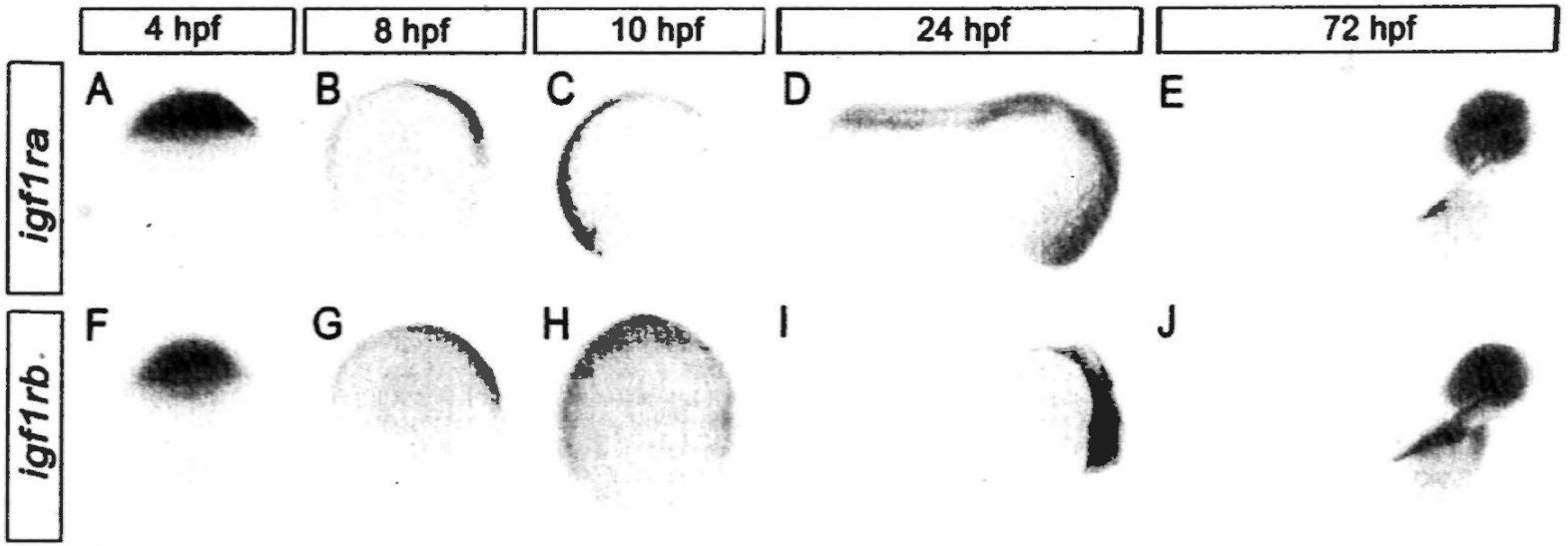

Fig. 5-5 The expression of *igf1ra* and *igf1rb* during early development of zebrafish.

(A-E) The results of whole mount *in situ* hybridization showing the spatial expression of *igf1ra* during embryogenesis, all are lateral view. A: 4 hpf (sphere stage); B: 8 hpf (75% epiboly stage); C: 10 hpf (bud stage); D: 24 hpf (prim-5 stage); E: 72 hpf (protruding-mouth stage).

(F-J) The results of whole mount *in situ* hybridization showing the spatial expression of *igf1rb* during embryogenesis, all are lateral view. F: 4 hpf (sphere stage); G: 8 hpf (75% epiboly stage); H: 10 hpf (bud stage); I: 24 hpf (prim-5 stage); J: 72 hpf (protruding-mouth stage).

Chapter 6 Summary

6.1 Overview

It is widely accepted that the development and function of ovarian follicles are tightly controlled by gonadotropins (follicle-stimulating hormone (FSH) and luteinizing hormone (LH)), which are secreted by the pirtuitary. The coordinated activity of these hormones is essential for the fine-tuning of ovarian functions. Increasing evidence also indicates that a network of locally produced growth factors such as insulin-like growth factors (Igfs), epidermal growth factor (Egf), and the activin-follistain system is also involved in the regulation of ovarian follicles. Among these growth factors, the Igf system has increasingly received attention.

