Expression and Function Analysis of Kit System in the Ovary of Zebrafish, *Danio rerio*

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of the Requirements for the Degree of
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Abstract of thesis entitled:

Expression and Function Analysis of Kit System in the Ovary of Zebrafish, *Danio rerio*

Submitted by YAO Kai

for the degree of Doctor of Philosophy

at the Chinese University of Hong Kong

In the ovary of mammals, the primordial follicles contain all female gametes which are dormant and arrested at the prophase stage of meiosis I. These follicles are composed of small, non-growing and immature oocytes in the center and one single layer of squamous pre-granulosa cells in the periphery. Upon stimulation by factors from both inside and outside of the oocytes, a small number of primordial follicles will be activated (recruitment) and progress into the next stage of folliculogenesis. In this process, some follicles undergo growth, maturation and ovulation, while others are lost from the follicle pool by atresia. To date, a variety of growth factors have been documented to participate in the folliculogenesis including the recruitment of primordial follicles, proliferation and differentiation of granulosa and theca cells, and growth, survival, maturation and ovulation of oocytes. Among these intraovarian growth factors, Kit system has received increasing attention in recent years.

Kit ligand (also named stem cell factor, SCF) is a pleiotropic growth factor with diverse biological functions. It exerts effects on target cells by binding to its cognate tyrosine kinase receptor, Kit. In mammals, accumulated evidence has demonstrated important roles for Kit ligand and Kit in gametogenesis, melanogenesis and haematopoiesis. However, very little is known about Kit system in other vertebrates. In the present study, we used zebrafish as the model to investigate the expression, regulation and function of the Kit system in the ovary.

The zebrafish has two homologues of Kit ligand (*kitlga* and *kitlgb*) and Kit (*kita* and *kitb*) instead of one copy for each as in mammals. The present study proposed the origin of these homologues in the zebrafish by phylogenetic and chromosome synteny analyses, and provided further evidence for neo- or subfunctionalization for both Kit ligands and Kit receptors in the zebrafish ovary. All four Kit system members exhibited distinct and significant changes in mRNA expression during folliculogenesis, particularly in the periovulatory period before and after final oocyte maturation and ovulation.

Then we further studied the spatial localization of each member within the follicle. The present study demonstrated that *kitlga* and *kitb* are exclusively expressed in the follicle layer, while *kitlgb* and *kita* only in the oocyte. Using CHO cell line as a bioreactor, we produced recombinant zebrafish Kitlga and Kitlgb. Analysis in mammalian COS-1 cells and zebrafish primary follicle cells confirmed their biological activity and binding specifity. Two opposite paracrine pathways of Kit system in the zebrafish ovary have been shown. Kitlga from the follicle cells preferably activates Kita in the oocyte in spite of the weak response of Kitb to it. Kitlgb from the oocyte, however, exclusively activates Kitb in the follicle cells without any effects on Kita.

Finally, as the first step to study the regulation of Kit system, we found that IGF-I was a potent regulatory factor that up-regulated the expression of *kitlga* in zebrafish follicle cells. The stimulation involved transcription but not translation, indicating that the *kitlga* gene is a direct downstream target of IGF-I. The effect of IGF-I on *kitlga* was exerted via PI3K-Akt but not MAPK pathway. In contrast, the MAPK pathway may play a negative role in controlling *kitlga* expression.

On the other hand, cAMP is involved in regulating the expression of *kitlga* in zebrafish follicle cells. Two cAMP-activated effectors, PKA and Epac, have reverse

effects. PKA promotes but Epac inhibits the expression of *kitlga*, which was identified by the respective activator. The effect of forskolin and H89 on IGF-I-induced expression of *kitlga* suggests a cross-talk between the two signaling pathways. Both hCG and PACAP inhibited IGF-I-induced *kitlga* expression, indicating that they may have negative regulation through cAMP signaling pathways in the full-grown follicles.

As the first study of Kit system in fish ovary, the present study will not only provide insight into the function of Kit system in the zebrafish ovary, but also contribute to our understanding of the roles of Kit system in vertebrate ovary.

摘要

在哺乳動物卵巢中，原始卵泡（primordial follicle）包含所有的雌性配子。它們都處於靜止狀態，停留在第一次減數分裂前期。這些卵泡是由位於中央的卵子和周圍的粒細胞（granulosa cell）前體組成。卵子體積小，處於非生長期，尚未成熟，粒細胞前體則是單層鱗片狀。一小部分原始卵泡一旦被來自卵子內或外的因子激活，它們就將進入卵泡發生（folliculogenesis）過程。在這個過程中，一些卵泡會歷經生長，成熟並最終排卵（ovulation），但另一些則由於閉鎖（atresia）而消失。時至今日，許多生長因子已經被證實參與了卵泡發生過程，包括原始卵泡的激活，粒細胞和膜細胞（theca cell）的增殖分化，卵子的生長，存活，成熟與排出。在這些卵巢內的生長因子中，Kit system 在近年受到了越來越多的關注。

Kit 配體（Kit ligand，也稱為 stem cell factor，SCF）是一個具有廣泛功能的生長因子。它通過與其酪氨酸激酶受體 Kit 結合而發揮作用。哺乳動物中，Kit 配體和受體在配子形成（gametogenesis），黑素形成（melanogenesis）和造血作用（haematopoiesis）中發揮着重要作用。然而，在其他脊椎動物中，關於 Kit system 的信息則非常少。因此，我們在這裡用斑馬魚作為模式動物，研究了 Kit system 在卵巢中的表達，調控和功能。

與哺乳動物僅有一個配體或受體基因不同，斑馬魚有兩個同源物（homologue），配體有 *kitlga* 和 *kitlgb*，而受體有 *kita* 和 *kitb*。借助於進化樹和染色體同源性分析，我們研究了它們的起源，並且發現，這四個基因的表達在卵泡發生特別是在卵子成熟和排出的前後過程中發生顯著變化並且彼此不同。

我們接下來研究了這些基因在卵泡中的空間分佈。結果顯示，*kitlga* 和 *kitb* 僅在卵泡細胞層（follicle layer）表達而 *kitlgb* 和 *kita* 則僅在卵子中表達。使用 CHO 細胞系作為生物反應器，我們製成了重組的斑馬魚 Kitlga 和 Kitlgb。通過在哺乳動物 COS-1 細胞系和斑馬魚原代卵泡細胞中的分析，我們鑑定了重組蛋

白的生物活性和結合特異性。結果表明，Kit system 在斑馬魚的卵巢中形成了兩條相反的旁分泌（paracrine）途徑。來自卵泡細胞的 Kitlga 主要激活卵子上的受體 Kita，而 Kitb 對 Kitlga 僅存在較弱的反應。來自卵子的 Kitlgb 則特異的激活卵泡細胞上的 Kitb，並且對 Kita 沒有任何效應。

最後，我們研究了 Kit system 的調控。我們發現，在斑馬魚卵泡細胞中，胰島素樣生長因子 I（IGF-I）對 *kitlga* 的表達有很強的促進作用。這種作用僅涉及轉錄水平，而與翻譯水平無關，表明 IGF-I 直接促進了 *kitlga* 的轉錄。磷脂酰肌醇-3-激酶-蛋白激酶 B（PI3K-Akt）途徑介導了 IGF-I 對 *kitlga* 的調控，相反，有絲分裂原活化蛋白激酶（MAPK）途徑在 *kitlga* 的表達中可能起到了負調控作用。

另一方面， 環化腺核苷一磷酸（cAMP）也參與了 *kitlga* 在斑馬魚卵泡細胞中的表達調控。兩個 cAMP 激活的效應蛋白--蛋白激酶 A（PKA）和 cAMP 激活之交換蛋白（Epac）--有相反的效應。用相應的激活因子激活 PKA 和 Epac 的作用，結果顯示 PKA 促進而 Epac 抑制 *kitlga* 的表達。腺苷酸環化酶激活因子 forskolin 和 PKA 抑制物 H89 對 IGF-I 誘導的 *kitlga* 表達的效應表明了兩條信號傳導途徑之間的相互作用。人絨毛膜促性腺激素（hCG）和垂體腺苷酸環化酶激活肽（PACAP）對 IGF-I 誘導的 *kitlga* 的表達的抑製作用則表明它們可能通過 cAMP 信號途徑對 *kitlga* 的表達產生負調控作用。

本研究不僅能對 Kit system 在斑馬魚卵巢中的功能提供見解，而且也有助於理解 Kit system 在脊椎動物卵巢中的作用。

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Symbols and abbreviations

Symbols

alpha	α
beta	β

Abbreviations

17α, 20β-dihydroxy-4-pregnen-3-one	DHP
Actinomycin D	AD
Activation loop	AL
Activin receptor	acvr
Activin βA subunit	*inhba*
Activin βB subunit	*inhbb*
Adenylate cyclase	AC
Protein kinase B	PKB
Catalytic loop	CL
Chinese hamster ovary	CHO
Complementary deoxyribonucleic acid	cDNA
Cumulus-oocyte complexes	COC
Cyclic adenosine 3', 5'-monophosphate	cAMP
Cycloheximide	CH
Days postcoitus	dpc
Deoxyribonucleic acid	DNA
Deoxyribonucleotide triphosphate	dNTP
Dibutyryl-cAMP	db-cAMP

Dimethylsulfoxide	DMSO
Early vitellogenic	EV
Elongation factor 1-alpha	*ef1a*
Epidermal growth factor	EGF
Epidermal growth factor receptor	EGFR
Exchange protein-activated directly by cAMP	Epac
Extracellular domain	ED
Fetal bovine serum	FBS
Follicle stimulating hormone	FSH
Follistan	*fst*
FSH receptor	*fshr*
Full-grown	FG
Germinal vesicle breakdown	GVBD
Glycine-asparagine-asparagine-lysine	GNNK
Growth differentiation factor 9	*gdf9*
Growth hormone	GH
Gunanine nucleotide exchange factor	GEF
Hepatocyte growth factor	HGF
Human chorionic gonadotropin	hCG
In vitro fertilization	IVF
Insulin-like growth factor-I	IGF-I
Juxtamembrane domain	JD
Keratinocyte growth factor	KGF
Kinase insert domain	KI
Leukaemia inhibitory factor	LIF

Luteinizing hormone	LH
Luteinizing hormone receptor	LHCGR
Macrophage colony-stimulating factor	M-CSF
Mast cell growth factor	MGF
Messenger ribonucleic acid	mRNA
Mid-vitellogenic	MV
Mitogen-activated protein kinase	MAPK
Phosphatidylinositol 3,4,5-trisphosphate	PIP_3
Phosphatidylinositol 3-kinase	PI3K
Phosphatidylinositol 4,5-bisphosphate	PIP_2
Phosphatidylinositol-dependent protein kinase 1	PDK1
Pituitary adenylate cyclase activating polypeptide	PACAP
Platelet-derived growth factor	PDGF
Previtellogenic	PV
Primary growth	PG
Primordial germ cell	PGC
Protein kinase A	PKA
Ras guanine nucleotide exchange factor	Ras-GEF
Receptor tyrosine kinase	RTK
Reverse transcription polymerase chain reaction	RT-PCR
Rho-GTPase activating protein	Rho-GAP
Ribonucleic acid	RNA
Sodium dodeocyl sulphate polyacrylamide gel electrophoresis	SDS-PAGE
Sp-6-Phe-cAMPS	6-Phe-cAMP

Sp-8-pCPT-2'-O-Me-cAMPS	8-CPT-cAMP
Src homology 2	SH2
Steel	*Sl*
Steel factor	SF
Stem cell factor	SCF
Transmembrane domain	TD
Truncated forms of Kit	tr-kit
white spotting	*W*

Chapter 1

General Introduction

In the ovary of mammals, the primordial follicles contain all female gametes which are dormant and arrested at the prophase stage of meiosis I. These follicles are composed of small, non-growing and immature oocytes in the center and one single layer of squamous pre-granulosa cells in the periphery. Upon stimulation by factors from both inside and outside of the oocytes, a small number of primordial follicles will be activated (recruitment) and progress into the next stage of folliculogenesis. In this process, some follicles undergo growth, maturation and ovulation, while others are lost from the follicle pool by atresia. Folliculogenesis occurs cyclically throughout female's reproductive life span until the continuous activation and apoptosis of primordial follicles exhaust the original pool, which is followed by menopause (1). In spite of the extensive studies on ovarian endowment, primordial follicle activation and folliculogenesis, the underlying mechanisms involved in these processes are still not fully understood.

To date, a variety of growth factors have been documented to participate in the recruitment of primordial follicles, proliferation and differentiation of granulosa and theca cells, and growth, survival, maturation and ovulation of oocytes. Among these intraovarian growth factors, Kit system has received increasing attention in recent years.

Kit ligand (also named stem cell factor, SCF) is a pleiotropic growth factor with diverse biological functions. It exerts effects on target cells by binding to its cognate tyrosine kinase receptor, Kit. Both Kit ligand and Kit are actively expressed by a variety of cell lineages in both embryos and adults. Accumulated evidence has

demonstrated important roles for Kit ligand and Kit in gametogenesis, melanogenesis and haematopoiesis (2).

Previous reports have described the functions of Kit ligand and Kit based on the studies of natural mouse mutants. Kit ligand and Kit are encoded by the *steel* (*Sl*) and *white spotting* (*W*) loci, respectively (3-6). Lines of evidence from mice carrying mutations at these loci suggest roles for Kit ligand and Kit in primordial germ cell (PGC) survival, migration and proliferation in the embryo, as well as in follicle development in the adult (7-13). For example, due to the failure of PGC migration to the genital ridge, the Sl^{d}/Sl^{d} mice is infertile (8, 9). In Sl^{pan}/Sl^{pan} or Sl^{con}/Sl^{con} mice, the failure of follicle recruitment from dormant pool also leads to infertility, despite the successful migration of PGC (11, 12). Interestingly, in Sl/Sl^{t} mice, follicle growth can progress to the antral stage, but these animals have limited fertility with sporadic ovulation (14).

1.1 Structure of Kit ligand and Kit

1.1.1 Structure of Kit ligand

Kit ligand is one of four-helix-bundle cytokines (15). Members of this family have little similarity at the amino acid level; however, they can be aligned based on their secondary structures (16). Kit ligand exists as both soluble (KITL1) and membrane-associated (KITL2) forms, which are derived from alternative mRNA splicing and proteolytic processing (17, 18). In the mouse, the precursor for KITL1 contains 273 amino acids, constituting the signal peptide (1-25), the extracellular domain (26-214), the transmembrane domain (215-237), and the intracellular domain (238-273). Following removal of the signal sequence, proteolysis at the cleavage site encoded by exon 6 leads to the release of the soluble KITL1 (residues 26–189) (18). As a consequence of alternative splicing, KITL2 contains 28 fewer amino acids due to

the deletion of the exon 6 and therefore the loss of the cleavage site. Without proteolysis, KITL2 remains primarily anchored to the membrane (Fig. 1-1). However, it has been reported that KITL2 may also be cleaved at an alternative site to release a soluble form (17). Kit ligand functions as a non-covalent homodimer.

1.1.2 Structure of Kit

Kit belongs to the type III receptor tyrosine kinase (RTK) family (19, 20). Other type III RTKs include the platelet-derived growth factor (PDGF) receptors α and β, the macrophage colony-stimulating factor receptor (M-CSF) (21), and the Fl cytokine receptor (Flt3). Receptor tyrosine kinases all share the same topology: an extracellular ligand-binding domain, a single transmembrane segment, and a cytoplasmic kinase domain. The type III RTKs are characterized by the presence of five immunoglobulin-like domains in their extracellular domain and a cytoplasmic protein kinase domain with an 80-amino acid insert (22).

According to the evidence from mutagenesis studies, epitope mapping with site-specific Kit antibodies and crystal structure analyses, Kit ligand binds to Kit at immunoglobulin-like domains 1, 2 and 3 (Fig. 1-2) (15, 23). Treatment with antibodies against the first two immunoglobulin-like domains results in inhibition of Kit activity (23), and the ability of Kit ligand to bind the receptor is significantly reduced when the third immunoglobulin-like domain is deleted (23). The Kit mutant, which contains only the first three immunoglobulin-like domains, binds Kit ligand to the same degree as the complete receptor protein (24). However, Kit ligand-induced dimerization of Kit requires the fourth immunoglobulin-like domain (Fig. 1-2) (25).

A number of Kit isoforms have been reported (26-29). Kit transcript can be alternatively spliced to yield isoforms characterized by the residues glycine-asparagine-asparagine-lysine (GNNK) in the juxtamembrane region of the

extracellular domain (26, 27). These variants have been reported to be different in the timing and level of Kit tyrosine kinase activity (30). Truncated forms of Kit (tr-kit) lacking extracellular and transmembrane domains have been found in spermatids (29). It is interesting that microinjection of mouse oocytes with tr-Kit strongly activates the Src-family of kinases and induces resumption of meiosis in MII-arrested oocytes (31). Therefore, tr-Kit may be related to oocyte activation in fertilization. Finally, a soluble form of Kit, consisting of a part of the extracellular domain (KitS), has been detected in human serum (28). KitS can bind Kit ligand with high affinity and therefore regulate the activity of Kit ligand *in vivo* by preventing its binding to membrane-associated Kit (28).

1.2 Kit ligand and Kit expression in the ovary

1.2.1 Kit ligand and Kit expression in oogenesis

During embryonic development, mouse primordial germ cells (PGCs) display small amounts of Kit mRNAs as early as embryonic day 7.5 (32). Oogonia in mitosis express high levels of Kit mRNA but the expression ceases when oogonia enter meiosis (32). Consequently, Kit mRNA is absent between embryonic days 13.5 and 15.5 until late fetuses (embryonic day 17.5) when a very limited expression (restricted to a few oocytes at the most advanced stages) can be detected using *in situ* hybridization (33). Hence, in mice, the expression of Kit is proposed to be related to PGCs migration and mitosis of oogonia.

In the fetal ovaries of mice, Kit ligand appears to be expressed along the migration pathway of PGCs as early as embryonic day 9 (6, 34). Expression is also detectable in the genital ridge (6). Once PGCs colonize in the developing genital ridge (at embryonic day 12.5), the expression of Kit ligand along the migratory pathway disappears and is restricted to the gonad. These findings imply that the somatic cells

expressing Kit ligand guide migration of germ cells expressing Kit towards the genital ridge. Within the gonad, Kit ligand is not detectable at embryonic day 14.5 (34), demonstrating a synchronized expression of Kit ligand and Kit in the developing gonad.

1.2.2 Kit ligand and Kit expression in folliculogenesis

In postnatal mouse ovaries, expression of Kit becomes detectable at birth and abundant in primordial and growing oocytes in the ovaries of young mice (5, 8 and 10 days of age) (33). Furthermore, ovaries from older mice (17 days of age) contain follicles from primordial to antral stages, in which all oocytes express *Kit*. Results from *in situ* hybridization showed that the oocytes of primordial and later stage follicles uniformly express Kit protein and mRNA, though the expression decreases in antral follicles (33). Finally, starting at days 14–17, Kit protein and mRNA in theca cells become detectable (33, 35, 36).

On the other hand, Kit ligand protein and mRNA expression are detectable in the granulosa cells of follicles at all stages. Its expression is very low in primordial and primary follicles and increases in the granulosa cells of three layered follicles (days 8–13), which is the time when theca cells differentiate from the stroma cells. Expression of Kit ligand remains high in early antral follicles (11), but decreases as follicular growth progresses towards the late antral stage (37). In antral follicles, Kit ligand mRNA is high in the mural granulosa cells but low within the cumulus cells (11, 35).

1.3 Function of Kit ligand and Kit

1.3.1 Function in primordial germ cells

Oogenesis begins with the formation of PGCs during the early stages of

embryonic development. As a cluster of alkaline phosphatase-positive cells at the base of the allantois (38), PGCs can first be detected at embryonic day 7.5 (E7.5). By E9.0, PGCs migrate from the extraembryonic mesoderm and become incorporated into the hindgut. Between E9.0-E9.5, PGCs emerge from the dorsal side of the gut and migrate laterally to colonize in the developing ovary (39) where PGCs lose their motility and ability to proliferate and enter the meiosis. Then, the oocytes enclosed in a layer of somatic cells become arrested in prophase I at the diplotene stage of the first meiosis, marking the formation of primordial follicles. Those oocytes not surrounded by granulose cells, however, are lost by apoptosis (40, 41). There are lines of evidence in support of the involvement of Kit ligand and Kit in PGC migration, proliferation and survival (39).

As described above, Kit ligand can be detected in the somatic cells along the migration pathway of PGCs and progressively increases with proximity to the genital ridge (6, 34). Given the high level of Kit expression in PGCs, it is likely that the interaction between Kit ligand and Kit plays a role in directing their migration to the genital ridges. This hypothesis is supported by the observation from mice carrying mutations at *Sl* or *W* loci. In Sl^d/Sl^d mice, germ cells are absent in the ovaries because of an alteration in the migration of PGCs towards the presumptive ovary (7). In W^e/W^e mice, there is no increase in the number of PGCs between E8.5 and E10.5 due to the migration of PGCs to ectopic sites (42). Furthermore, some recent reports demonstrated a differential requirement for Kit ligand in PGC migration. Kit ligand does not seem to be necessary for PGC migration to the hindgut but is partially required for their subsequent migration from the hindgut. As for PGC proliferation, Kit ligand appears to be partially required for the proliferation of PGCs in the hindgut, but is absolutely indispensable for the proliferation afterwards after they leave the hindgut (43). Kit ligand has also been shown to promote germ cell proliferation *in*

vitro (29, 44).

Kit ligand also functions as a survival-promoting factor for PGCs both *in vivo* and *in vitro* (10, 29, 45-47). The activation of Kit has been shown to negatively regulate Fas-mediated apoptosis in germ cells. In W^v/W^v mice, the oocytes are absent in ovaries, whereas there are many oocytes in the ovaries carrying mutations at both the *Kit* and *Fas* alleles (W^v/W^v:*Fas-/-*). Furthermore, Kit ligand downregulates the expression of Fas ligand in fibroblasts *in vitro* (47). These observations indicate that Kit ligand may facilitate germ cell survival by regulating the activity of the Fas ligand. It is likely that the failure of germ cell establishment in the ovaries of W^v/W^v mutant mice is due to the death of PGCs but not their inability to migrate to the genital ridges. Studies have also proven that Kit ligand, in synergy with IGF-I and leukaemia inhibitory factor (LIF), promotes the survival of oocytes at the meiotic stage in fetal mouse ovaries cultured *in vitro* (46).

1.3.2 Function in primordial follicle activation and early follicle development

In mammals, the female reproductive lifespan is determined, to a great degree, by the number of primordial follicles within the ovary and the rate of primordial follicle activation. So far, there is abundant evidence in support of a role of Kit ligand and Kit in the latter, although the mechanism responsible for the exit of follicles from the resting pool remains largely unknown.

The first set of data showing this effect was generated by observations of natural mutants. In two lines of mice carrying mutation at *Sl* locus (Sl^t and Sl^{pan}), the ovaries only contain a limited number of primordial follicles and the follicles rarely grow to primary stage (11, 14). Most of these mutants are therefore sterile. In another study demonstrating the importance of Kit ligand and Kit for follicle activation, newborn mice were injected with a function-blocking antibody against Kit (ACK2) at selected

time-points during the first 2 weeks of life (48). Neutralization of Kit resulted in serious disturbances in initial follicle recruitment, primary follicle growth, antrum formation and granulosa cell proliferation. When the injection at birth was omitted, however, the initiation of follicle growth proceeded almost normally, demonstrating that there is a narrow period (from birth to day 2) when the interaction between Kit ligand and Kit is of importance for recruitment. On the other hand, using an *in vitro* culture of postnatal rat ovaries in the presence or absence of Kit ligand or the blocking antibody (ACK2), Parrott and Skinner showed that the activation of rat primordial follicles was promoted by Kit ligand (49). In this experiment, the number and developmental stage of follicles in the cultured ovaries were compared with those in the ovaries freshly isolated from 4-day old rats. Sections from the freshly isolated ovaries contained 68% primordial follicles and 32% growing follicles. After a 14-day culture in vitro, however, the number of primordial follicles declined to 50%, with the remaining proportion of follicles in the growing pool. This spontaneous activation could be completely blocked by the co-culture of ovary with neutralizing antibody of Kit, suggesting that the endogenous Kit ligand was in effect. Furthermore, in the ovaries treated with Kit ligand, only 17% of the follicles remained in the resting pool, while 83% initiated growth, and this effect was also inhibited by neutralizing antibody. These findings suggest that Kit ligand is sufficient for the induction of primordial follicle activation.

In addition to activation of primordial follicles, the interaction of Kit ligand and Kit has also been reported to have stimulatory effects on oocyte growth in early follicle development (50, 51). It has been demonstrated that addition of Kit ligand (10–50 ng/ml) significantly accelerated the growth of collagen-enclosed oocytes in vitro with a double growth rate observed at the concentration of 50 ng/ml. Moreover, oocytes from prepubertal mouse appeared to have the ability to increase Kit ligand

mRNA accumulation in the monolayered granulosa cells (50). This finding was further confirmed by Joyce *et al* (37). In another study using a multistep culture system for mouse oocytes from embryos at 15.5–16.5 days postcoitus (dpc), Klinger and De Felici identified three distinct stages of oocyte growth: (I) the initial stage in which the oocyte growth is induced directly by Kit ligand without involving gap junctions with the granulosa cells; (II) the second phase in which the oocyte growth depends on the combined actions of Kit ligand and contacts with the granulosa cells; and (III) the third phase of Kit ligand-independent but granulosa cell-dependent growth (51). On the basis of these data, we can conclude that the oocyte increases expression of Kit ligand in the surrounding granulose cells and the increased Kit ligand in turn further stimulates oocyte growth.

Similar to the situation described for PGCs, Kit ligand appears to be involved in promoting the survival and inhibiting apoptosis of both primordial (52) and preantral follicles (48, 53). In mouse ovaries cultured *in vitro*, Kit ligand inhibited oocyte apoptosis in the primordial follicles (52). Kit ligand up-regulates the expression of anti-apoptotic proteins Bcl-2 and Bcl-cL, while down-regulates that of pro-apoptotic factor Bax. Kit ligand initiates these anti-apoptotic effects via the phosphoinositide-3 kinase pathway (52). Further evidence shows that inhibition of the interaction between Kit ligand and Kit with antibody against Kit promotes oocyte death *in vitro* (53). Further studies are required to elucidate the underlying mechanism for the role of Kit ligand in oocyte survival during early follicle development.

1.3.3 Function in theca cell differentiation in preantral follicles

Preantral follicles have the ability to stimulate differentiation of the stromal–interstitial cells into theca cells by a paracrine mechanism (54). Kit ligand may be involved in this process since Kit ligand appears to promote the formation of

theca cell layers around small ovarian follicles (55), stromal–theca cell proliferation in a dose-dependent manner (55, 56) and androstenedione production in the absence of gonadotropins (56). On the other hand, theca cell-derived keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF) can stimulate Kit ligand expression, and Kit ligand, in turn, can stimulate theca cell-derived KGF and HGF expression. This positive feedback loop between thecal cells and granulosa cells may play a significant role in controlling ovarian follicular development and mediating gonadotropin actions (57).

1.3.4 Function in later stages of folliculogenesis

The interaction between Kit ligand and Kit has also been proven to be relevant for oocyte maturation. When fully grown rat oocytes were cultured in the presence of Kit ligand, a significant, albeit transient, delay in the progression of spontaneous meiotic maturation was observed. The inhibitory effects of Kit ligand can be specifically blocked by ACK2, an antibody to the extracellular domain of the Kit, as described above. These findings suggest a possible role of Kit ligand in the maintenance of meiotic arrest throughout oocyte growth (58). On the other hand, when antisense oligonucleotides for Kit were injected into the meiotically arrested rat oocytes to decrease Kit expression, an increased ability of these oocytes to resume meiosis compared with those microinjected with missense oligonucleotides or buffer alone was observed (59). Furthermore, human chorionic gonadotropin (hCG) induced a drop of Kit ligand to undetectable levels in cumulus cells and an increases to high levels in mural cells (58). It is therefore hypothesized that the LH surge *in vivo* may cause a decrease in the production of Kit ligand by those granulosa cells adjacent to the oocyte, thereby allowing meiosis to resume. These findings in rats demonstrate that the interaction between Kit ligand and Kit appears to prevent resumption of

meiosis within follicles. However, the role of Kit ligand in oocyte maturation may be different in other species. *In vitro* culture of mouse cumulus-oocyte complexes (COC) demonstrated that treatment with Kit ligand enhanced first polar body extrusion in a dose-dependent manner. In addition, the effects of Kit ligand were accompanied with an increase in cyclin B1 synthesis, which is important for the progression of meiotic maturation after germinal vesicle breakdown (GVBD). A possible effect of Kit ligand on cytoplasmic maturation has been suggested by the positive correlation between the Kit ligand concentrations in follicular fluid and pregnancy rate after *in vitro* fertilization (IVF) (60). Such an effect has been further confirmed by an *in vitro* study in which addition of Kit ligand to cultured mouse follicles for 12 days significantly increased the proportion of oocytes reaching the 2-4-cell stage after IVF (53). These discrepancies in different species indicate that further studies using different models are required before a role for Kit ligand in oocyte maturation can be firmly established.

1.4 Signaling

1.4.1 Kit and PI3K/Akt pathway

Phosphatidylinositol 3-kinase (PI3-kinase or PI3K) belongs to lipid kinases. It can catalyze the production of phosphatidylinositol 3,4,5-trisphosphate (PIP_3) by phosporylating phosphatidylinositol 4,5-bisphosphate (PIP_2) *in vivo*. PI3K was first identified by its association with two viral oncoproteins: v-Src and the middle T antigen of polyoma virus (61). The PI3-kinases are heterodimers and composed of regulatory (p85) and catalytic (p110) subunits. Various isoforms of the catalytic subunit (p110α, p110β, p110γ and p110δ) have been isolated, and the regulatory subunits that associate with p110α, p110β and p110δ are p85α and p85β (62). From the N to C-terminus, the typical regulatory subunit consists of an SH3 domain, a

Rho-GTPase activating protein (Rho-GAP) homology domain, and two SH2 domains. The typical catalytic subunit contains a Ras-binding domain, a phosphatidylinositol kinase accessory (PIKa) domain, and a catalytic domain. PI3-kinase is involved in many aspects of cell physiology including cell growth, cycle entry, migration, and survival (63).

PI3-kinase can be activated by a variety of growth factors (61) including Kit ligand (64, 65). By its SH2 domains, the p85 regulatory subunit can bind to phosphotyrosine residues of activated protein-tyrosine kinase receptors, and the interaction between p85 and p110 leads to the allosteric activation of the latter. Using Chinese hamster ovary (CHO) cells transfected with mutant or wild type human Kit, Lev et al. showed that treatment with Kit ligand led to a rapid association of PI3-kinase with phosphorylated Kit and co-precipitation of Kit and PI3-kinase by antibody against Kit. On the contrary, PI3-kinase is absent in precipitates from mutant Kit lacking the kinase insert domain. Additionally, PI3-kinase can only bind to phosphorylated kinase insert domain, but not unphosphorylated fusion protein. The most important tyrosine residues for PI3-kinase binding are Y721 and Y730 (64). Using COS-1 cells transiently expressing mutant forms of mouse Kit, Serve et al. determined more precisely the residues in the kinase insert domain responsible for the binding of PI3-kinase. They showed that PI3-kinase associated with the wild type, and the Y703F, Y730F, and Y747F mutants after Kit ligand treatment but not Y721F, despite the fact that all of these mutants exhibit autophosphorylation like wile type. These findings indicate that phosphotyrosine 721 plays a key role for the binding of PI3-kinase (65).

It is well known that Akt (also named protein kinase B, PKB), a protein serine/threonine kinase, acts downstream of PI3-kinase (66). Binding and activation of PI3-kinase occur at or near the plasma membrane where PI3-kinase phosphorylates

PIP_2 to PIP_3. The latter, in turn, activates phosphatidylinositol-dependent protein kinase 1 (PDK1) that leads to the phosphorylation and activation of Akt. Moreover, Akt contains a PH domain that binds to PIP_3. This binding brings Akt to the membrane and approaches PDK1.

One substrate of Akt is Bcl2 antagonist of cell death (Bad), a pro-apoptotic protein that promotes cell death. Blume-Jensen et al. demonstrated that, in response to Kit ligand, Akt becomes activated and mediates phosphorylation of Bad. Following phosphorylation, Bad no longer promotes apoptosis. And in the human osteosarcoma cell line (U2-OS), the Kit Y721F mutant is unable to protect cells against Bad-induced apoptosis (66).

The physiological role of Kit-mediated activation of PI3-kinase has been demonstrated in two studies by transgenic mice expressing Kit carrying a Y719F mutation (corresponding to Y721F in human Kit). Blume-Jensen et al. showed that the mutation completely disrupted PI3-kinase binding to Kit and reduced Kit ligand-induced PI3-kinase dependent activation of Akt by 90%. The mutant males are sterile due to a block in spermatogenesis, with initially decreased proliferation and subsequent extensive apoptosis occurring at the spermatogonial stem-cell level. In another study, Kissel et al. also demonstrated that in mutant females, follicle development was impaired at the cuboidal stages resulting in reduced fertility (67). These findings together suggest that Y719 (Y721 in human) serves as the docking site for PI3-kinase, and Akt plays an important role in the survival and growth of primordial germ cells (68).

1.4.2 Kit and Ras/Erk pathway

A number of studies have demonstrated a critical role of the Ras/Erk pathway in cell division and survival (69). Ras is a small G-protein that can cycle between an

active GTP-bound form and an inactive GDP-bound form. The role of Ras in the Ras/Erk cascade has been well characterized. Receptor tyrosine kinases (RTKs) activate Ras through association with the Ras guanine nucleotide exchange factor (Ras-GEF, or Sos), which can facilitate exchange of GDP to GTP. Ras-GEF forms a complex with the adapter protein Grb2. Grb2 contains one SH2 domain between two SH3 domains. So this complex in turn associates via the SH2 domain to phosphorylated tyrosine residues in RTKs. Thus, the Grb2-Ras-GEF complex is recruited near the plasma membrane, where it can act on Ras. Activated Ras interacts with the serine/threonine kinase Raf-1, leading to its activation. Raf-1 kinase (MAPKKK) then activates the dual-specificity kinases Mek1 and Mek2 (MAPKK) by phosphorylation (70). The targets of MAPKK are serine/threonine kinases Erk1 and Erk2 (mitogen-activated protein kinase, MAPK), which are activated through phosphorylation by Mek1/2 (18). The activated MAPKs are then translocated to the nucleus (71), where they regulate the activity of various transcription factors through phosphorylation (72).

Kit ligand is one of the growth factors that can activate the Ras-Erk pathway. Using a GST-fusion protein containing the SH2 domain of Grb2, Thommes et al. showed that this construct could bind to Kit in Kit ligand-stimulated cells. To demonstrate the binding specificity, they showed that the phosphopeptides corresponding to the sequences encompassing Y703 and Y936 inhibited GST-Grb2 SH2 binding to Kit, whereas the unphosphorylated peptides failed to inhibit the binding. It was therefore proposed that the binding of Grb2 to Kit might link Kit ligand signaling to the Ras/MAPK pathway (73).

1.5 Objectives of the present study

In the present study, we used zebrafish as the model to investigate the expression,

regulation and function of the Kit system in the ovary.

Different from the situation in mammals, two forms of Kit ligand (*kitlga* and *kitlgb*) and Kit (*kita* and *kitb*) have been identified in the zebrafish. This is likely due to the specific genome duplication in fish evolution (3R hypothesis). In the first part, we investigated the tissue distribution of Kit system in the zebrafish. After confirming the existence of each member of Kit system in the ovary, we cloned a full-length cDNA for zebrafish *kitb*, the only member of the Kit system whose full-length cDNA was not available. Phylogenetic and chromosome synteny analyses were performed to identify the origin of different forms of Kit system. With the aim to understand differential functions of different ligand and receptor isoforms, we performed real-time qPCR to analyze temporal expression profiles of all members of zebrafish Kit system during ovarian folliculogenesis with particular emphasis on the periovulatory period.

After analyzing the temporal expression profiles of Kit system during folliculognesis, we further studied the spatial localization of each member within the follicle in the second part. To demonstrate the functional relationship between different isoforms of Kit ligand and Kits in different compartments of the follicle, recombinant zebrafish Kit ligand proteins (rzfKitlga and rzfKitlgb) were produced by stably transfected CHO cells. Using these proteins, we further characterized the ligand-receptor specificity by transiently expressing the receptors (*kita* and *kitb*) in the COS cells and treating them with rzfKitlga or rzfKitlgb. This was followed by examining the phosphoryaltion of Kita and Kitb using Western blot analysis.

In the last parts, we performed a series of experiments using primary follicle cell culture to investigate the regulation of Kit system, particularly Kitlga which was shown to be expressed exclusively in the follicle layer. It is well known in mammals that both IGF-I and Kitlg have important roles in folliculogenesis including oocyte

growth, survival and final maturation, granulosa cell proliferation, and steroidogenesis in theca cells. This raises an interesting question of whether any functional relationship exists between these two growth factor systems. After demonstrating a stimulatory effect of IGF-I on the expression of *kitlga* in cultured follicle cells, we further studied the signaling pathways involved in IGF-I effects.

In addition to IGF-I, we also studied the effect of cAMP on *kitlga* expression in cultured follicle cells because accumulated evidence has demonstrated that cAMP may be involved in regulation of *kitlga*. Additionally, we further dissected the cAMP pathway and demonstrated that cAMP might differentially regulate *kitlga* expression by activating PKA and Epac, which stimulates and suppresses *kitlga* expression, respectively. Preliminary experiments showed that gonadotropins (hCG) and PACAP may exert their regulatory effects on *kitlga* expression via the cAMP pathways.

As the first study of Kit system in fish ovary, the present study will not only provide insight into the function of Kit system in the zebrafish ovary, but also contribute to our understanding of the roles of Kit system in vertebrate ovary.

Membrane associated KITLG

Soluble KITLG

Proteolytic processing

Alternative splicing

Fig. 1-1 Structures of two forms of KITLG. Kit ligand exists as soluble and membrane-associated forms that are derived from alternative splicing and proteolytic processing.

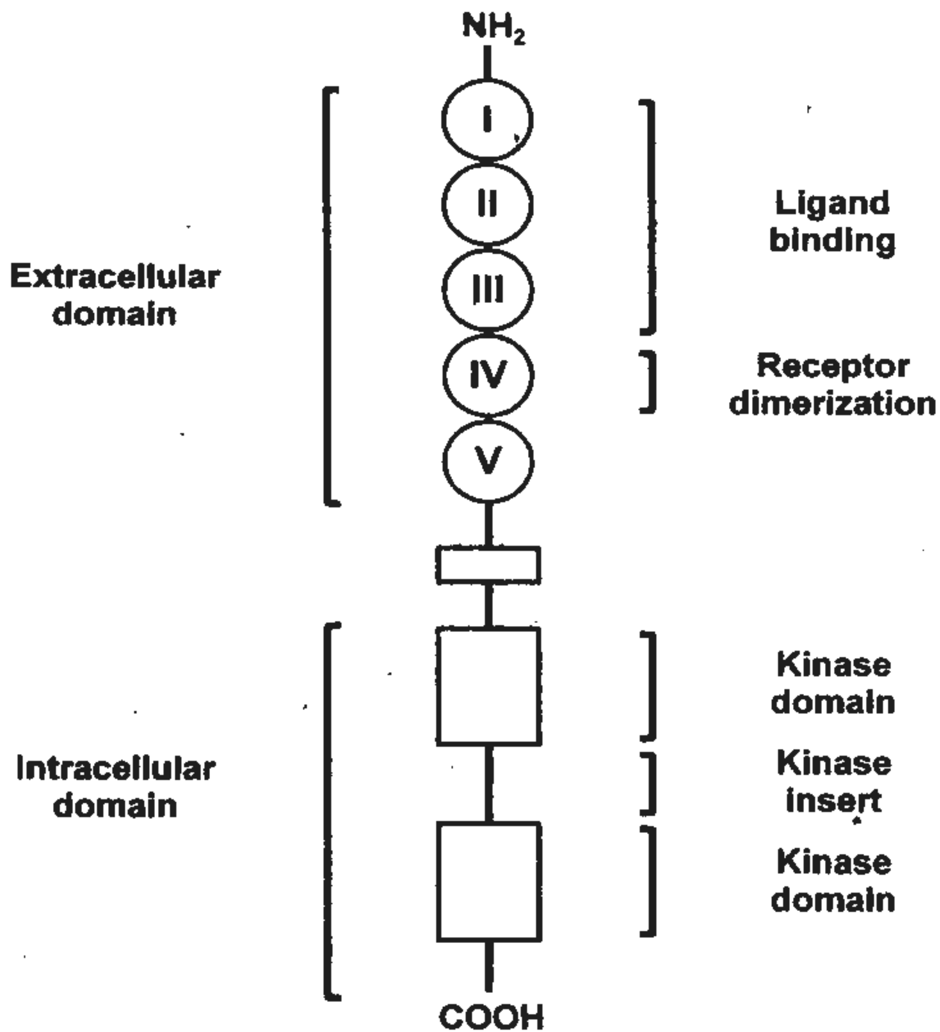
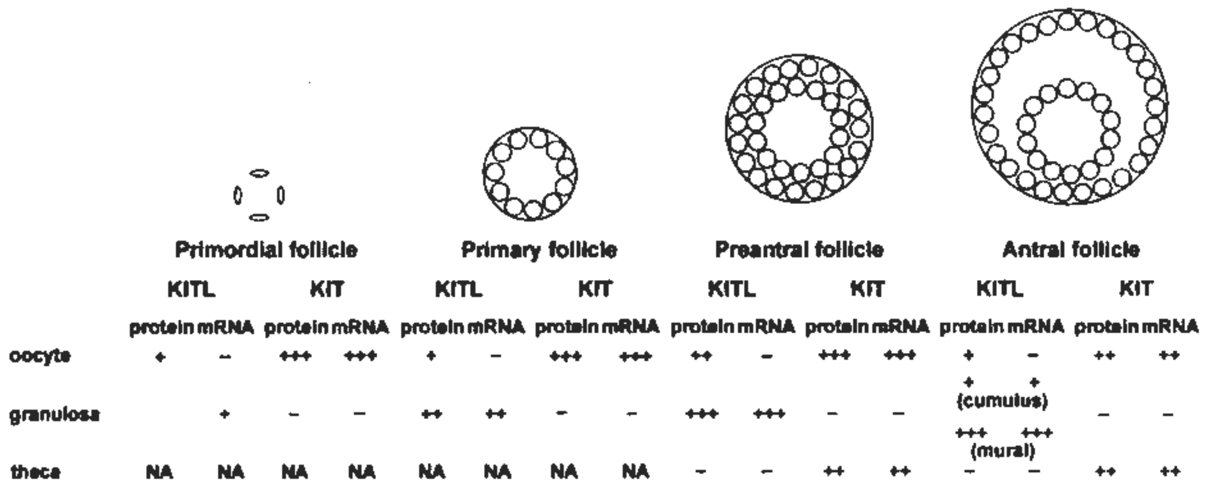


Fig. 1-2 Structure of KIT. Kit is composed of an extracellular ligand-binding domain containing five immunoglobulin like (Ig-like) domains, a single transmembrane segment, and a cytoplasmic kinase domain separated by a large kinase insert.