In mammals, the Igf system includes two ligands (Igf1 and Igf2), six Igf-binding proteins (Igfbp1 to Igfbp6), and two types of receptors, the Igf type 1 receptor (Igf1r) and the Igf type 2 receptor. Both Igf1 and Igf2 are mitogenic, low molecular weight peptides that are structurally related to proinsulin. These peptides stimulate cellular responses mainly through Igf1r; in contrast, the mannose-6-phosphate receptor is primarily responsible for clearing release of Igf2 (Le Roith et al., 2001). Activation of the Igf signaling pathway occurs when Igf ligands bind Igf1r, which leads to the activation of a number of downstream signals, including the MAPK and PI3-k/Akt pathways. Considerable experimental evidence has indicated an essential role for Igfs in the ovary (Yoshimura, 2003).

The expression of the genes encoding Igf1 and Igf2 in the ovary has now been clearly established in several mammalian species, including rat (Adashi et al., 1997), sheep (Perks et al., 1995; Teissier et al., 1994), cow (Perks et al., 1999), pig (Yuan et al., 1996) and human (Zhou and Bondy, 1993). It was believed that Igf1 is the primary subtype of Igf in rodents. In contrast, in the human, the Igf2 expression level is high in granulosa and thecal compartments, and Igf2 expression is increased in granulosa cells as follicle size increases, whereas Igf1 is only weakly detectable,

suggesting the importance of Igf2 in the human ovarian Igf system (el-Roeiy et al., 1993; Yoshimura, 2003; Zhou and Bondy, 1993). Igf1 and Igf2 have been demonstrated to be potent regulators of many aspects of ovarian development and function. They stimulate the release of progesterone, testosterone, and estradiol in ovarian cells of different mammals (Sirotkin, 2010). The effects of Igf1 on oocyte maturation has also been demonstrated in many mammal species, including rabbit (Lorenzo et al., 1996), pig (Grupen et al., 1997), sheep (Guler et al., 2000), and cow (Sakaguchi et al., 2000).

In fish, previous reports have demonstrated the expression of the Igf system (including the Igf peptides, Igf receptors, and Igf-binding proteins) in fish gonads. Igf1 mRNA and protein have been demonstrated to be present in the ovary of several fish species including seabream (Kagawa et al., 1995), tilapia (Berishvili et al., 2006) and sturgeon (Wuertz et al., 2007). Functional analysis revealed that Igf1 can induce meiotic resumption in common carp (Mukhejee et al., 2006) and striped bass (Weber and Sullivan, 2000) and can also induce oocyte maturation competence in white bass (Weber and Sullivan, 2000). Igf2 mRNA and protein have also been studied in the ovaries of tilapia (Schmid et al., 1999b) and seabream (Radaelli et al., 2003). Igf2 can induce oocyte maturation in striped bass (Weber and Sullivan, 2000). These results indicate an important role for the Igf system in fish ovarian function. However, the precise mechanisms of action for this system in the fish gonad are still poorly understood. More recently, a novel teleost Igf (Igf3) has been indentified by our lab, interestingly, the expression of Igf3 is restricted to the gonad in zebrafish and tilapia. It has been proposed that Igf3 plays a more important role in fish reproduction, further highlighting the role of the Igf system in fish reproduction (Wang et al., 2008). In this dissertation, we studied the Igf system especially the Igf3, by assessing its expression, regulation and function in the ovary. In addition, the expression of the Igf system in early development was also investigated.

6.2 Contributions of the present study

6.2.1 The expression patterns of both Igf3 mRNA and protein in the zebrafish ovary

Using RACE assays, two *igf3* transcripts were characterized in zebrafish. These two transcripts, namely *igf3 tv1* and *igf3 tv2*, have different 5'-UTRs and translation initiation sites but produce the same mature peptide. During early development, *igf3 tv2* is highly expressed after the pre-midblastula transition; however, the expression of *igf3_tv1* is low throughout embryogenesis. In adult zebrafish, *igf3 tv1* is exclusively expressed in the gonads, whereas *igf3 tv2* cannot be detected in any adult zebrafish tissue. The different expression patterns of the two *igf3* transcripts in zebrafish suggest that *igf3_tv1* functions exclusively in the adult gonads whereas *igf3 tv2* functions primarily during early development.