	Primordial follicle				Primary follicle				Preantral follicle				Antral follicle			
	KITL		KIT		KITL		KIT		KITL		KIT		KITL		KIT	
	protein	mRNA	protein	mRNA	protein	mRNA	protein	mRNA	protein	mRNA	protein	mRNA	protein	mRNA	protein	mRNA
oocyte	+	–	+++	+++	+	–	+++	+++	++	–	+++	+++	+	–	++	++
granulosa		+	–	–	++	++	–	–	+++	+++	–	–	+ (cumulus) +++ (mural)	+ (cumulus) +++ (mural)	–	–
theca	NA	NA	NA	NA	NA	NA	NA	NA	–	–	++	++	–	–	++	++

Fig. 1-3 Expression of Kit ligand and Kit during folliculogenesis in mice (modified from Hutt, 2006). Relative expression levels: +, low; ++, moderate; +++, high. NA = Not applicable.

Chapter 2

Existence of Two Isoforms of Kit (Kita and Kitb) and Kit Ligands (Kitlga and Kitlgb) in the Follicle and Evidence for Their Functional Divergence during Folliculogenesis

2.1 Introduction

As an important growth factor, Kit ligand (or stem cell factor, SCF) and its receptor Kit have received increasing attention in recent years, especially its roles in the gonads and embryonic development. In mammals, the Kit system consists of one ligand (*Kitl* on *Sl* locus in mice) and one major cognate receptor (*Kit* on *W* locus in mice) (3-6, 74). It is well known that Kit belongs to the type III tyrosine kinase family (15, 19, 20) and its binding by Kit ligand leads to a quick dimerization and activation of the receptor (75), which then induces a series of cellular responses via a cascade of signal transduction (22).

The Kit system plays critical roles in vertebrate life because it is involved in almost all aspects of development and growth (2, 6, 76, 77). In addition to its well-characterized functions in melanogenesis (78) and hematopoiesis (79), the importance of the Kit system in reproduction is also well recognized in mammals. The interaction between Kit ligand and its receptor Kit has been implicated in primordial germ cell (PGC) migration, survival and proliferation during embryonic development (39), as well as spermatogenesis and oogenesis in the adults (76, 78). In mouse embryos, *Kit* is expressed in PGCs as early as 7.5 dpc (32, 33), while *Kitl* is expressed by the microenvironment along the migratory pathway of PGCs from 9 dpc onward (6, 67, 80, 81). Mutation in either *Kit* or *Kitl*, therefore, may lead to failure of PGC migration to the gonads. In addition to directing PGC migration, the Kit system also

promotes PGC proliferation and survival during migration. The number of PGCs in a homozygous *Kit* mutant (W^e/W^e) is much lower than that in the heterozygous or wild type mice (42).

The Kit system also plays important roles in postnatal development of gonads. In mouse testis, the Sertoli cells express *Kitl* whereas multiple other types of cells express *Kit* mRNA, including type A and B spermatogonia, primary spermatocytes and Leydig cells (33, 80). In the ovary of postnatal mouse, *Kit* is expressed primarily by the oocytes and theca cells, while the expression of its ligand *Kitl* is restricted to the granulosa cells (32, 33, 82). The expression level of *Kit* is high from primordial to antral follicles in folliculogenesis (82-84); in contrast, the expression level of *Kitl* in the granulosa cells is low in primordial follicles, but gradually increases with oocyte growth and maturation of oocytes (35, 78, 83). The indispensable role of the Kit system in postnatal gonadal development has been further confirmed in mice with a point mutation in *Kit* (67).

Despite that very little is known about Kit system in other vertebrates, its existence has been demonstrated in fish, particularly the zebrafish (85, 86). Interestingly, in contrast to mammals which have one gene for Kit and Kit ligand in the genome, respectively, there exist two genes for Kit (*kita* and *kitb*) and Kit ligand (*kitlga* and *kitlgb*) in the zebrafish genome. This is likely due to the specific genome duplication in fish evolution (3R hypothesis) (87-92). Since most duplicated genes have been lost during fish evolution due to natural selection (nonfunctionalization), the existence of duplicated genes in the genome would suggest either functional partition of the original gene (subfunctionalization) or acquisition of new functions (neofunctionalization) (22, 52, 88, 89, 93-97). As for the function in zebrafish reproduction, the significance of the two ligands and receptors remains entirely unknown.

As the first step towards understanding Kit system and its function in fish reproduction, the present study was undertaken using the zebrafish as the model to characterize the two forms of Kit ligand (*kitlga* and *kitlgb*) and Kit (*kita* and *kitb*) in the ovary with particular emphasis on the differential functions of the ligand and receptor isoforms. After cloning the cDNA for zebrafish *kitb*, we performed phylogenetic and chromosome synteny analyses on members of the Kit system followed by examining their tissue distribution. We then analyzed temporal expression profiles of all members of zebrafish Kit system during ovarian folliculogenesis, focusing particularly on the periovulatory period. Although the exact roles played by different members of the Kit system in the ovary still remain elusive, the evidence from the present study strongly suggests functional differentiation for the two isoforms of both Kit and Kit ligand.

2.2 Materials and Methods

2.2.1 Animals and chemicals

Zebrafish (*Danio rerio*) were obtained from local tropical fish market and maintained in flow-through aquaria at 28 ± 1°C on a photoperiod of 14L:10D with light on at 8:00. The fish was fed twice a day with the commercial tropical fish feed Otohime S1 (Marubeni Nisshin Feed Co., Tokyo, Japan) and once with frozen artemia. All experiments performed were licensed by the Government of the Hong Kong SAR and endorsed by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong. All common chemicals used were purchased from Sigma (St. Louis, MO), USB Corporation (Cleveland, OH), GE Healthcare (Waukesha, WI) or Merck (Whitehouse Station, NJ), enzymes from Promega (Madison, WI) and culture medium from Gibco Invitrogen (Carlsbad, CA), unless otherwise indicated.

2.2.2 *Cloning and sequencing of zebrafish kitb cDNA*

The cDNA sequences of zebrafish *kitlga*, *kitlgb* and *kita* were obtained from GenBank (AY929068, AY929069 and NM_131053). For *kitb*, a partial genomic sequence was available in the Ensembl Databank (ENSDARG00000056133; http://www.ensembl.org/Danio_rerio/) and the exons of *kitb* were predicted by CBS PREDICTION SERVERS (http://www.cbs.dtu.dk/services/) and SOFTBERRY (http://www.softberry.com/berry.phtml). Specific primers were then designed based on the predicted sequence for amplification of the coding sequence. The amplicon was then cloned into pBluescript II KS(+) for sequencing. The sequencing reaction was performed with the BigDye Terminator Cycle Sequencing Kit v3.1 and analyzed on the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

2.2.3 *Phylogenetic and chromosome synteny analyses*

Sequences of Kit system proteins from different vertebrate species were aligned using ClustalW. Phylogenetic trees were constructed using Maximum-Likelihood (ML, 100 runs) method (Phylip, version 3.6). The tBLASTn search was performed in Ensembl and NCBI databanks (http://www.ensembl.org/index.html & http://www.ncbi.nlm.nih.gov/BLAST/) using zebrafish Kit ligands or receptors as query sequences to search for the corresponding orthologues in genome databases of human, medaka, takifugu, and stickleback. The synteny was then determined according to the genetic information of their chromosome linkage from Ensembl and NCBI databanks.

2.2.4 *Isolation of ovarian follicles*

After anesthetization on ice and decapitation, ovaries were removed from 10 to 20 female zebrafish and placed in a 100-mm petri dish containing 60% Leibovitz

L-15 medium (Invitrogen). The follicles of different stages were manually isolated and grouped into six developmental stages: primary growth (PG, stage I), previtellogenic (PV, stage II), early vitellogenic (EV, early stage III), mid-vitellogenic (MV, mid-stage III), full-grown (FG, late stage III) stage as well as mature (GVBD, stage IV) stage. The staging of the follicles was based on our previous report (98).

To collect follicles of FG and later stages in periovulatory period, several groups of fish with similar body size were set up (about 10 males and 10 females per group) in different tanks one day before follicle collection for sampling at different times (5:00, 6:00, 7:00 and 8:00). The ovaries from each time point were quickly dispersed in L-15 medium, and full-grown (FG) but immature follicles (5:00 and 6:00), mature but non-ovulated follicles (7:00) and ovulated eggs (8:00) were isolated for RNA extraction.

2.2.5 Follicle incubation

To obtain follicles that matured spontaneously in vitro, the immature FG follicles (~ 0.65 mm) were isolated and incubated as previously reported (99). In brief, the follicles isolated from 10 to 20 gravid female zebrafish were placed in a 24-well plate (~ 40 follicles per well) with 1 ml medium in each well. After incubation for 6 hr at 28°C, the follicles had undergone germinal vesicle breakdown (GVBD), which is a visible morphological marker for oocyte maturation (99), were separated from the immature ones.

2.2.6 Embryo collection

To follow the temporal expression patterns of the Kit system beyond fertilization, we collected embryos of different developmental stages according to the method published previously (100) for qPCR analysis.

2.2.7 Total RNA isolation and RT

Total RNA was extracted from cultured follicle cells with Tri-Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol and our previous study (101). The RT was then performed at 37°C for 2 h in a volume of 10 μl containing 0.5 μg of oligo(dT), 1× M-MLV RT buffer, 0.5 mM each deoxyribonucleotide triphosphate (dNTP), 0.1 mM dithiothreitol, and 100 U of M-MLV RT (Invitrogen, Carlsbad, CA).

2.2.8 Quantification of gene expression by real-time qPCR

Real-time quantitative PCR was performed to quantify the expression of *kitlga*. The template for the standard curve was prepared by PCR amplification of cDNA fragment with specific primers. After purification with a PCR Purification Kit (Qiagen, Valencia, CA), the amplified DNA amplicons were quantified with the software Quantity One (Bio-Rad, Hercules, CA) using the Mass Ruler DNA marker (MBI Fermentas, Hanover, MD) as the standard, and the copy numbers of the DNA molecules were calculated before use as templates to construct standard curves in real-time quantitative PCR. All PCR reactions were performed in a total volume of 30 μl containing 10 μl template (RT reaction mix diluted at 1:15), 1× PCR buffer, 0.2 mM each dNTP, 2.5 mM $MgCl_2$, 0.75 U of Taq polymerase, 0.5× EvaGreen (Biotium, Hayward, CA), and 20 nM fluorescein (Bio-Rad) on the iCycler iQ Real-time PCR Detection System (Bio-Rad). The amplification protocol was 30 sec at 94°C, 30 sec at 60°C, and 30 sec at 72°C, with a signal detection period of 7 sec at 80°C. A melt curve analysis was performed at the end of the reaction to check the reaction specificity.

2.2.9 Data analysis

The ratio of target gene expression to that of the internal control *ef1a* was calculated and then expressed as the fold change compared to the control or reference group. All values were expressed as the mean ± SEM and the data were analyzed by one-way ANOVA followed by Dunnett's test using Prism 5 on Macintosh OS X (GraphPad Software, San Diego, CA). All experiments were performed at least twice to ensure repeatable results.

2.3 Results

2.3.1 Bioinformatics analysis of the Kit system in the zebrafish

In the zebrafish, the Kit system includes two ligands, *kitlga* and *kitlgb*, and two receptors, *kita* and *kitb*. For *kitb*, we obtained two EST sequences of *kitb* from the GenBank and retrieved their corresponding genomic sequences. Using the tools CBS PREDICTION SERVERS and SOFTBERRY for gene prediction and tBlastn search in NCBI and Ensembl databases for *kitb* sequences from other teleosts, we obtained the deduced amino acid sequence of zebrafish *kitb*, which allowed us to clone the cDNA subsequently. The sequence of the cloned *kitb* cDNA has been deposited in the GenBank (GQ994993). Sequence analysis with the above tools suggested that both *kita* and *kitb* were members of the classical receptor tyrosine kinase family. We have further confirmed this by expressing the two receptors in the COS cells and examining their responses to recombinant zebrafish Kitlga and Kitlgb by Western analysis (Chapter 3).

Amino acid sequence analysis demonstrated that the two receptors (Kita and Kitb) are highly conserved, especially in the intracellular domains, with sequence identity being 44.6%, 60.9%, 73.7%, 21.9%, 100% and 90% over the extracellular domain (ED), transmembrane domain (TD), juxtamembrane domain (JD), kinase

insert domain (KI), catalytic loop (CL) and activation loop (AL), respectively. In contrast, the two ligands exhibit relatively higher sequence variation with ~30% sequence identity (Fig. 2-1). In the zebrafish genome, the genes coding for Kita and Kitb have 21 and 20 exons, respectively, whereas those for Kitlga and Kitlgb have 9 and 8 exons, respectively (Fig. 2-2).

Phylogenetic analysis revealed that zebrafish *kitlga* was clustered with those of takifugu, tetraodon and stickleback, so were *kitlgb*, *kita* and *kitb* (Fig. 2-3). Synteny analysis on the Kit system in human and teleosts showed that a variety of genes on human chromosome 12 containing *KITLG* had respective homologues on zebrafish chromosome 4 containing *kitlgb* and chromosome 25 containing *kitlga* (Fig. 2-4A). This conserved synteny was also apparent among teleost chromosomes including chromosome 25, group XIX, ultracontig 72 for *kitlga* and chromosome 4, group IV, chromosome 23 for *kitlgb* in zebrafish, stickleback and medaka, respectively (Fig. 2-5A and B). Despite that zebrafish chromosome 20 with *kitb* had no corresponding conserved regions of human chromosome 4 with *KIT* and zebrafish chromosome 20 with *kita* (Fig. 2-4B), the synteny was much more evident among chromosome 20, group VIII and chromosome 4 for *kita* and chromosome 1, group IX and chromosome 1 for *kitb* in zebrafish, stickleback and medaka, respectively (Fig. 2-5C and D).

2.3.2 *Tissue distribution of Kit ligands and receptors*

As a growth factor family important in development, Kit ligand and Kit are anticipated to act in a wide range of cells and tissues. RT-PCR analysis of zebrafish tissues (brain, gill, kidney, liver, muscle, ovary and testis) demonstrated that *kitlga* and *kita* existed in all tissues examined including the ovary and testis. Similarly, *kitlgb* and *kitb* also exhibited a wide tissue distribution; however, these two genes were not expressed in the liver (Fig. 2-6). The expression of all Kit system members in the

gonads highlights their involvement and importance in zebrafish reproduction.

2.3.3 *Temporal expression profiles of the Kit system during folliculogenesis*

To provide clues to the roles of the Kit system in the zebrafish ovary, we further examined their temporal expression profiles in folliculogenesis. Interestingly, the two isoforms of Kit and Kit ligand exhibited significantly different expression profiles during follicle growth and maturation.

As the orthologue of mammalian Kit ligand in the zebrafish, *kitlga* showed a dramatic increase in expression during the transition from the primary growth phase to fast secondary growth phase, or from the primary growth stage (PG, stage I) to previtellogenic stage (PV, cortical alveolus stage or stage II). After reaching the peak level at EV stage, its expression declined steadily afterwards. Interestingly, the expression of *kitlga* dropped significantly to a marginal level in the ovulated oocytes, which were free of the somatic follicle cells. In contrast, *kitlgb* showed a significant drop in expression during the PG-PV transition and its level remained stable afterwards (Fig. 2-7A).

The expression profiles of the two receptors during folliculogenesis were even more impressive as compared to the ligands. The expression of *kita* was relatively stable from PG to FG; however, its level surged dramatically in the ovulated eggs after final maturation. As for *kitb,* its expression dropped significantly during the transition from PG to PV and remained very low afterwards, similar to *kitlgb*; however, *kitb* expression level surged at the FG stage. Interestingly, the increase of *kitb* expression occurred before final maturation whereas *kita* expression surged in the ovulated eggs after maturation. In the ovulated eggs, *kitb* expression was barely detectable (Fig. 2-7B).

2.3.4 Expression profiles of the Kit system during in vivo and in vitro oocyte maturation

The results presented above strongly implicated Kit system in early and late stages of folliculogenesis, especially the period before and after final oocyte maturation. To provide additional evidence for the role of the Kit system in oocyte maturation, ovulation, or even postovulatory event such as fertilization, we further examined the expression profiles of the Kit system in vivo in the FG follicles or ovulated eggs before and after maturation. In our laboratory, we adopted a 14L:10D lighting scheme with the light on at 8:00 and off at 22:00. According to our experience, the FG follicles that have undergone final maturation but not ovulation can normally be seen in the ovaries sampled at 7:00 (one hour before light-on) whereas the ovaries collected at 8:00 often contain ovulated eggs. In this experiment, we sampled FG follicles at 5:00 (-3 hr, immature), 6:00 (-2 hr, immature), 7:00 (-1 hr, mature) and 8:00 (0 hr, ovulated) for quantitative determination of Kit system expression. The mature but non-ovulated follicles and the ovulated eggs could be easily distinguished by their distinct morphologies. When placed in the medium, the chorion of the ovulated eggs became quickly detached from the oocyte surface and expanded, which did not happen in the mature but non-ovulated follicles (Fig. 2-8). In addition, due to the presence of the somatic follicle layer, the non-ovulated follicles were often seen with some young follicles attached.

During the periovulatory period, all Kit system members displayed significant variation in expression before and after maturation. At 6:00, which was one hour before GVBD was noticed, the expression level of *kitlga* increased dramatically compared to the level at 5:00. Its expression dropped significantly at 7:00 in the GVBD or mature follicles and continued to decrease to very low level in the ovulated eggs at 8:00. In contrast, *kitlgb* expression was low at 5:00 and 6:00 but increased at

7:00 in the mature follicles although the increase was not statistically significant. Interestingly, its level continued to increase and surged in the ovulated eggs collected at 8:00 (Fig. 2-8).

The two Kit receptors also exhibited distinct and significant changes during this period. There was a marginal increase in *kita* expression at 7:00 in GVBD follicles compared with the immature FG follicles sampled at 5:00 and 6:00; however, its expression rose dramatically in the ovulated eggs collected at 8:00, similar to the pattern of *kitlgb*. In contrast, *kitb* expression started to increase at 6:00 and continued to rise to the maximal level at 7:00 in the GVBD follicles. Interestingly, no *kitb* transcript could be detected in the ovulated eggs at 8:00 (Fig. 2-8).

Among the four genes examined, *kitlga* was the only one that exhibited a decreased expression in the mature follicles at 7:00 whereas the other three genes all increased their expression although the increase was not statistically significant for *kitlgb* and *kita* due to their exceedingly high expression level in the ovulated eggs at 8:00 (Fig. 2-8). To test if the expression changes of these genes observed at 7:00 in the GVBD follicles were related to the event of final maturation, we did another experiment to compare the expression levels of the target genes in the immature FG follicles and mature GVBD follicles isolated at the same time of 7:00. As shown in Fig. 2-9, compared with the levels in the immature FG follicles, *kitlga* decreased its expression in the mature GVBD follicles whereas all the other three genes (*kitlgb, kita* and *kitb*) exhibited an increased expression, consistent with the results obtained above between the immature FG follicles collected at 6:00 and mature GVBD follicles at 7:00.

To further confirm these observations, we went on to carry out an in vitro experiment by incubating immature FG follicles in vitro, during which some follicles would undergo spontaneous maturation as we reported previously (98, 99). After 6-hr

incubation, the follicles that matured in vitro were separated from the immature follicles, and the expression levels of the Kit system were determined and compared. Similar to the in vivo data described above, *kitlga* expression decreased whereas *kitlgb* and *kita* expression both increased in the GVBD follicles that matured spontaneously in vitro. However, different from the in vivo observation, the expression of *kitb* did not show any change during the in vitro spontaneous maturation (Fig. 2-9).

2.3.5 Developmental profiles of Kit system transcripts during embryogenesis

The significant changes of Kit system transcripts during folliculogenesis, especially in late stages of development, raise an interesting question about their levels beyond fertilization. In this experiment, we examined the expression profiles of the ligands (*kitlga* and *kitlgb*) and receptors (*kita* and *kitb*) during the entire embryogenesis. We used FG follicles as the control and reference point. Since the expression of housekeeping gene *ef1a* changed its expression dramatically during embryogenesis, we did not normalize target gene expression to it in this experiment (Fig. 2-10).

Consistent with the marginal expression level detected in the ovulated eggs, *kitlga* had nearly undetectable expression at the 4-cell stage after fertilization; but its level increased steadily and dramatically after the sphere stage, reaching the highest at the hatching stage. By comparison, *kitlgb* had a detectable expression at the 4-cell stage and its level started to rise significantly at the 8-somite stage. As for the receptors, *kita* transcript was abundant at the 4-cell stage, consistent with its high expression level in the ovulated eggs. However, this high expression level did not last for long period, and it decreased significantly at sphere stage and continued to decline to nearly undetectable level at the shield stage. The expression quickly went up again

at the bud stage and continued to rise afterwards. Different from *kita* but similar to *kitlgb*, *kitb* had nearly undetectable expression at the 4-cell stage and its level remained very low through the bud stage; however, the expression elevated significantly beyond the 8-somite stage.

It is worth noting that the four target genes exhibited distinct expression levels at the 4-cell stage after fertilization. Compared to the control of immature FG follicles, *kitlga* and *kitb* expression was nearly undetectable at this stage, whereas *kitlgb* and *kita* both had detectable mRNA levels with *kita* expression being even higher than that in the FG follicles, reflecting its high level of expression in the unfertilized ovulated eggs (Fig. 2-10).

2.4 Discussion

It is well documented that the Kit system plays important roles in reproduction. During prenatal embryonic development, the Kit system is essential for primordial germ cell migration, proliferation and survival. In postnatal period, the system continues to regulate gonadal development and gametogenesis in adults. Most of these studies, however, have been carried out in mammalian models, and very little is known about the Kit system in reproduction of other non-mammalian vertebrates.

In the present study, we demonstrated that the Kit system in the zebrafish consists of two pairs of homologues for the ligand (*kitlga* and *kitlgb*) and receptor (*kita* and *kitb*), respectively. This might be caused by genome duplication in vertebrate evolution. To date, it has been proposed that whole genome duplication has occurred three times in vertebrate lineage (3R hypothesis) (87-89, 91, 92). The third round has been proposed to be specific to ray-finned fish, which accounts for the complexity and diversity of teleost fish compared to other vertebrates. After genome duplication, the duplicated genes might have different fates: nonfunctionalization,

subfunctionalization, and neofunctionalization (22, 52, 88-90, 93-97). Our phylogenic and chromosome synteny analyses in the zebrafish suggest that the duplicated genes of Kit and Kit ligand in the zebrafish are likely derived from the third genome duplication. Both forms of zebrafish Kit receptors (*kita* and *kitb*) seem to be functional and the expression patterns of *kita* and *kitb* in zebrafish embryos partially recapitulates that of mouse *Kit* (86). However, in contrast to the well-documented function of the Kit system in mammalian PGCs, in situ hybridization in the zebrafish failed to demonstrate the expression of *kita* or *kitb* in the PGCs (85, 86). Despite this, our data in the present study showed that all members of the zebrafish Kit system, including the ligands (*kitlga* and *kitlgb*) and receptors (*kita* and *kitb*), are expressed in the ovary, suggesting potential roles for the system in controlling ovarian development and function.

Although we do not understand the importance of the Kit system in zebrafish reproduction, the expression profile analysis in the present study provided lines of evidence for the involvement of the Kit system in the zebrafish ovarian folliculogenesis. Members of the Kit system displayed dramatic changes in their expression during folliculogenesis from follicle recruitment to final maturation. While *kitlgb* expression remained more or less stable during follicle growth and maturation, *kitlga* exhibited a remarkable increase in expression during the transition from PG to PV, an important period in fish folliculogenesis that marks the recruitment of the follicles from the gonadotropin-independent primary growth phase to gonadotropin-dependent secondary growth phase. This strongly implicated Kitlga in initiating follicle activation for vitellogenesis. This potential function of zebrafish Kitlga appears to agree with that of its counterpart in mammals. Kit ligand and its receptor have been well demonstrated in mammals to play a critical role in early follicle development, particularly the activation of primordial follicles. Injection of

neonatal mice with a Kit specific antibody disturbed the initial follicle recruitment, primary follicle growth, and formation of antral follicles (48). This has also been demonstrated by in vitro experiments. When cultured in the presence of Kitl, the ovaries from neonatal rats contained significantly higher number of activated follicles, which could be blocked by specific Kit antibody (49). Furthermore, in both mice and humans, KITL has a higher expression level in the primary follicles than that in primordial follicles whereas KIT expression level remains relatively stable and high from primordial to preantral follicles compared to that in the antral follicles (35, 78, 83, 89, 94). This is somewhat similar to what we observed in the zebrafish ovary where *kitlga* had high expression in the early stages of secondary growth phase whereas *kita* maintained a more or less contact expression level until after final maturation. The expression of *kitb* was somehow different from that of *kita* in that its level dropped during the PG to PV transition, remained stable and low through MV stage, but surged in the FG stage before final maturation.

The most interesting changes of Kit system expression during folliculogenesis, particularly the two receptors, occurred in the periovulatory period, *i.e.*, the stage before and after final oocyte maturation. Despite its extremely low expression level in early periods of follicle growth, *kitb* dramatically increased its expression in the FG stage prior to maturation. However, its level plunged to nearly zero in the ovulated eggs. This could be due to the loss of the somatic follicle layer during ovulation. A recent study in our laboratory showed that *kita* and *kitb* expression had distinct localization in the follicle with *kita* being expressed exclusively in the oocyte, similar to the situation in mammals, whereas *kitb* expression restricted to the surrounding follicle cells (Chapter 3). In contrast to *kitb*, *kita* expression was even lower in the FG follicles than the earlier stages. However, its mRNA abundance surged in the ovulated eggs after final oocyte maturation. In view of the distinct location of *kita* and *kitb* in

the follicle, the reverse patterns of their expression in the FG follicles strongly imply different roles for Kit signaling pathways in the two follicle compartments in preparing the follicles for the final maturation. It is interesting to speculate that an increased signaling in the follicle cells by *kitb* and decreased signaling by *kita* in the oocyte may be beneficial to oocyte maturation or ovulation.

Further evidence for differential functions of the Kit system members in oocyte maturation, ovulation, or even postovulatory events came from in vivo and in vitro experiments focusing on FG follicles and ovulated oocytes. Both ligands and receptors exhibited significant but different patterns of expression in the periovulatory period. A significant increase in *kitlga* at 6:00, one hour before GVBD became visible, strongly suggests a potential role for this ligand in oocyte maturation, but its action may be transient because its expression level dropped significantly in the follicles undergoing GVBD at 7:00. In contrast to *kitlga*, all other members of the family including *kitlgb*, *kita*, and *kitb* demonstrated an increase in expression in the GVBD follicles at 7:00, particularly *kitb*. Interestingly, *kita* level continued to rise in the ovulated eggs at 8:00, in agreement with the result described above for developmental profiles during folliculogenesis. We are not sure about what happened to *kitb* at 8:00 because of the lack of follicle cells in the ovulated eggs. It was evident that the changes occurring at 7:00 were associated with the event of final oocyte maturation because when comparing mature follicles to immature ones collected at the same time, *kitlga* had significantly lower whereas all others had higher expression levels in the GVBD follicles. Similar results were obtained in vitro for *kitlga*, *kitlgb*, and *kita* using follicles that spontaneously matured in culture. Interestingly, despite its increase in expression in the GVBD follicles that matured in vivo, *kitb* did not show any difference in its expression between immature and mature follicles in vitro. The reason for this discrepancy is unknown. It could be that the increased expression of

kitb in the mature follicles in vivo is induced by certain endocrine hormones, which are lacking in the in vitro incubation.

Although both in vivo and in vitro data obtained in the present study support an involvement of the Kit system in the final oocyte maturation and probably postovulatory events as well, the exact roles played by the system, particularly the differential functions of different ligand and receptor isoforms remain entirely unknown. We have recently established two cell lines producing recombinant zebrafish Kitlga and Kitlgb, which will provide critical tools for future studies on the physiological functions of the system in final oocyte maturation, ovulation, and postovulatory events including follicle cell apoptosis and fertilization. In mammals, the Kit system has been reported to exert an inhibitory effect on oocyte maturation or meiosis resumption. Microinjection of Kit antisense oligonucleotides into the rat oocytes stimulated the oocytes to resume meiosis (59). In agreement with this, the oocytes cultured in the presence of Kit ligand were delayed in their resumption of meiosis and the inhibitory effects could be blocked by a specific antibody against Kit extracellular domain (58, 59). Furthermore, the membrane-associated form of Kit ligand has been evidenced to maintain meiotic arrest in rat oocytes in vitro (102) and hCG-induced GVBD was accompanied by a transition of Kit ligand from membrane-associated form to soluble form and the loss of both forms in cumulus granulosa cells (37, 59). Given that the membrane-associated Kit ligand is more capable of activating Kit receptor than the soluble form (103-105), this transition would relieve the inhibition of the oocyte by Kit ligand. Whether or not Kit ligands play similar roles in the zebrafish ovary is unknown; however, the possibility exists because *kitlga* exhibited a higher expression level in immature FG follicles than that in GVBD follicles both in vivo and in vitro.

The possibility that the Kit system may also be involved in the postovulatory

events is raised by our observation that *kitlgb* and *kita* both started to increase their expression during oocyte maturation and the levels continued to rise in the ovulated eggs. The exact roles played by them after ovulation are entirely unknown. They could be involved in postovulatory follicle cell functions such as proliferation, differentiation and apoptosis, fertilization, or even early embryonic development. These would be interesting issues to address in the future.

Despite that the level of *kita* expression reached the highest in the ovulated eggs, it dropped sharply after fertilization, which has led us to speculate that *kita* may play a functional role in early developmental events such as fertilization. An interesting question that arises from this speculation would be the sources of the ligand that activates Kita during these events. One possibility is that the ovulated eggs could produce their own ligands, which may in turn exert an autocrine influence on the eggs themselves. According to our data in the present study, *kitlgb* expression also surged further in the ovulated eggs, which may serve as the ligand to stimulate Kita in the same cell. To further elucidate this mechanism, future studies on ligand-receptor specificity would be essential. In mammals, it is generally accepted that Kit ligand is primarily expressed in the somatic granulosa cells whereas its receptor Kit is produced by the oocyte, suggesting a follicle cell-to-oocyte signaling. However, the expression of both *Kitl* and *Kit* has been demonstrated by sensitive RT-PCR in mouse fetal oocytes (51). Similar evidence has also been reported in humans that *KITL* and *KIT* were both detectable in the oocytes of primordial, primary and preantral follicles (106). Further evidence for an autocrine action of the Kit system on the oocytes came from the experiment that treatment of oocytes with a Kit ligand antibody led to apoptosis in the oocytes (51). In addition to the oocyte, there have also been lines of evidence supporting autocrine roles of Kit system in other cell types including aortic endothelial and smooth muscle cells (107), neural crest cells (108), mast cells

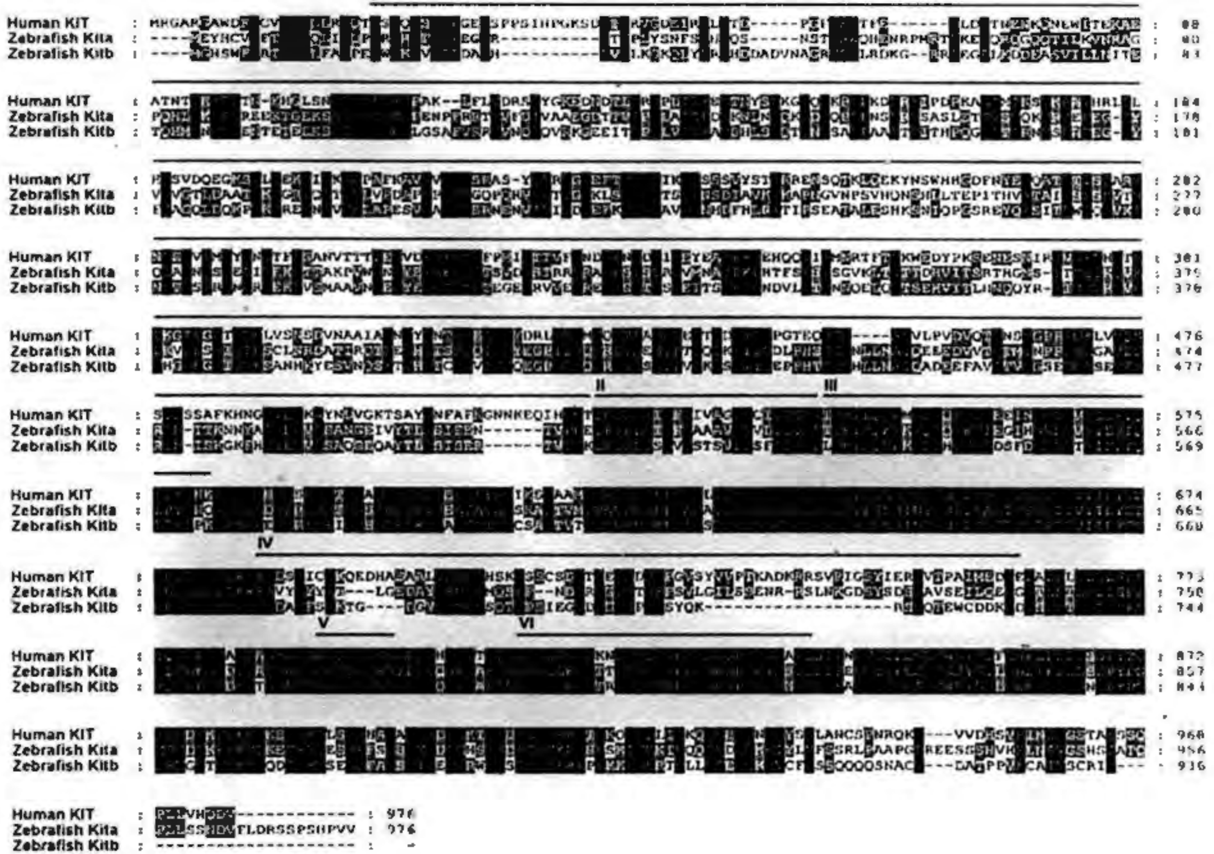
(109-111), and ovarian surface epithelial cells (55). The second potential source of Kit ligand after ovulation would be the sperm that fertilizes the ovulated egg; however, we do not have any evidence at this moment for the expression of *kitlga* and/or *kitlgb* by the zebrafish sperm cells, which would be an interesting issue to address in the future. If proven, such an interaction of sperm-anchored Kit ligand with oocyte-associated Kita may play a role in fertilization. Indeed, it has been shown in mammals that the membrane-associated Kitl produced by cultured somatic cells facilitates the adhesion of primordial germ cells that express Kit, which can be blocked specifically by antibodies against Kit or Kitl (112).

In summary, the zebrafish has two homologues of Kit ligand (*kitlga* and *kitlgb*) and Kit (*kita* and *kitb*) instead of one copy for each as in mammals. The present study proposed the origin of these homologues in the zebrafish by phylogenetic and chromosome synteny analyses, and provided further evidence for neo- or subfunctionalization for both Kit ligands and Kit receptors in the zebrafish ovary. All four Kit system members exhibited distinct and significant changes in mRNA expression during folliculogenesis, particularly in the periovulatory period before and after final oocyte maturation and ovulation. Based on our observations, the Kit system may play important roles during follicle recruitment from primary growth to secondary growth phase and late stages of follicle development such as final maturation and ovulation. The function may even extend to postovulatory events such as fertilization and early embryonic development. Future studies of critical importance to our understanding the Kit system in the zebrafish would include ligand-receptor specificity, bioactivities of each ligand, and differential regulation of different forms of Kit and Kit ligand. Albeit in an early stage, our study has set a strong foundation for future studies in the zebrafish and provided a broader prospect to understand the Kit system in vertebrate reproduction.

Table 2-1 Primers used in RT-PCR

Gene	Strand	Sequence	Expected size (bp)	Accession no.
ef1a	Sense	5'-GGCTGACTGTGCTGTGCTGATTG-3'	409	NM_131263
	Antisense	5'-CTTGTCGGTGGGACGGCTAGG-3'		
kitlga	Sense	5'-AATTCATCAAGAGATGCTAGGAC-3'	213	AY929068
	Antisense	5'-TGCAAACGGGATGGTGAGGAG-3'		
kitlgb	Sense	5'-ATGTTCCACATGAGGGAGGTTA-3'	235	AY929069
	Antisense	5'-TGCTCTCGACTGGATACAGG-3'		
kita	Sense	5'-CCGATCACACTGGGTCAGCC-3'	284	NM_131053
	Antisense	5'-TTGACCCAGACTGGCTTTGC-3'		
kitb	Sense	5'-CCAAACCCAACGACGTCTTG-3'	234	GQ994993
	Antisense	5'-CTTCCTGGGAAACAATCACT-3'		

A



B

Fig. 2-1 Multiple alignments of Kit system proteins of zebrafish and human (A, Kit; B, ligand Kit). Different functional domains of Kit are indicated by the overlines (I-VI: ED, extracellular domain; TD, transmembrane domain; JD, juxtamembrane domain; KI, kinase insert domain; CL, catalytic loop; AL, activation loop).

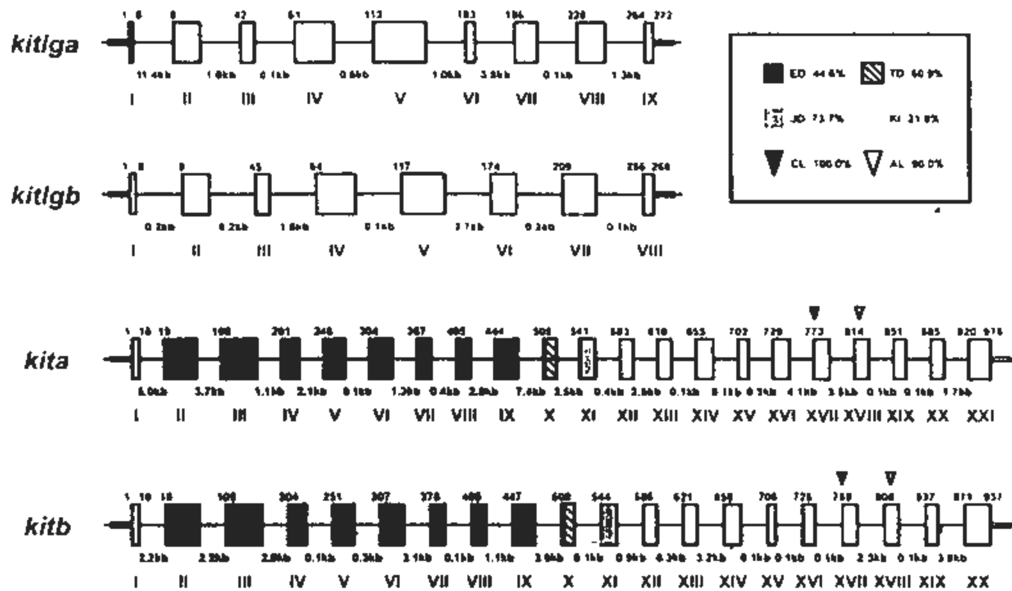


Fig. 2-2 Genomic structures of zebrafish Kit system members. Exons are boxed and labeled with Roman numbers whereas introns are represented by lines. The smaller boxes at both ends of each gene are UTRs. Lengths of introns are indicated in kb and numbers above exons represent amino acid positions. In *kita* and *kitb*, the functional domains ED, TD, JD and KI are indicated by filled boxes and the functional loops CL and AL by arrows. Percentage values are the identity of respective domains between the two receptors.

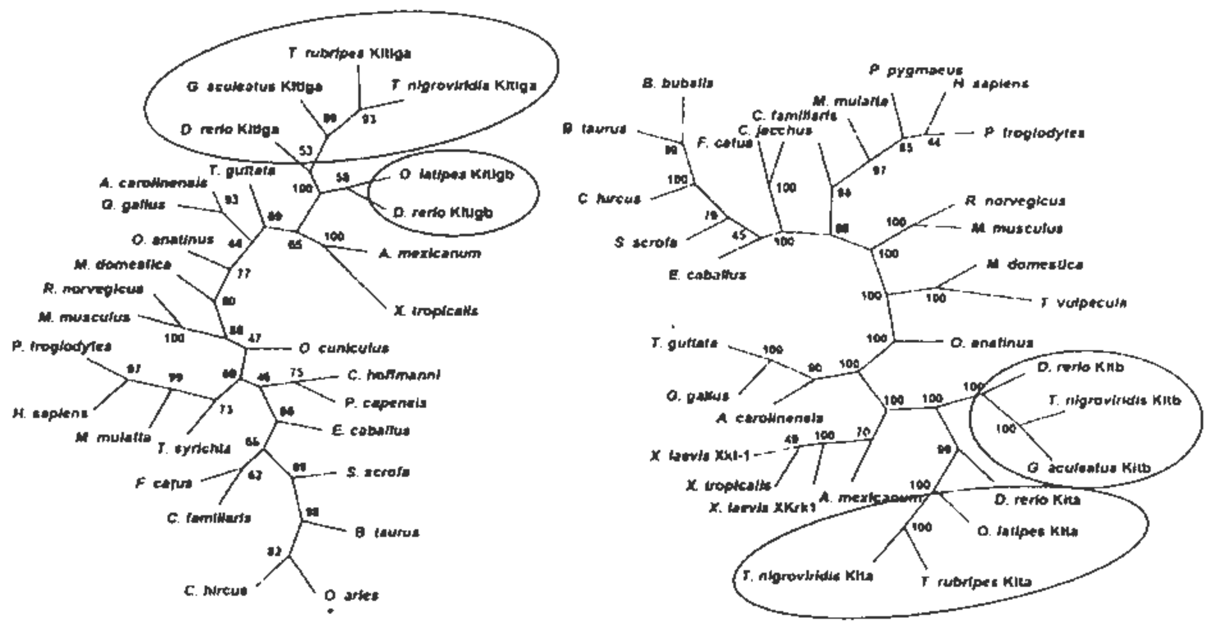


Fig. 2-3 Phylogenetic trees of Kit system proteins. Phylogenetic analysis was performed with Phylip (Joseph Felsenstein, Washington University). Numbers in the branches indicate the bootstrap values (%) from 100 replicates by the Maximum-Likelihood method. The sequence of zebrafish Kitb is based on the cDNA cloned in the present study whereas other sequences are extracted from NCBI (http://www.ncbi.nlm.nih.gov/) and Ensembl (http://www.ensembl.org/index.html) databases.