Using both real-time PCR and *in situ* hybridization, we detected a gradual increase in *igf3* expression during follicle development. The level of *igf3* mRNA is low in follicles at early stages. The mRNA level increases in the midvitellogenic (MV) stage, and reaches its peak in the full-grown (FG) stage. Western blot analyses, using a specific polyclonal antibody against Igf3 generated in our laboratory also demonstrate the gonad-specific expression pattern of Igf3 particularly in the ovary. Immunofluorescence staining shows that the Igf3 protein is predominantly expressed in the follicular cell layer in both the MV- and FG-stage follicles. The expression of Igf3 correlates with that of the LH receptor which also exhibits a gradual increase in expression level during zebrafish follicle growth and is also restricted to the follicular cell layer (Kwok et al., 2005).

6.2.2 The regulation of *igf3* by gonadotropin in the ovary and its role in the zebrafish oocyte maturation

Gonadotropin is the major regulator of ovarian development and function in

many organisms. The results of the present study show that *igf3* in the zebrafish ovary is strongly stimulated by hCG treatment. In this study, such results were demonstrated not only using ovarian fragments in a time- and dose-dependent manners but also using isolated follicles in a dose- and stage-dependent manner. The action of hCG on the follicles is particularly prominent on follicles at the FG stage. Gonadotropin is known to regulate target gene expression primarily via activation of the cAMP-PKA pathway (Leung and Steele, 1992). Our results also show that both 8-Br-cAMP and IBMX mimic the effects of hCG on follicles, a putative cAMP response element (CRE) can actually be found on the promoter of the *igf3* genes in zebrafish (Fig. 6-1), suggesting that cAMP is likely a second messenger involved in the action of hCG in stimulating *igf3* expression in ovarian follicles. These results also suggest that Igf3 serves as a downstream factor in gonadotropin signaling in ovarian follicles.

Recombinant zebrafish Igf3 was then expressed and purified from a bacterial system. The bioactivity of this recombinant protein was tested on two cell lines. Using this protein, we have demonstrated for the first time that Igf3 exerts a potent action in stimulating oocyte maturation in zebrafish. Treatment of zebrafish follicles with recombinant Igf3 significantly induced oocyte maturation in a time- and dose-dependent manner. Interestingly, early-stage follicles (including EV and MV follicles) also had a response to Igf3 treatment. However, the follicles in these early stages cannot undergo oocyte maturation based on treatment with hCG or DHP alone (Pang and Ge, 2002a).

6.2.3 Mechanism of the action of Igf3 on oocyte maturation

We first tested the possibility of a requirement for steroidogenesis in Igf3-induced oocyte maturation. Igf3 stimulates GVBD via a steroid-independent pathway, as demonstrated here. These results are consistent with other studies on Igf1

that reported the failure to block Igf1-induced GVBD with inhibitors of cholesterol side-chain cleavage enzymes or 3β-HSD in fish ovarian follicles (Kagawa et al., 1994; Kagawa et al., 1995; Negatu et al., 1998; Weber and Sullivan, 2000).

We also demonstrated that Igf3 stimulates oocyte maturation through Igf1r in zebrafish. The effects of Igf3 on oocyte maturation were diminished by two specific Igf1rs inhibitors in a dose-dependent manner. Furthermore, we also demonstrated that Igf1rs can be activated by Igf3 in a time-dependent manner.

The potential downstream signaling pathways involved in Igf3-induced oocyte maturation were analyzed. The present study provided direct evidence that PI3 kinase, PDE3 and MAP kinase are necessary for Igf3-mediated oocyte maturation in zebrafish. This evidence helps us understand the molecular mechanism of Igf3 on oocyte maturation. However, one limitation of this study is that all experiments were performed in intact ovarian follicles. Therefore, we do not know whether Igf3 activates these downstream pathways to stimulate the oocyte maturation via Igf1rs taking place in the oocyte or follicle cell layer. This limitation exists because of the technical difficulties of removing the follicle cell layer surrounding the oocytes. Although Thomas and colleagues have reported the application of denuded oocytes in zebrafish (Pang and Thomas, 2010; Peyton and Thomas, 2011), we have not yet applied this technique in our laboratory. However, it has been reported previously that oocyte maturation could be stimulated by Igf1 in denuded oocytes of *Cyprinus carpio* (Paul et al., 2009), suggesting a direct action of Igf1 on the oocyte. It is therefore possible that Igf3 might also exerts its action and activates downstream pathways directly on the oocyte. Definitive proof or disproof of this point will await a solution to the technical problem of separating follicle cells in zebrafish.