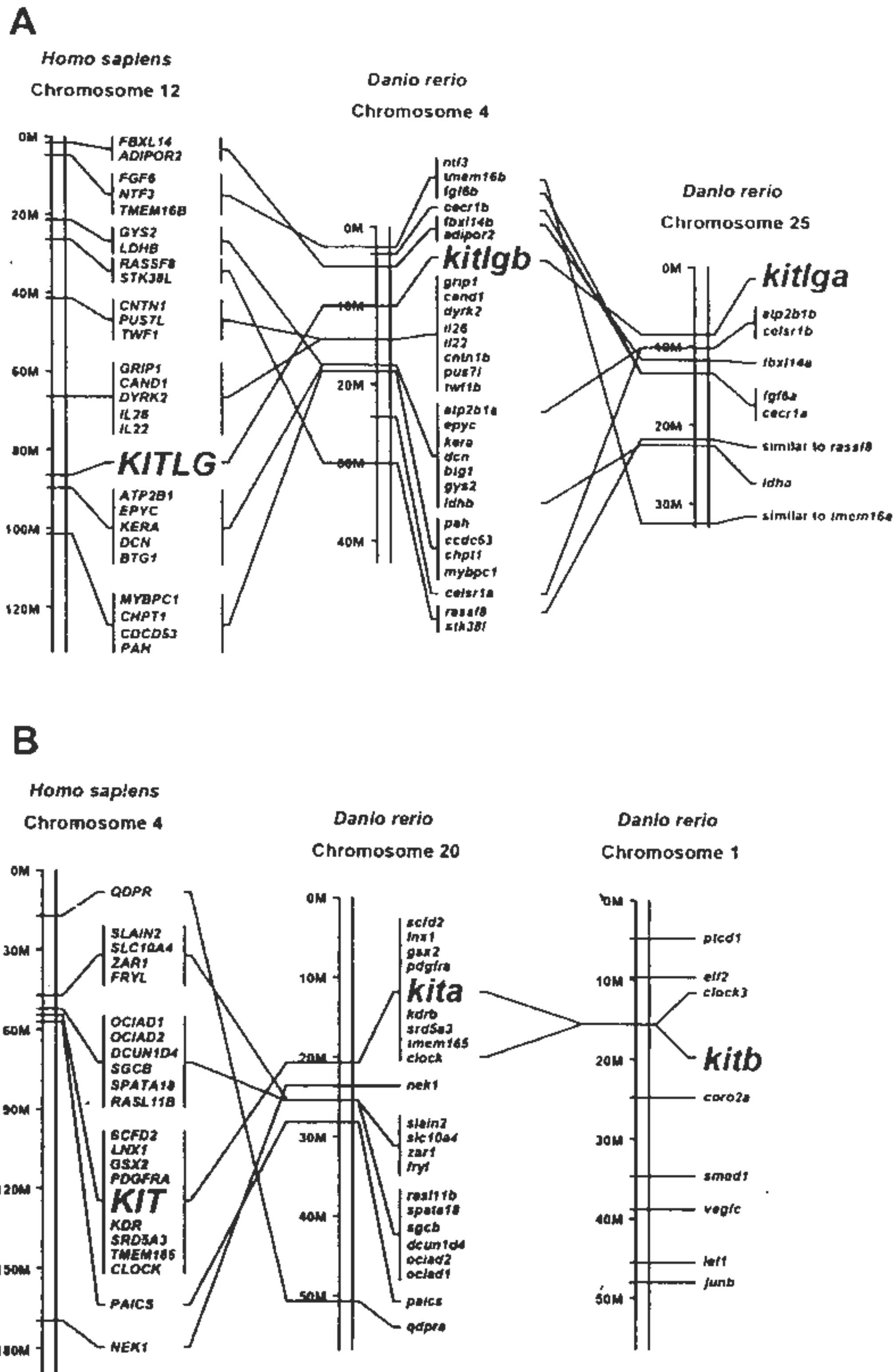


Fig. 2-4 Synteny analysis of human and zebrafish Kit system (A, Kit ligand; B, Kit). The approximate loci of the genes in the genomes were obtained from NCBI and Ensembl databases.

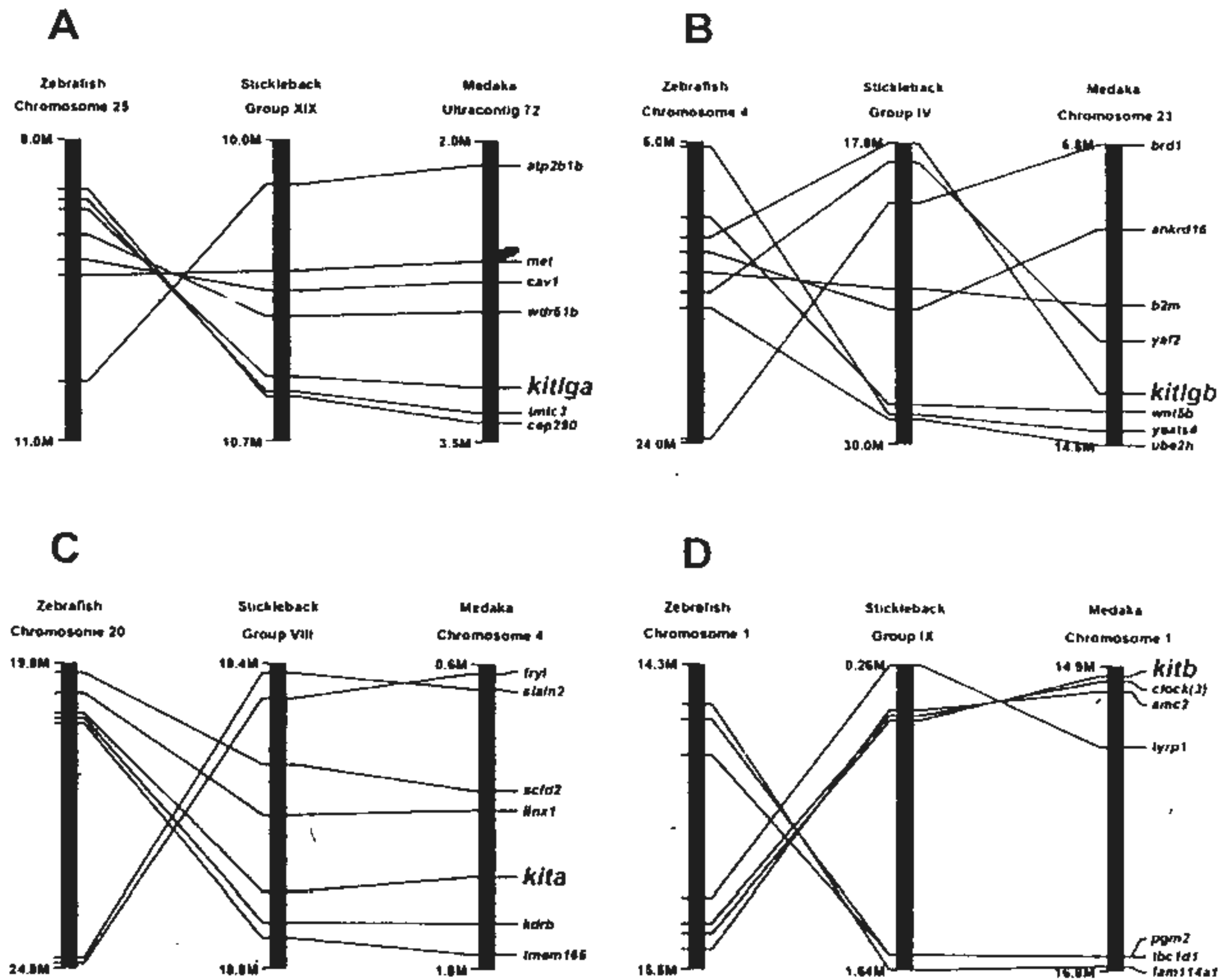


Fig. 2-5 Comparison of synteny in zebrafish, stickleback and medaka (A, Kitlga; B, Kitlgb; C, Kita; D, Kitb). The approximate loci of the genes in the genomes were obtained from NCBI and Ensembl databases.

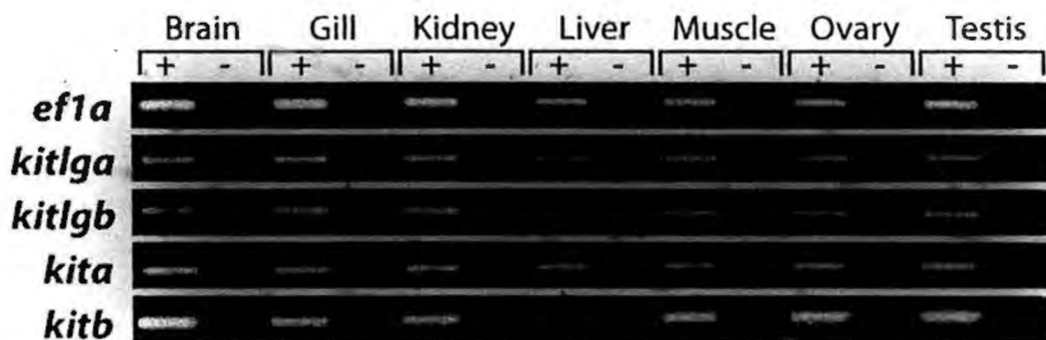
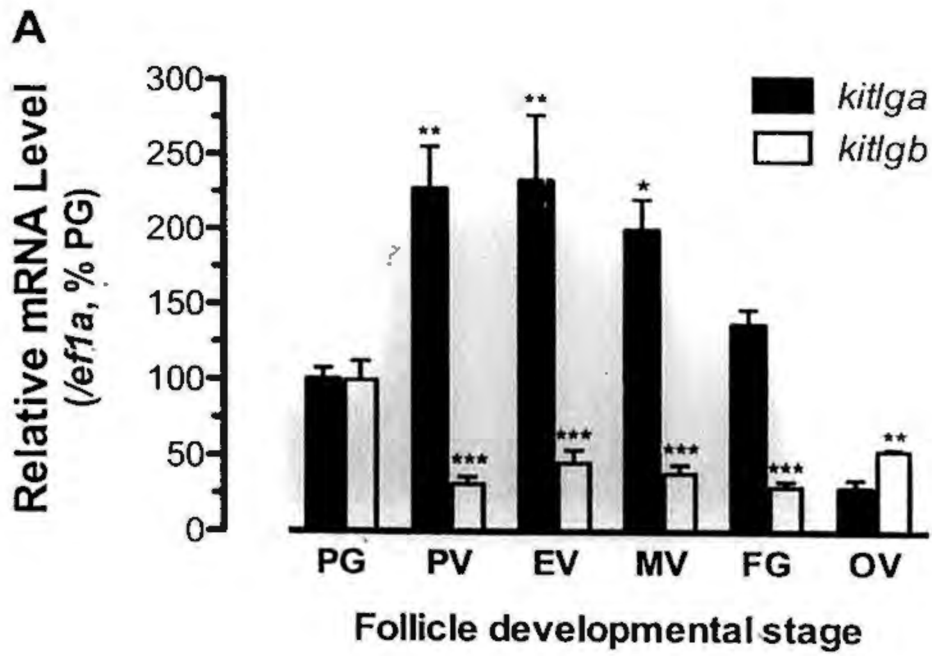


Fig. 2-6 Tissue distribution of Kit ligand (*kitlga* and *kitlgb*) and Kit (*kita* and *kitb*). Semi-quantitative detection of Kit system expression in the brain, gill, kidney, liver, muscle, ovary and testis. +, RT with reverse transcriptase; -, RT without reverse transcriptase.


A
Relative mRNA Level
(/ef1a, % PG)
300
250
200
150
100
50
0
kitlga
kitlgb
**
**
*
***
***
***
***
**
PG
PV
EV
MV
FG
OV
Follicle developmental stage

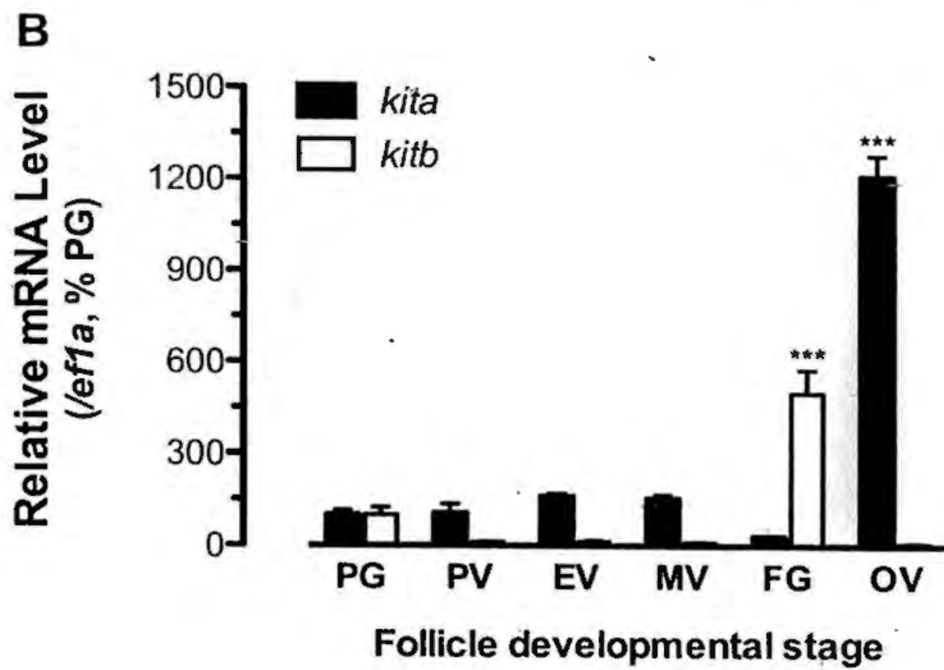
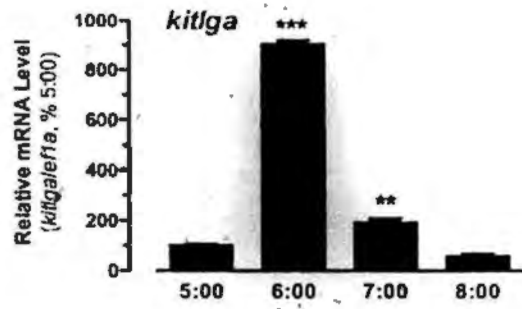
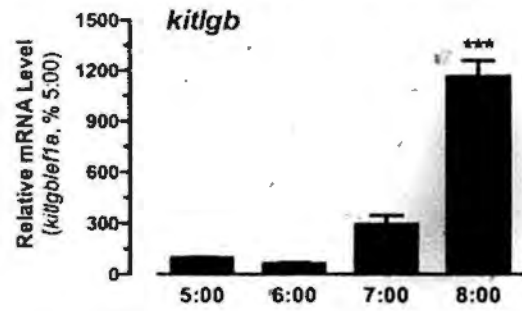
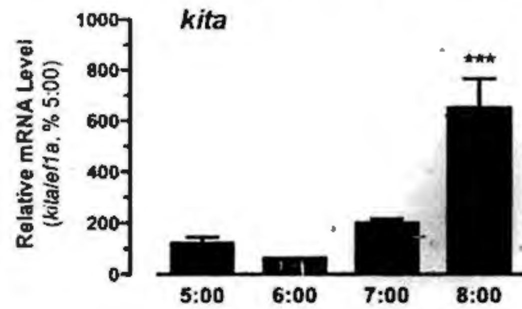
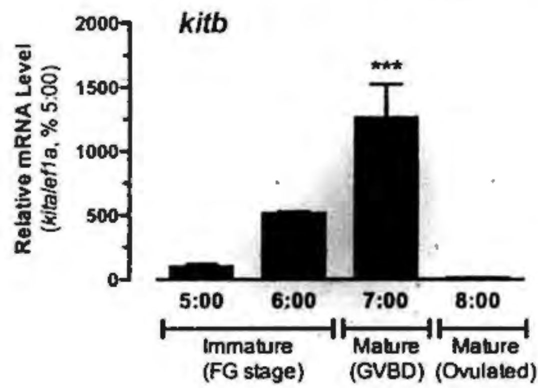

B
Relative mRNA Level
(/ef1a, % PG)
1500
1200
900
600
300
0
kita
kitb
***
***
PG
PV
EV
MV
FG
OV
Follicle developmental stage

Fig. 2-7 Temporal expression profiles of the Kit system during folliculogenesis. The expression levels at different developmental stages were determined by real-time qPCR. The values are the mean ± SEM (n = 3) from a representative experiment. *, P < 0.05; **, P < 0.01; ***, P < 0.001 compared to the level of each gene at PG stage. PG, primary growth (Stage I); PV, pre-vitellogenic (Stage II); EV, early-vitellogenic (early Stage III); MV, mid-vitellogenic (mid-Stage III); FG, full-grown stage (late Stage III); OV, ovulatory stage (Stage V).


kitlga
Relative mRNA Level
(kitlga/ef1a, % 5:00)
1000
800
600
400
200
0
***
**
5:00
6:00
7:00
8:00


kitlgb
Relative mRNA Level
(kitlgb/ef1a, % 5:00)
1500
1200
900
600
300
0
***
5:00
6:00
7:00
8:00


kita
Relative mRNA Level
(kita/ef1a, % 5:00)
1000
800
600
400
200
0
***
5:00
6:00
7:00
8:00


kitb
Relative mRNA Level
(kita/ef1a, % 5:00)
2000
1500
1000
500
0
***
5:00
6:00
7:00
8:00
Immature
(FG stage)
Mature
(GVBD)
Mature
(Ovulated)

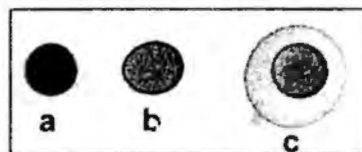
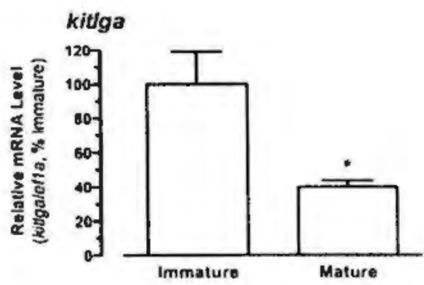
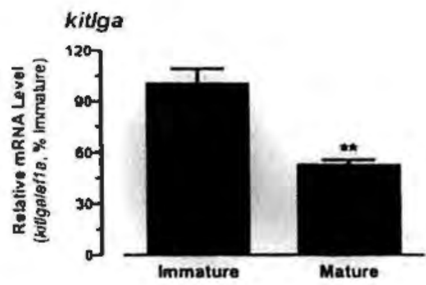
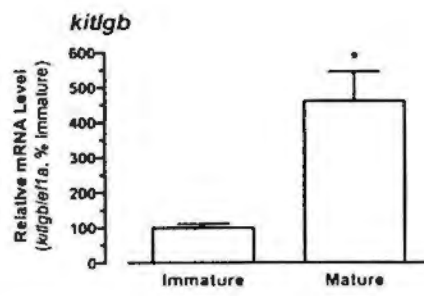
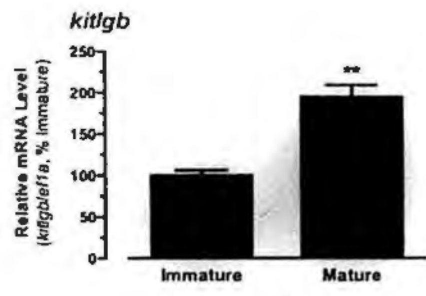

a
b
c

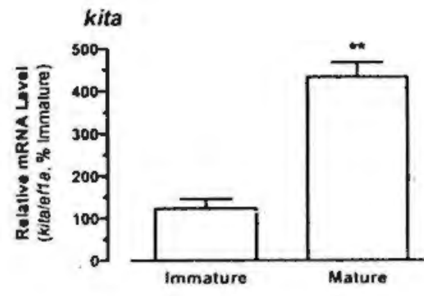
Fig. 2-8 Periovulatory expression profiles of the Kit system in vivo. Follicles were collected at different time points (5:00, 6:00, 7:00 and 8:00). Follicles sampled at 5:00 and 6:00 were immature FG follicles (Stage III, ***a*** at the bottom) while those sampled at 7:00 and 8:00 were GVBD follicles (***b*** at the bottom) and ovulated eggs (*c* at the bottom), respectively. The expression levels at different time points were determined by real-time qPCR and the values presented are the mean ± SEM (n = 3) from a representative experiment. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. 5:00.

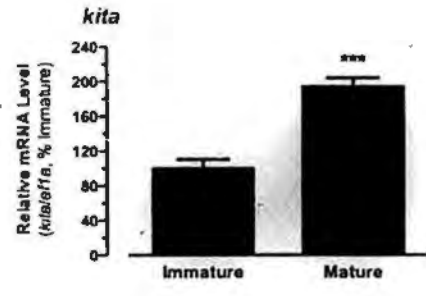

kitlga
Relative mRNA Level
(kitlga/ef1a, % Immature)
120
100
80
60
40
20
0
*
Immature
Mature

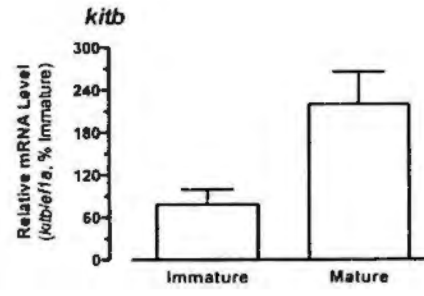

kitlga
Relative mRNA Level
(kitlga/ef1a, % Immature)
120
90
60
30
0
**
Immature
Mature


kitlgb
Relative mRNA Level
(kitlgb/ef1a, % Immature)
600
500
400
300
200
100
0
*
Immature
Mature


kitlgb
Relative mRNA Level
(kitlgb/ef1a, % Immature)
250
200
150
100
50
0
**
Immature
Mature


kita
Relative mRNA Level
(kita/ef1a, % Immature)
500
400
300
200
100
0
**
Immature
Mature


kita
Relative mRNA Level
(kita/ef1a, % Immature)
240
200
160
120
80
40
0
***
Immature
Mature


kitb
Relative mRNA Level
(kitb/ef1a, % Immature)
300
240
180
120
60
0
Immature
Mature

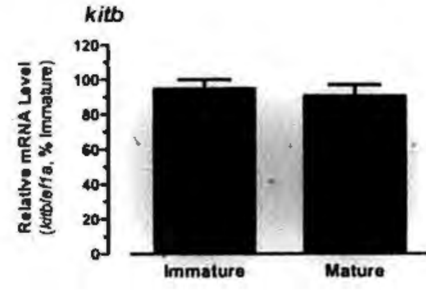

kitb
Relative mRNA Level
(kitb/ef1a, % Immature)
120
100
80
60
40
20
0
Immature
Mature

Fig. 2-9 (Left) Comparison of Kit system expression between immature and mature follicles in vivo. Immature FG follicles and mature GVBD follicles were isolated at 7:00 am for RNA extraction and qPCR analysis. (Right) Comparison of Kit system expression between immature and mature follicles in vitro. FG follicles were incubated for 6 hr in vitro followed by separation of the immature and mature follicles for qPCR analysis. The values are the mean ± SEM ($n \geq 3$) from a representative experiment. *, $P < 0.05$; **, $P < 0.01$ vs. immature follicles.

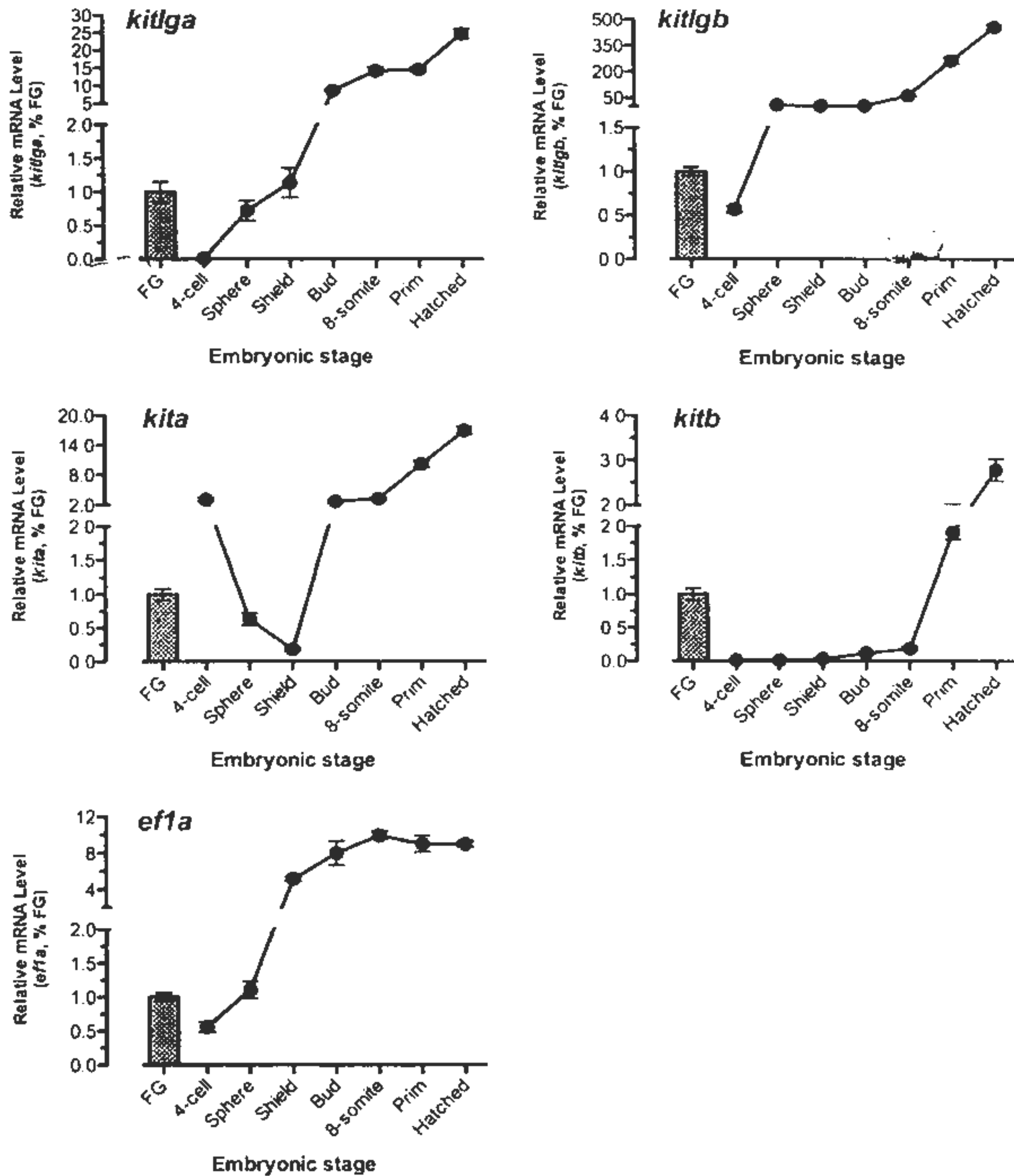


Fig. 2-10 Developmental profiles of Kit system transcripts during embryogenesis. Embryos were collected at different time points after fertilization for real-time RT-PCR analysis and levels of gene in full-grown follicles were used as a control. The values are the mean ± SEM (n = 3–4) from a representative experiment. Different letters indicate statistical significance ($P < 0.05$).

Chapter 3

Intrafollicular Distribution and Receptor Specificity of Zebrafish Kit System: Evidence for the Existence of a Kit/Kit ligand-mediated Bidirectional Communication System in the Follicle

3.1 Introduction

Kit ligand, also referred to as stem cell factor (SCF), mast cell growth factor (MGF) and steel factor (SF), is the product of the *Steel* (*Sl*) locus in mice (4-6). The *White Spotting* (*W*) locus encodes its receptor, Kit, which belongs to type III receptor tyrosine kinase family (3, 5, 6, 15, 19, 20). In mouse ovary, Kit ligand is derived from granulosa cells while Kit is restricted in oocytes and theca cells (78, 83), suggesting a paracrine signaling from the granulosa cells to the theca cells and oocytes through Kit ligand.

It has been documented that Kit ligand is involved in the growth and survival of oocytes (48, 50, 51, 53) and maintaining the arrest of oocyte meiosis (58, 59) by direct activation of Kit on the oocytes. These effects account for the support of follicle cells to the development of oocytes, which has been accepted as an example of granulosa cell-to-oocyte communication for a long time. On the other hand, lines of evidence have demonstrated that Kit ligand is also mitogenic for granulosa cells despite the lack of its receptors in these cells (53, 113). Interestingly, this effect depends on the existence of oocytes. Given the localization of Kit in oocytes instead of granulosa cells, it has been postulated that an oocyte-derived factor may mediate the mitogenic effect of Kit ligand on granulosa cells (114).

Additionally, Kit ligand is also involved in the interaction between granulosa cells and theca cells due to the existence of Kit in the latter (35). Kit ligand has been

shown to promote the formation of theca layer by influencing the recruitment of theca cells (55, 57). In addtion, Kit ligand also promotes the proliferation of theca cells (55, 56) and plays a role in the production of androgen from theca cells. Together with the Kit ligand-upregulated expression of aromotase in granulosa cells, the increased androgen production obviously contributes to the output of estrogen such as estradiol (52, 53, 56, 115).

As demonstrated in Chapter 2, different from the situation in mammals, the Kit system in zebrafish includes two forms of ligands (*kitlga* and *kitlgb*) and receptors (*kita* and *kitb*) due to the specific genome duplication in fish evolution. We have analyzed their expression during folliculogenesis, which implied an important role of the Kit system, particularly in the late stages. However, it is still unknown how the two forms of ligands and receptors interact in the follicle. To address this question, we first studied the distribution of Kit system in the follicle in the present study. This was followed by analyzing receptor specificity for both ligands and receptors using recombinant zebrafish Kitlga and Kitlgb produced by the Chinese hamster ovary (CHO) cells and receptors Kita and Kitb expressed in the COS cells. Our results provided strong evidence for a Kit-mediated bidirectional communication system in the zebrafish ovarian follicle, which could be part of the complex interplay between the oocyte and the follicle cells in the development of follicles.

3.2 Materials and Methods

3.2.1 Animals and chemicals

Zebrafish (*Danio rerio*) were obtained from a local tropical fish market and maintained in flow-through aquaria at 28 ± 1°C on a photoperiod of 14L:10D, with lights on at 8:00. The fish was fed twice a day with the commercial tropical fish feed Otohime S1 (Marubeni Nisshin Feed Co., Tokyo, Japan) and once with frozen artemia.

All experiments performed were licensed by the Government of the Hong Kong Special Administrative Region and endorsed by the Animal Experimentation Ethics Committee of The Chinese University of Hong Kong. Unless otherwise indicated, all common chemicals used were purchased from Sigma (St. Louis, MO), USB Corporation (Cleveland, OH), GE Healthcare (Waukesha, WI), or Merck (Whitehouse Station, NJ); enzymes from Promega (Madison, WI); and culture medium from Gibco Invitrogen (Carlsbad, CA). Mouse KITL (mKITL) was purchased from Invitrogen (Carlsbad, CA). mKITL was first dissolved in water and then diluted to the desired concentrations with the medium before use. Antibodies for phospho-KIT (Cat. sc-18076), HRP-linked anti-goat IgG (Cat. sc-2056) and HRP-linked anti-rabbit IgG (Cat. sc-2374) were from Santa Cruz (Santa Cruz, CA) and those for β-actin (Cat. 4967L), p44/42 MAP Kinase (9102L) and phospho-p44/42 MAP Kinase (Cat. 9101L) were from Cell Signalling Technology (Danvers, MA).

3.2.2 Construction of expression constructs

Gene-specific primers (Table 3-1) flanking the open reading frame (ORF) of five genes (mouse *Kit*, zebrafish *kitlga*, *kitlgb*, *kita* and *kitb*) were used to amplify the ORF of each gene from the ovary. Restriction sites for *Hind* III and *Xho* I (for mouse *Kit*, zebrafish *kitlga*, *kitlgb* and *kita*) or *Not* I and *Xho* I (for zebrafish *kitb*) were respectively added to the 5′-end of the sense and antisense primers for subsequent cloning. The Kozak sequence (5′-GCCGCCACC-3′) was included in all sense primers before the start codon ATG to enhance translation efficiency (116). PCR was performed for 36 cycles in a volume of 30 μl containing 10 μl template (RT reaction mix diluted at 1:15), 1× PCR buffer, 0.2 mM each dNTP, 2.5 mM $MgCl_2$, 0.2 mM each primer, and 1.5 U *Pfu* polymerase with the profile of 30 sec at 94°C, 30 sec at 60°C, and 4 min at 72°C. The PCR products were double digested with *Hind* III or

Not I and *Xho* I and cloned into pCMV-Script vector (Stratagene, CA) for mouse *Kit*, zebrafish *kita* and *kitb* or pcDNA5/FRT vector (Invitrogen) for zebrafish *kitlga* and *kitlgb* at *Hind* III or *Not* I and *Xho* I sites downstream of the CMV promoter to generate five constructs: pCMV/mKIT, pCMV/zfKita, pCMV/zfKitb, pcDNA5/FRT/zfKitlga and pcDNA5/FRT/zfKitlgb. All the expression constructs were sequenced to confirm sequence fidelity. The sequencing reaction was performed with the BigDye Terminator Cycle Sequencing Kit v3.1 and analyzed on the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

To generate the receptor chimeras containing mouse KIT intracellular domain (mKITID) and zebrafish Kita or Kitb extracellular domains (zfKitaED or zfKitbED), the above mouse *Kit* antisense primer and the sense primer corresponding to mKITID domain with an extra sequence complementary to 3′-end of zfKitaED or zfKitbED were used in PCR to produce mKITID fragment. Similarly, the antisense primers corresponding to zfKitaED or zfKitbED domains with an extra sequence complementary to 5′-end of mKITID and the above zebrafish *kita* or *kitb* sense primers were used to produce zfKitaED or zfKitbED fragments. The mKITID fragment was then mixed with zfKitaED or zfKitbED fragments and used as templates in PCR without primers to produce zfKitaED-mKITID or zfKitbED-mKITID. The chimeric products were then amplified with corresponding 5′or 3′-end primers. After double digestion with *Hind* III and *Xho* I, the PCR products were cloned into pCMV-Script vector to generate pCMV/zfKitaED-mKITID and pCMV/zfKitbED-mKITID (Fig. 3-7).

3.2.3 Cell culture and transfection of COS-1 and Flp-In CHO cells

The primary follicle cell culture of zebrafish ovary was performed according to our previous report (101). Briefly, the ovaries from about 20 female zebrafish were

isolated and dispersed in a 100-mm petri dish containing 60% Leibovitz L-15 medium (Invitrogen). The full-grown follicles were removed by sieving, followed by washing with medium M199 (Invitrogen) for about five times. Afterwards, the follicles were cultured in M199 supplemented with 10% fetal calf serum (Hyclone, Logan, UT, USA) at 28°C in 5% CO_2 for 6 days for the proliferation of follicle cells. Then, the follicle cells were harvested by trypsinization and plated in 24-well plates at the density of about 2.5×10^5 cells/well for 24 h. The cells were then starved with M199 without serum for 24 h before treatment.

The COS-1 cells were cultured in DMEM (Dulbecco Modified Eagle Medium) medium containing antibiotics (streptomycin, 100 μg/ml; penicillin, 100 U/ml) and 10% FBS at 37°C with 5% CO_2. The expression plasmid pCMV/mKIT, pCMV/zfKita, pCMV/zfKitb, pCMV/zfKitaED-mKITID and pCMV/zfKitbED-mKITID were transfected into the COS-1 cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The expression vector pCMV-Script was used as the control. Twelve hours after transfection, the cells were subcultured into 24-well plates at the density of 10^5 cells/well. The medium was changed to serum-free medium after 24-h incubation, and the incubation continued for 24 h before drug treatment.

Flp-In CHO cells (Invitrogen) were cultured in Ham F-12 medium containing antibiotics (streptomycin, 100 μg/ml; penicillin, 100 U/ml) and 10% FBS at 37°C with 5% CO_2. The cells were subcultured in 10-cm culture dishes (Falcon, Franklin Lakes, NJ) and allowed to grow to 25% confluence before transfection. The expression constructs pcDNA5/FRT/zfKitlga and pcDNA5/FRT/zfKitlgb (0.5 μg) were cotransfected into the CHO cells separately using Lipofectamine 2000 (Invitrogen) together with 4.5 μg of plasmid pOG44 (Invitrogen) that encodes a recombinase to facilitate homologous recombination of the expression plasmids at the specific FRT site.

3.2.4 Production of recombinant zfKitlga and zfKitlgb

The CHO cells stably transfected with expression constructs, pcDNA-Kitlga or pcDNA-Kitlgb, were screened by hygromycin B (Invitrogen) at 500 μg/ml for one month and then diluted serially to 96-well plates at a density of 2-3 cells/well. After about one week, the wells containing single colonies were labeled and expanded until the cells grew to half confluence. Each clone was analyzed by RT-PCR for plasmid integration. The positive clones were then used to produce recombinant proteins according to the protocol described in our previous reports (117). Briefly, the cells were subcultured into 750 ml flasks (Falcon) in 50 ml Ham F12 medium supplemented with 10% FBS and allowed to grow to about 90% confluence at 37°C. The FBS-containing medium was then replaced with 50 ml FBS-free medium and the culture temperature was reduced to 28°C. After a further 5-day incubation, the medium was harvested and concentrated by 200 folds with the Labscale TFF System with Pellicon XL Biomax 8 filter (Millipore, Bedford, MA).

3.2.5 Total RNA isolation and RT

Total RNA was extracted from cultured follicle cells with Tri-Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacture's protocol and our previous study (101). The RT was then performed at 37°C for 2 h in a volume of 10 μl containing 0.5 μg of oligo(dT), 1× M-MLV RT buffer, 0.5 mM each deoxyribonucleotide triphosphate (dNTP), 0.1 mM dithiothreitol, and 100 U of M-MLV RT (Invitrogen, Carlsbad, CA).

3.2.6 Separation of oocytes and follicle layers

The full-grown follicles were pretreated in Cortland's medium without calcium

and magnesium ions but with Na_2EDTA (1 mM) for about one hour to reduce the cohesion between the oocyte and the follicle layer. The follicle layer was then carefully peeled off with fine forceps without damaging the oocyte. The isolated follicle layers and the denuded oocytes from five follicles were pooled and subject to RT-PCR analysis.

3.2.7 Northern blot analysis

Northern blot hybridization was performed according to our previous reports (118). In brief, total RNA (20 μg) from the cells was separated in a 1% denaturing agarose gel containing 2.2 M formaldehyde, transferred to a positively charged nylon membrane (Roche, Mannheim, Germany), and UV cross-linked with the GS Gene Linker (Bio-Rad, Hercules, CA). The membrane was then hybridized with DIG-labeled cRNA probes in vitro transcribed from the cloned zebrafish *kitlga* or *kitlgb* cDNA, detected with the Chemiluminescent Detection Kit according to the manufacturer's protocol (Roche), and analyzed on the Lumi-Imager F1 workstation (Roche).

3.2.8 Quantification of gene expression by real-time qPCR

Real-time quantitative PCR was performed to quantify the expression of *kitlga* and *kitlgb* in transfected CHO cells. The template for the standard curve was prepared by PCR amplification of cDNA fragment with specific primers. After purification with a PCR Purification Kit (Qiagen, Valencia, CA), the amplified DNA amplicons were quantified with the software Quantity One (Bio-Rad, Hercules, CA) using the Mass Ruler DNA marker (MBI Fermentas, Hanover, MD) as the standard, and the copy numbers of the DNA molecules were calculated before use as templates to construct standard curves in real-time quantitative PCR. All PCR reactions were

performed in a total volume of 30 µl containing 10 µl template (RT reaction mix diluted at 1:15), 1× PCR buffer, 0.2 mM each dNTP, 2.5 mM $MgCl_2$, 0.75 U of Taq polymerase, 0.5× EvaGreen (Biotium, Hayward, CA), and 20 nM fluorescein (Bio-Rad) on the iCycler iQ Real-time PCR Detection System (Bio-Rad). The amplification protocol was 30 sec at 94°C, 30 sec at 60°C, and 30 sec at 72°C, with a signal detection period of 7 sec at 80°C. A melt curve analysis was performed at the end of the reaction to check the reaction specificity.

3.2.9 Western blotting

The cells were lysed by adding 1× SDS sample buffer (62.5 mM Tris-HCl, pH 6.8 at 25°C, 1% w/v SDS, 10% glycerol, 5% 2-mercaptoethanol, 100 µl per well of 24-well plate). Then the plate was shaken immediately for a few times and the extract from each well transfered to a microcentrifuge tube. All samples were heated to 95–100°C for 5 minutes, cooled on ice and microcentrifuged for 5 minutes. Western blotting was performed according to the manufacture's protocol (Cell Signalling Technology, Danvers, MA). Briefly, samples (about a half for 24-well plate) were loaded and separated in the 12.5% SDS–PAGE gel in 1× running buffer (25 mM Tris base, 0.2 M glycine, 0.1% w/v SDS), followed by blotting to the nitrocellulose membrane (Bio-Rad) using blotting buffer (25 mM Tris base, 0.2 M glycine, 20% methanol). The membrane was incubated in 25 ml blocking buffer (1× TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk) for 1 h at room temperature and then incubated in 5 ml of diluted primary antibody (1:1000 in blocking buffer) at 4°C overnight. The membrane was washed three times for 5 min each with wash buffer (1× TBS, 0.1% Tween-20) and then incubated with HRP-conjugated secondary antibody (1:2000 in wash buffer) for 1 h at room temperature. The membrane was then washed again and equilibrated with the developing solution (Western Blotting

Luminol Reagent; Santa Cruz Biotechnology, Santa Cruz, CA). The signals were detected on the Lumi-Imager F1 Workstation (Roche, Mannheim, Germany).