6.2.4 The differential expression and regulation of *igf1*, *igf2a*, *igf2b* and *igf3* by gonadatropin in the zebrafish ovary

We found that all four *igfs* are expressed in the ovary of zebrafish and exhibit differential expression profiles during folliculogenesis. The present study demonstrated that hCG stimulated *igf2b* and *igf3* expression but suppressed *igf2a* expression. We also provid evidence that the effect of gonadotropin can be mimicked by IBMX, which increases the intracellular levels of cAMP, suggesting the involvement of cAMP in the gonadotropin-mediated regulation of the differential expression of *igf2a*, *igf2b* and *igf3*. *igf3* is the subtype of *igf* most sensitive to the regulation of gonadotropin and cAMP. These results also indicate that the regulation of *igf2a*, *igf2b* and *igf3* by gonadotropin through the cAMP pathway occurs in follicle cells. The regulation of *igfs* by gonadotropin and cAMP has been demonstrated in several other species including bird (Onagbesan et al., 1999), pig (Hammond et al., 1988), rabbit (Yoshimura et al., 1996b) and human (el-Roeiy et al., 1993). Recently, in our laboratory, we have introduced additional pharmacological compounds, including forskolin and 8-Br-cAMP, that could increase the intracellular cAMP concentration to confirm the notion that the regulation of these *igfs* by gonadotropin occurs through the cAMP pathway. Because PKA is the major downstream factor of cAMP, H89 (an inhibitor of the PKA pathway) will be used to further investigate whether regulation by gonadotropin is mediated by cAMP-dependent PKA. Moreover, we have prepared four recombinant Igfs proteins in a bacterial system, and the efficacy of these Igfs on oocyte maturation will be compared.

Taken together, our results strongly indicate that the Igfs, especially Igf3, serve as a downstream mediator of gonadotropin signaling in zebrafish ovarian follicles (Fig. 6-2).

6.2.5 The differential expression patterns of *igf1*, *igf2a*, *igf2b* and *igf3* during zebrafish embryogenesis

In addition, to supply direct information for the functional study of the four *igfs* and two *igf1rs* in early development, the expression patterns of the four Igfs during embryogenesis were analyzed by real-time PCR and/or whole mount *in situ* hybridization in zebrafish. Our results revealed dynamic temporospatial expression patterns for *igfs* and *igf1rs* during zebrafish embryogenesis. The unique and overlapping expression patterns of *igf1*, *igf2a*, *igf2b* and *igf3* suggest divergent functions of these *igfs* in the early development of zebrafish.

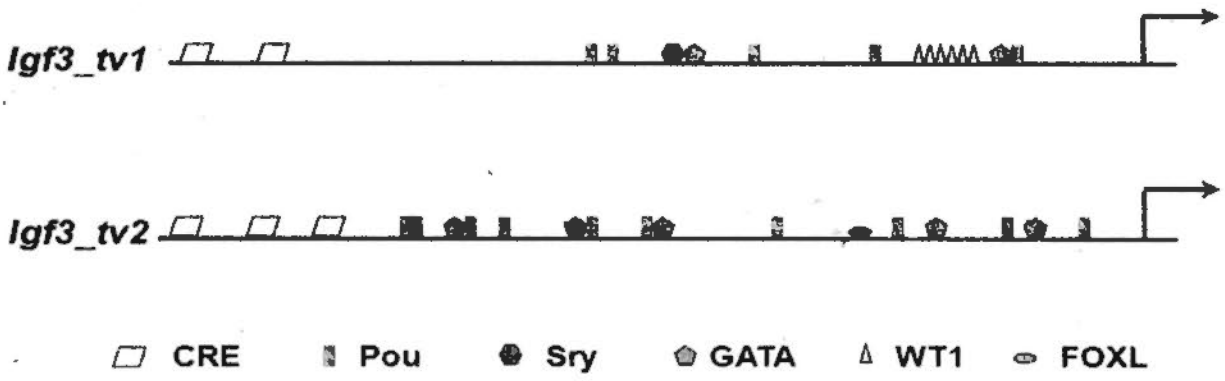

Fig. 6-1 The putative CRE in the promoter region of *igf3* transcript variants of zebrafish.