3.2.10 Data analysis

The ratio of phosphorylation levels to that of the internal control β-actin was calculated and then expressed as the fold change compared to the control. All values were expressed as the mean ± SEM and the data were analyzed by one-way ANOVA followed by Dunnett test using Prism 5 on Macintosh OS X (GraphPad Software, San Diego, CA).

3.3 Results

3.3.1 Spatial expression of Kit system within the follicle

Zebrafish ovarian follicle consists of two compartments: a developing oocyte in the center and a thin follicle layer of surrounding somatic cells containing the granulosa and theca cells. To understand the action modes of Kitlga and Kitlgb within the follicle, it is essential to find out the relative distribution of the two ligands and their potential receptors Kita and Kitb in the two compartments. We therefore performed the experiment by mechanically separating the follicle layer from the oocyte followed by examining the expression of Kit ligands and receptors in the two compartments. To ensure a clean separation of the two compartments, we used a gonadotropin receptor, *lhcgr*, and *gdf9* as the molecular markers for the follicle layer and oocyte, respectively. Consistent with our previous reports (100), *lhcgr* was exclusively expressed in the somatic follicle cells whereas the expression of *gdf9* was restricted in the oocyte (Fig. 3-1). Interestingly, *kitlga* was only expressed in the follicle cells and *kita* only in the oocyte, which is consistent with the situation in mammals. However, *kitb* was exclusively expressed in the follicle layer and *kitlgb*

only in the oocyte, which obviously differs from that in mammals.

3.3.2 Mouse KITL activates mouse Kit but not zebrafish Kita and Kitb

Considering that recombinant zebrafish Kit ligands were not available commercially, we first tested whether mouse KITL can be used to replace zebrafish Kit ligands for further functional study. As a positive control, the COS-1 cells transfected with the plasmid expressing mouse Kit protein were treated with mouse KITL. A strong KIT phosphorylation signal was observed by Western blot analysis, which demonstrated the feasibility of our experimental system. Zebrafish Kita and Kitb, however, had no significant response to mouse KITL (Fig. 3-2), suggesting that the mammalian molecule is not functional in the zebrafish system.

3.3.3 Production of recombinant zfKitlga and zfKitlgb

As shown above, Kit ligand shows strong species specificity in receptor recognition and activation as mouse KITL strongly activated its cognate receptor (mouse KIT) expressed in the COS-1 cells but failed to activate zebrafish Kita and Kitb. To study the biological functions of the Kit system in the zebrafish ovary and discriminate receptor specificity of Kitlga and Kitlgb and their differential roles in the follicle, the availability of zebrafish Kitlga and Kitlgb is indispensable. Given our previous work in producing recombinant zebrafish gonadotropins (117) and goldfish follistatin (119), we chose the Flp-In CHO cell system (Invitrogen) to produce recombinant zebrafish Kitlga and Kitlgb. After transfection, hygromycin B selection, and cloning by serial dilution, we obtained a number of stably-transfected clones. Some of these clones (C1-C9) were subject to further characterization by RT-PCR on the total RNA isolated from each clone (Fig. 3-3A and B) and Northern blot analysis for gene expression (Fig. 3-3C and D). As demonstrated in Fig. 3-3, all selected

clones for *kitlga* exhibited high levels of expression although the relative abundance of mRNA varied among these clones. On the other hand, there was only one clone which was positive for *kitlgb* expression both in PCR and in Northern analysis. The clone with the maximal expression level of *kitlga* and the only *kitlgb*-positive clone were chosen for large-scale production of recombinant Kitlga and Kitlgb according to our previous reports (117).

3.3.4 Receptor specificity of zebrafish Kit ligands and receptors

Recombinant zebrafish Kitlga and Kitlgb were then used to treat COS-1 cells transfected with the plasmids expressing receptor protein Kita or Kitb. The results showed that Kitlga preferentially activated Kita although it also activated Kitb weakly. Treatment with zfKitlga produced no signals in the cells transfected with control plasmids. Also, the control medium had no effect on the activation of either Kita or Kitb (Fig. 3-4). On the other hand, recombinant zebrafish Kitlgb specifically activated Kitb without any significant effects on Kita (Fig. 3-5). Dose response experiment showed that both zfKitlga and zfKitlgb activated their preferred receptors zfKita and zfKitb in a clear dose-dependent manner. The preparations of zfKitlga and zfKitlgb tested in the present study appeared to have different potencies with the EC50s of zfKitlga and zfKitlgb being 16.15 μl/ml and 238.3 μl/ml, respectively (Fig. 3-6). For future functional studies, we will define these doses as one unit (U).

To exclude the possibility that the ligand-receptor specificity demonstrated above resulted from differential recognition of the phosphorylated receptors by the antibody, we further examined the receptor specificity by using two receptor chimeras that contain the intracellular domain of mouse KIT and extracellular domains of zebrafish Kita or Kitb (zfKitaED-mKITID and zfKitbED-mKITID), respectively (Fig. 3-7). As demonstrated in Fig. 3-8, zfKitaED-mKITID responded specifically to zfKitlga but

not zfKitlgb. In contrast, zfKitlgb specifically activated zfKitbED-mKITID but not zfKitaED-mKITID (Fig. 3-9).

3.3.5 *Effects of zfKitlga and zfKitlgb on MAPK phosphorylation in cultured follicle cells*

The binding of KitL to Kit will lead to the activation of many intracellular signaling pathways (120). In addition to the above receptor specificity analysis in mammalian cell line, we performed further experiment to confirm the biological activity of recombinant Kitlga and Kitlgb and identify the activated signaling pathways in zebrafish follicle cells. As shown in Fig. 3-10, both zfKitlga and zfKitlgb significantly activated MAPK in a time-dependent manner in comparison with the stable phosphorylation level of MAPK in control cells. Different from phosphorylation level of MAPK in zfKitlga-treated cells, however, zfKitlgb showed stronger and more persistent effect on MAPK phosphorylation.

3.4 Discussion

In the past decade, accumulating evidence demonstrates that, instead of being a passive recipient, the oocyte plays an essential role in directing the development of ovarian follicles throughout folliculogenesis from the initial assembly of the primordial follicles to ovulation (121). It is now generally accepted that there exist extensive bidirectional communications between the oocytes and follicle cells, particularly the granulosa cells (121). A complex interplay of regulatory factors from both the follicle cells and oocytes governs the process of folliculogenesis.

Our previous work has demonstrated the existence of an intimate bidirectional communication between oocytes and peripheral follicle layer in the zebrafish ovary (95). Epidermal growth factor (EGF) and activin families represent two major groups

of ovarian factors involved in this process (122). The ligands of EGF family including EGF (*egf*), TGFα (*tgfa*), HB-EGF (*hbegf*) and betacellulin (*btc*) are exclusively or primarily expressed in the oocyte whereas their common receptor, EGFR (*egfr*), is exclusively expressed in the follicle layer, strongly suggesting an EGF ligand-mediated paracrine regulation of the somatic follicle cells by the oocyte. On the contrary, activin subunits, βA (*inhbaa*) and βB (*inhbb*), are exclusively expressed in the follicle layer while their receptors, *acvr1b*, *acvr2a* and *acvr2b*, are predominantly expressed in the oocyte, suggesting an activin-mediated follicle cell-to-oocyte signaling in the zebrafish follicle (122). The present study provided evidence for a potential Kit-mediated paracrine mechanism in the zebrafish follicle, which may mediate both follicle cell-to-oocyte and oocyte-to-follicle cell signaling.

Similar to the situation in mammalian models (78, 83), Kitlga was expressed only in the follicle layer and Kita only in the oocyte, suggesting a Kitlga/Kita-mediated paracrine signaling from the somatic follicle cells to the oocyte. This idea was further supported by the experiment on receptor specificity. Recombinant zfKitlga preferentially activated Kita but not Kitb. Different from the Kit system in mammals, there exist two forms of Kit ligands (Kitlga and Kitlgb) and receptor (Kita and Kitb) in teleosts (Chapter 2). In contrast to Kit ligand/Kit in mammals and Kitlga/Kita in the zebrafish, Kitlgb was found to exist in the oocyte and Kitb in the follicle layer, pointing to the possibility that there may be a unique Kitlgb/Kitb paracrine pathway in the zebrafish follicle that mediates the control of the follicle cells by the oocyte. Again, this idea was supported by the receptor specificity data that zfKitlgb specifically activated Kitb but not Kita. Different from Kita, however, Kitb still had weak response to zfKitlga, which has been demonstrated by both receptor assay in mammalian cell line and MAPK phosphorylation in zebrafish primary follicle cell culture. This weak interaction between zfKitlga and Kitb may be

analogous to the Kit-mediated granulosa-theca cell interaction in mammals (Chapter 2). In spite of this, the present study strongly supports the existence of a Kit-mediated bi-directional communication between the somatic follicle cells and the oocyte.

As demonstrated by synteny and phylogenetic analyses in Chapter 2, Kitlga/Kitlgb and Kita/Kitb were likely derived from the third round genome duplication specific to ray-finned fish (87, 88). Unlike many duplicated genes which possess redundant functions, both forms of Kit (Kita and Kitb) and Kit ligand (Kitlga and Kitlgb) in the zebrafish showed distinct expression patterns during folliculogenesis, particularly in periovulatory stages, implying functional differentiation of the Kit system in the ovary. This was further supported by the present study on the spatial distribution of the isoforms of Kit ligand and Kit in the follicle and receptor specificity. The oocyte is likely subject to the regulation by Kitlga derived from the follicle cells. It is therefore speculated that, in comparison with the expression of *kitlga* in full-grown follicles, the significant decline of its level in the mature follicles may be closely related to the oocyte maturation. On the other hand, the follicle cells may be subject to the regulation by Kitlgb from the oocyte as well as influence from Kitlga. In the light of the specificity of Kitlgb to Kitb and the high level of *kitb* expression after oocyte maturation and *kitlgb* before spawning (Chapter 2), we surmised that Kitlgb may be involved in triggering important events in follicle cells in this period.

It has been reported that the binding of Kit ligand to Kit can activate MAPK pathway in mammals (73, 123-126). So we monitored the change of MAPK phosphorylation levels in cultured follicle cells after the treatment with recombinant zebrafish Kit ligands, which can further confirm the biological activity of CHO cell-derived recombinant proteins in zebrafish. Both Erk1 and Erk2 demonstrated similar response. Both zfKitlga and zfKitlgb could significantly induce the

phosphorylation of MAPK, but zfKitlga was less potent, which is in agreement with the receptor binding analysis by zfKitlga and zfKitlgb. The activation of MAPK by zfKitlga and zfKitlgb in zebrafish primary follicle cells provides the evidence for their biological activity in the zebrafish ovary, making it possible to study the function of Kit system in zebrafish ovary by using these two recombinant proteins.

In summary, the present study demonstrated the distribution of all Kit system members in the zebrafish ovary. *kitlga* and *kitb* are exclusively expressed in the follicle layer, while *kitlgb* and *kita* only in the oocyte. Using CHO cell line as a bioreactor, we produced recombinant zebrafish Kitlga and Kitlgb. Analysis in mammalian COS-1 cells and zebrafish primary follicle cells confirmed their biological activity and binding specifity. Two opposite paracrine pathways of Kit system in the zebrafish ovary have been suggested. Kitlga from the follicle cells preferably activates Kita in the oocyte in spite of the weak response of Kitb to it. Kitlgb from the oocyte, however, exclusively activates Kitb in the follicle cells without any effects on Kita. This study also provided further evidence for the functional divergence of Kit system during folliculogenesis.

Table 3-1 Primers used in RT-PCR

Gene	Strand	Sequence	Accession no.
Kit	Sense	5'-GGCTGACTGTGCTGTGCTGATTG-3'	NM_021099
	Antisense	5'-CCCCTCGAGTCAGGCATCTTCGTGCACGA-3'	
	Sense	5'-CTCACCTACAAATATTTGCAGAA-3'	
kitlga	Sense	5'-AAGAAGCTTGCCGCCACCATGAATAATTCAAACATTTG-3'	AY929068
	Antisense	5'-CCCCTCGAGTTACACATCCATGATAATAT-3'	
kitlgb	Sense	5'-AATAAGCTTGCCGCCACCATGTTCCACATGAGGGAGGT-3'	AY929069
	Antisense	5'-CCTCTCGAGTTAGACCTCTGTGTCTGCAC-3'	
kita	Sense	5'-AATAAGCTTGCCGCCACCATGGAATATCACTGCGTTCT-3'	NM_131053
	Antisense	5'-CCCCTCGAGTCAGACTACAGGGTGACTTG-3'	
	Antisense	5'-CAAATATTTGTAGGTGAGCACAATCAGGATGAGAAC-3'	
kitb	Sense	5'-TATGCGGCCGCGCCGCCACCATGGGACACTCGTGGTTT-3'	GQ994993
	Antisense	5'-CCCCTCGAGTCAAGTAATCCTGCAAGATAC-3'	
	Antisense	5'-CAAATATTTGTAGGTGAGAACCACCAGAATGAAGCT-3'	

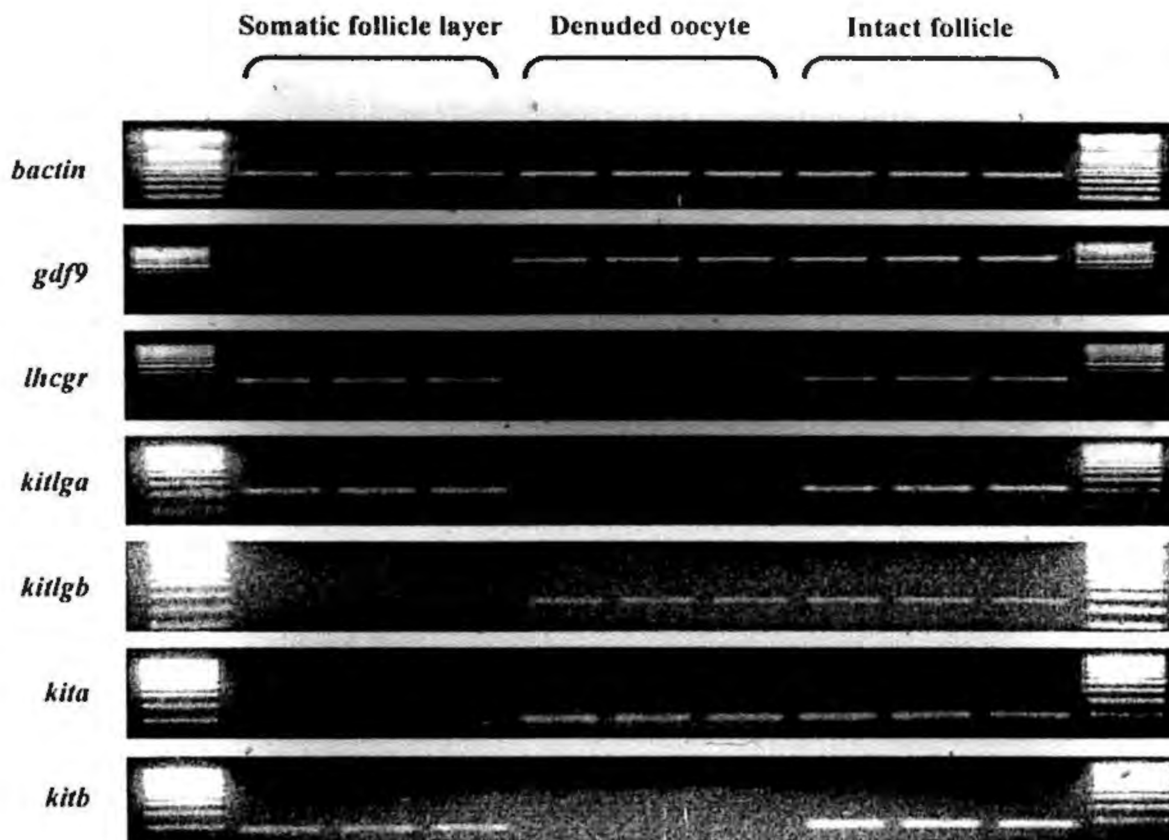
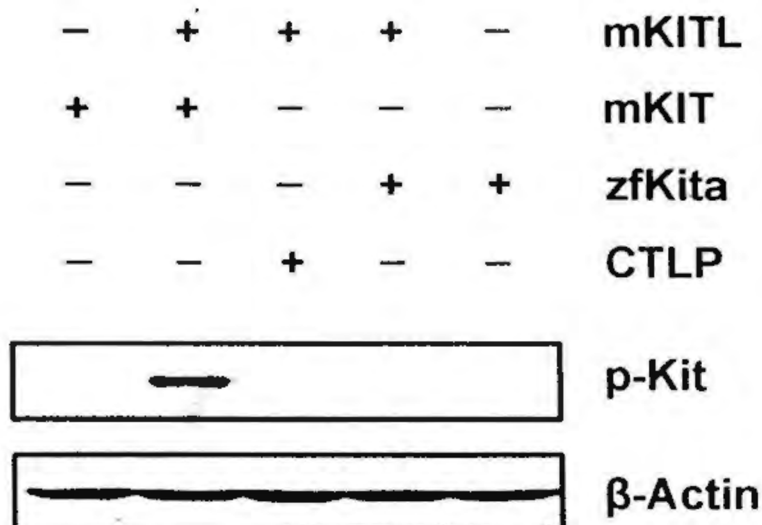


Fig. 3-1 Localization of Kit system within the FG follicle. The somatic follicle layer was separated from the oocyte followed by RNA extraction and semi-quantitative PCR detection in the two compartments (somatic follicle layer versus denuded oocyte). Each sample represents the total RNA pooled from 5 follicles. The housekeeping gene *bactin* was used as the internal control for all samples, whereas *gdf9* and *lhcgr* were used as the markers for denuded oocytes and somatic follicle layers respectively.

A



B

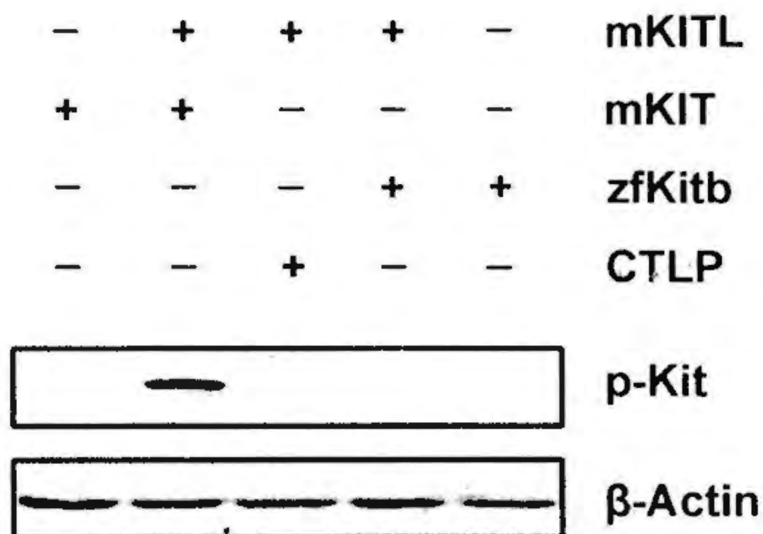
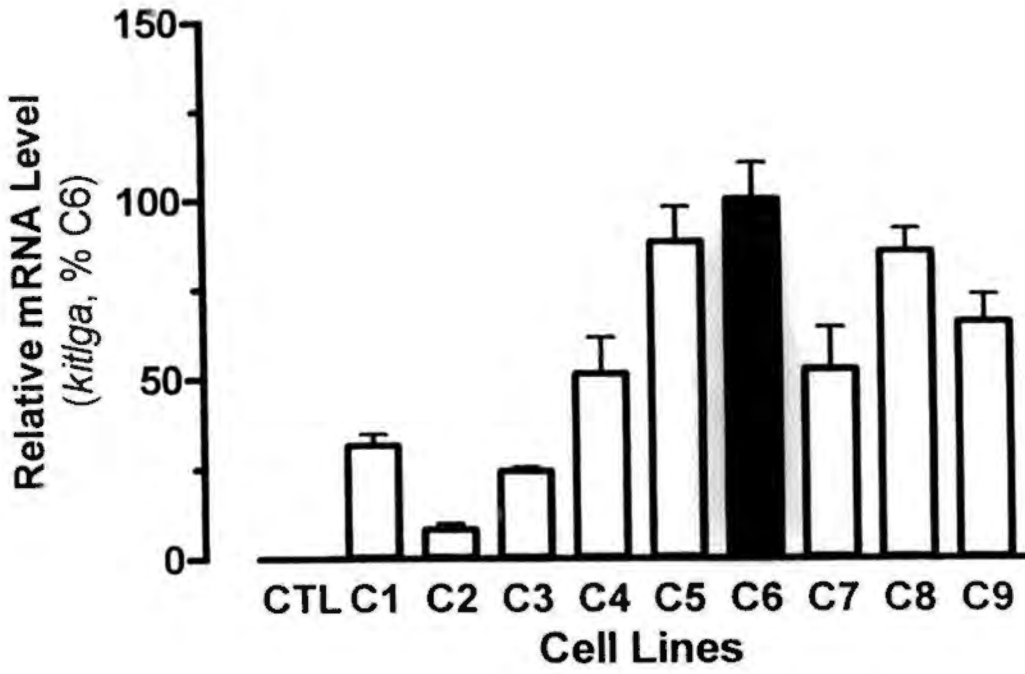
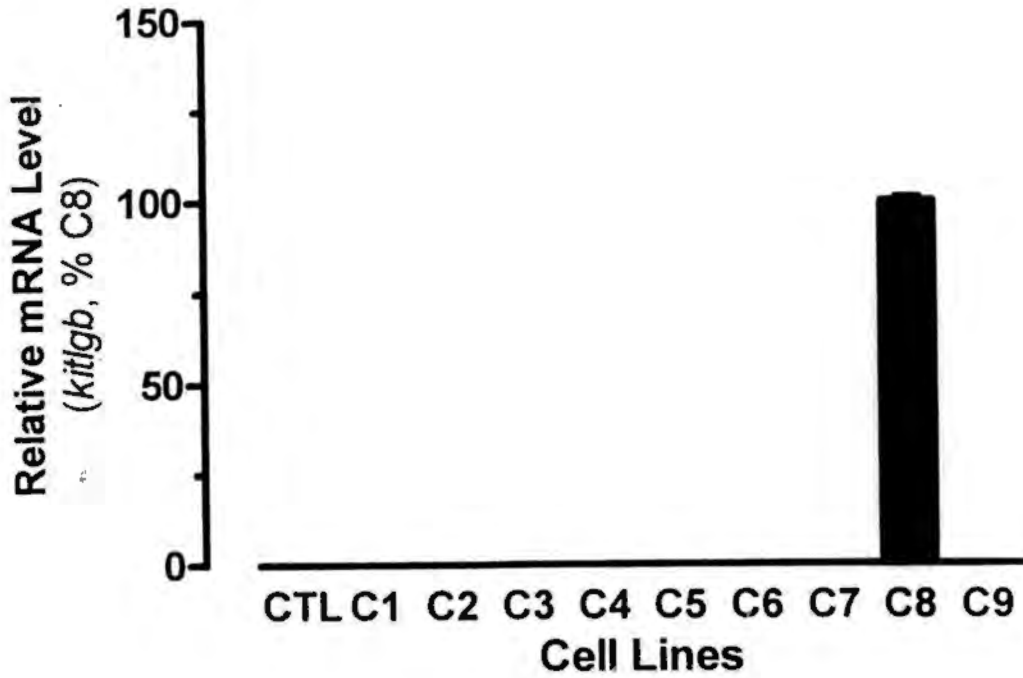


Fig. 3-2 Mouse KITL activated mouse Kit but not zebrafish Kita and Kitb. Plasmids expressing mouse KIT or zebrafish Kita (A) or Kitb (B) were transfected into COS-1 cells. After addition of recombinant mouse KITL for 10 min, the phosphorylation levels of Kit were detected by the antibody recognizing tyrosine phosphorylation sites in the intracellular domains. Plasmid pCMV-Script was used as a negative control. Actin was used as the internal control for all samples.