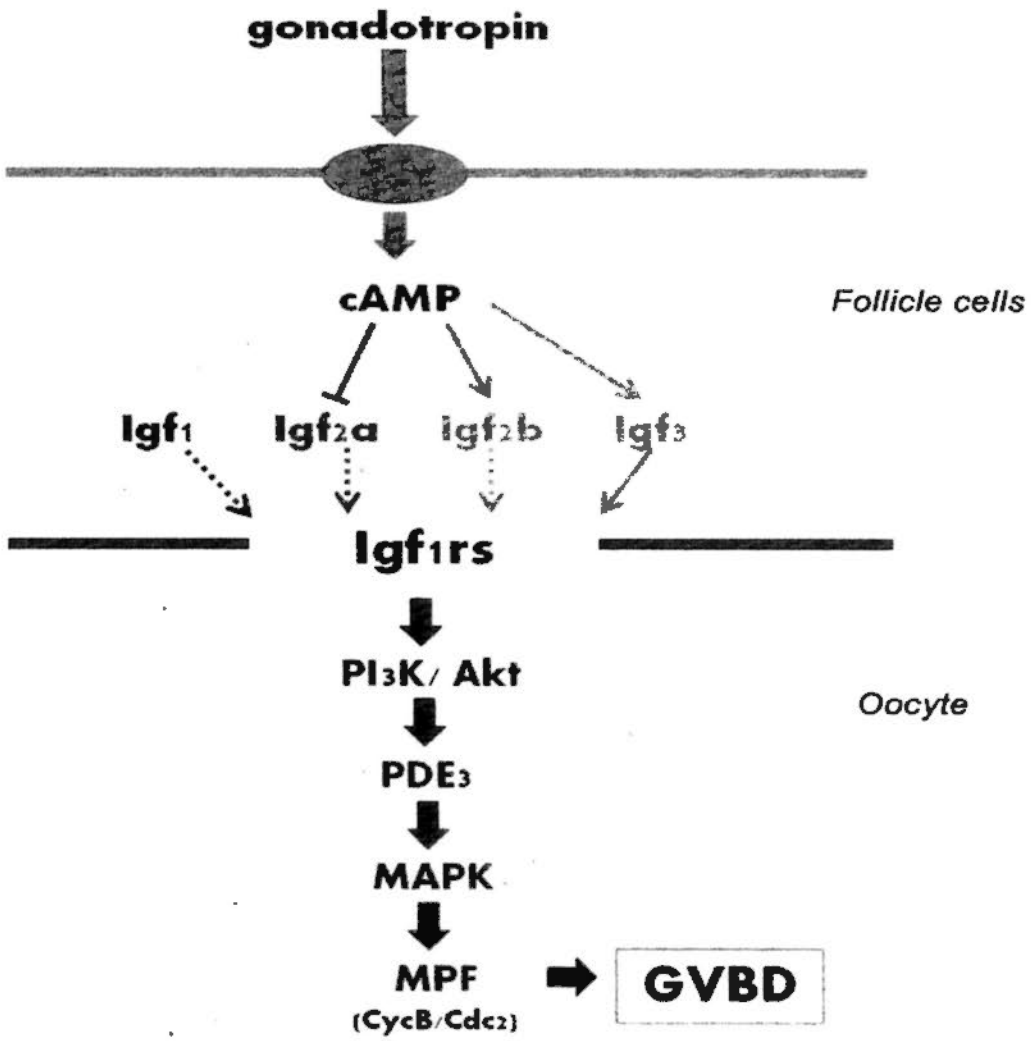

Fig. 6-1 A hypothesis on the mechanisms of intra-ovarian Igf system on oocyte maturation and their regulation by gonadotropin in zebrafish follicles.

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