A



B



C

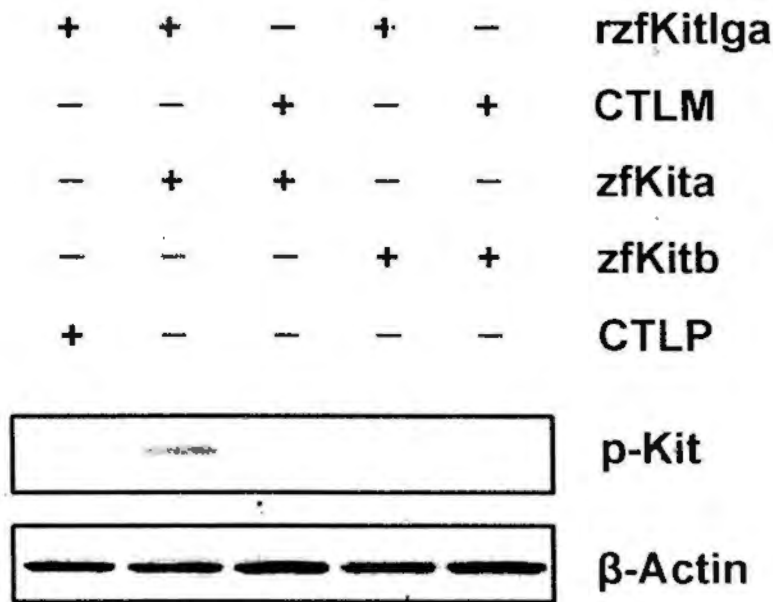
CTL C1 C2 C3 C4 C5 C6 C7 C8 C9

D

CTL C1 C2 C3 C4 C5 C6 C7 C8 C9

Fig. 3-3 Real-time qPCR (A and B) and Northern blot (C and D) analysis for the expression of *kitlga* and *kitlgb* in the established cell lines (C1 to C9). CTL, control CHO cells transfected with pcDNA5/FRT.

A



B

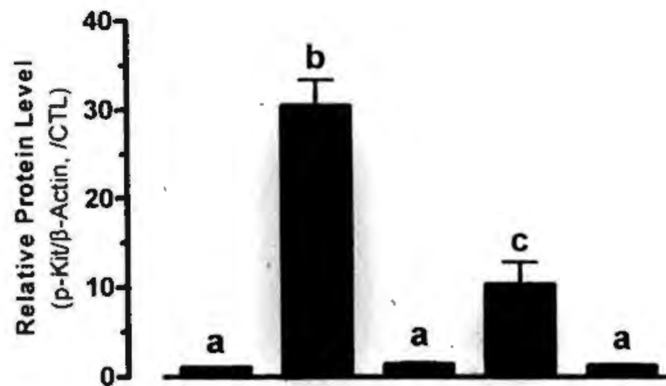
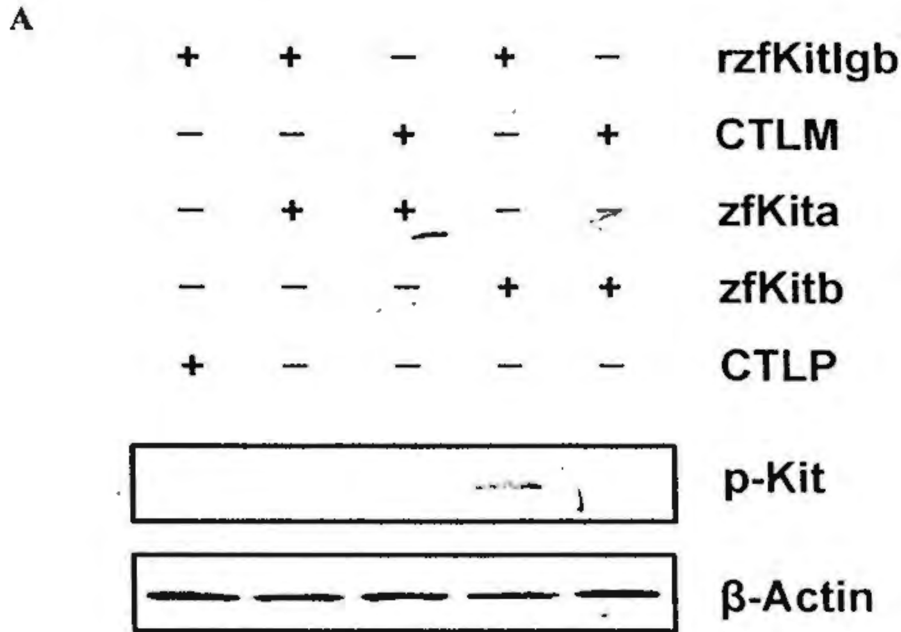


Fig. 3-4 Response of zebrafish Kita and Kitb to recombinant zebrafish Kitlga. (A) Western blot analysis (10-min treatment with recombinant proteins). (B) The densitometric analysis of the Western signals. CTLM, control medium from CHO cells transfected with the control plasmid pcDNA5/FRT. CTLP, control plasmid, pCMV-Script. The graph is the data normalized to the Actin and expressed as the fold change of the first group (mean ± SEM, n=3). Different letters indicate statistical significance ($P < 0.05$).

A



B

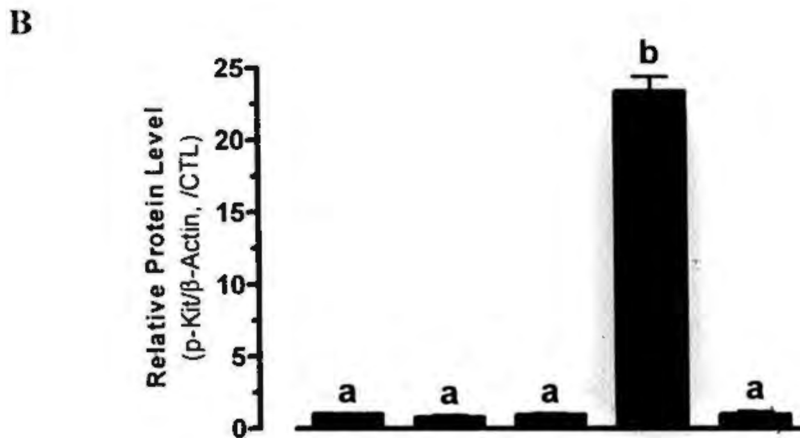


Fig. 3-5 Response of zebrafish Kita and Kitb to recombinant zebrafish Kitlgb. (A) Western blot analysis (10-min treatment with recombinant proteins). (B) The densitometric analysis of the Western signals. CTLM, control medium from CHO cells which was transfected with plasmid pcDNA5/FRT. CTLP, control plasmid, pCMV-Script. The graph is the data normalized to the Actin and expressed as the fold change of the first group (mean ± SEM, n=3). Different letters indicate statistical significance ($P < 0.05$). ** $P < 0.001$ vs. response of Kita to Kitlgb.

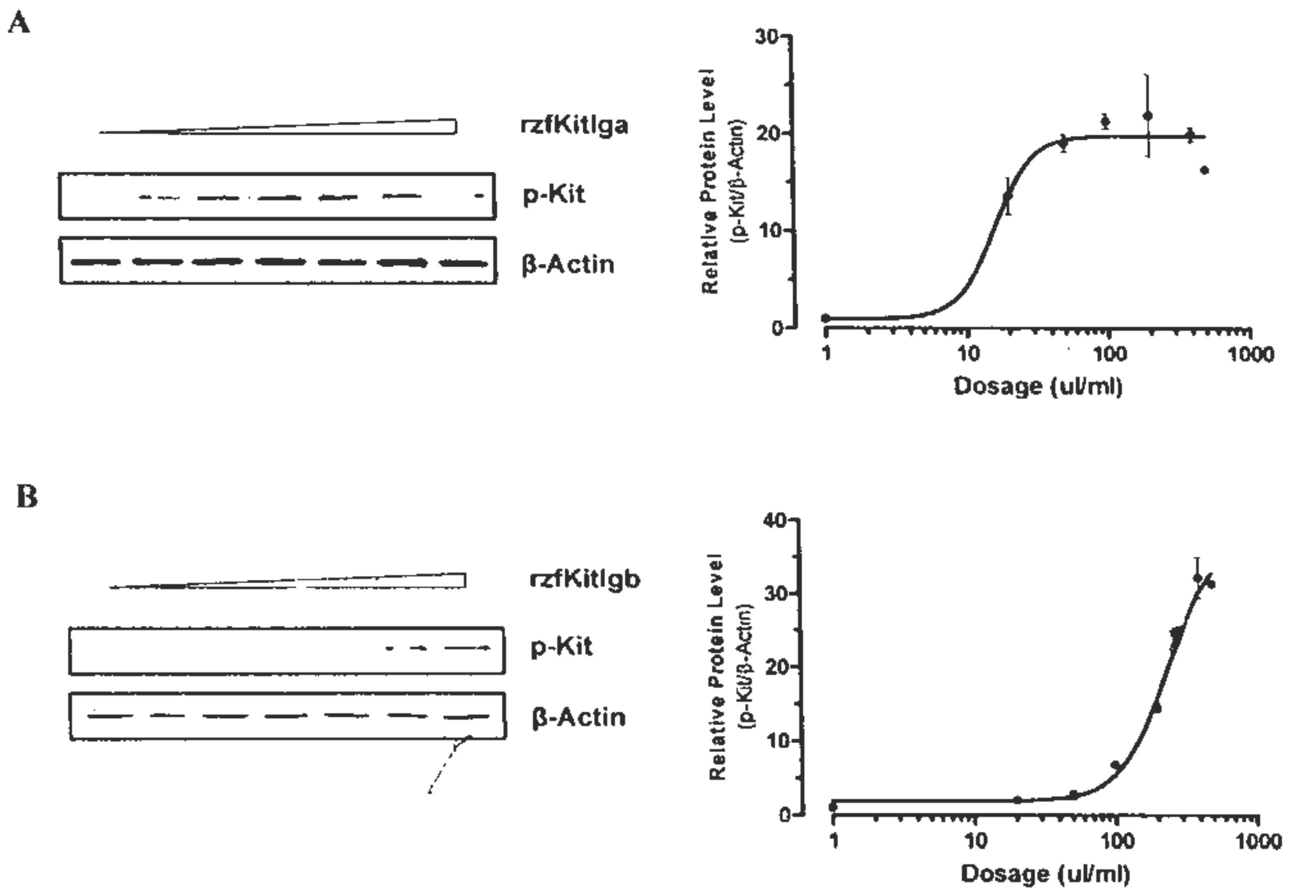


Fig. 3-6 Unit definition of recombinant zebrafish Kitlga (A) and Kitlgb (B). Western blot showed the dose response of Kita or Kitb to Kitlga or Kitlgb in transfected COS-1 cells. The unit was defined according to the value of the half maximal effective concentration (EC50, the concentration of a drug, antibody or toxicant which induces a response halfway between the baseline and maximum after some specified exposure time.).

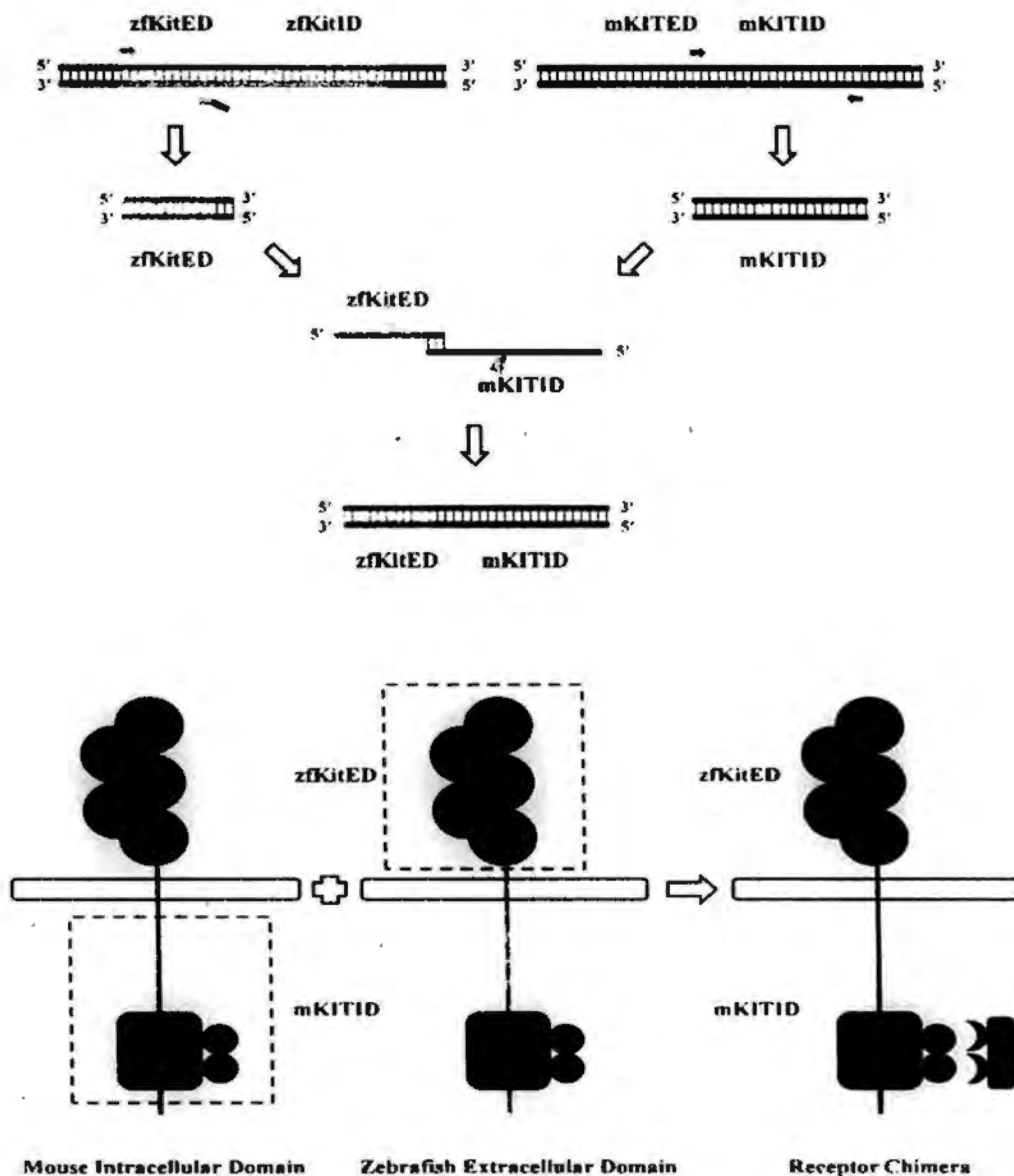
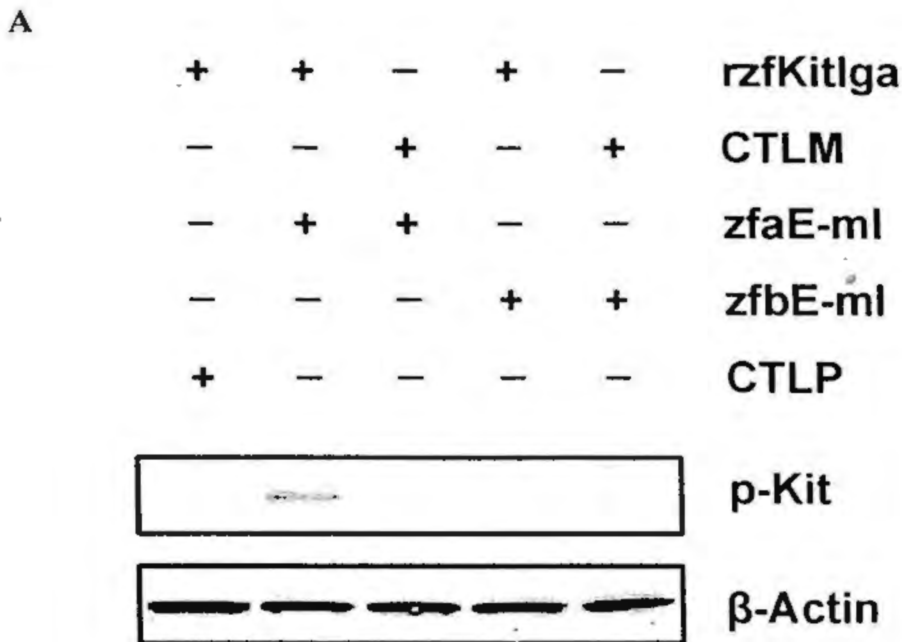


Fig. 3-7 The scheme of cloning process and receptor chimera constructs. The blue represents mouse Kit receptor and the green represents zebrafish Kita or Kitb. The yellow represents the cell membrane. The above of the membrane are extracellular domains of Kit receptor and below are intracellular domains. "P" represents the tyrosine phosphorylation site in the intracellular domains. The black represents vector sequences, the red represents the antibody recognizing the phosphorylated tyrosines and the arrows represent specific primers. mKITID, mouse KIT intracellular domain and zfKitED, zebrafish Kit extracellular domain.

A



B

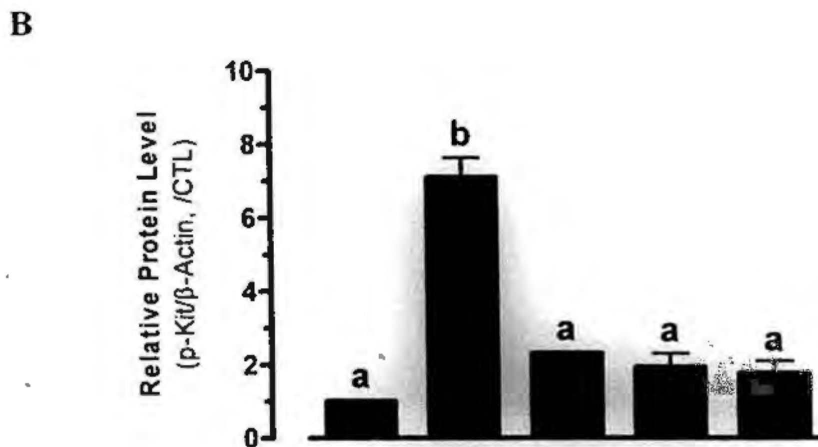
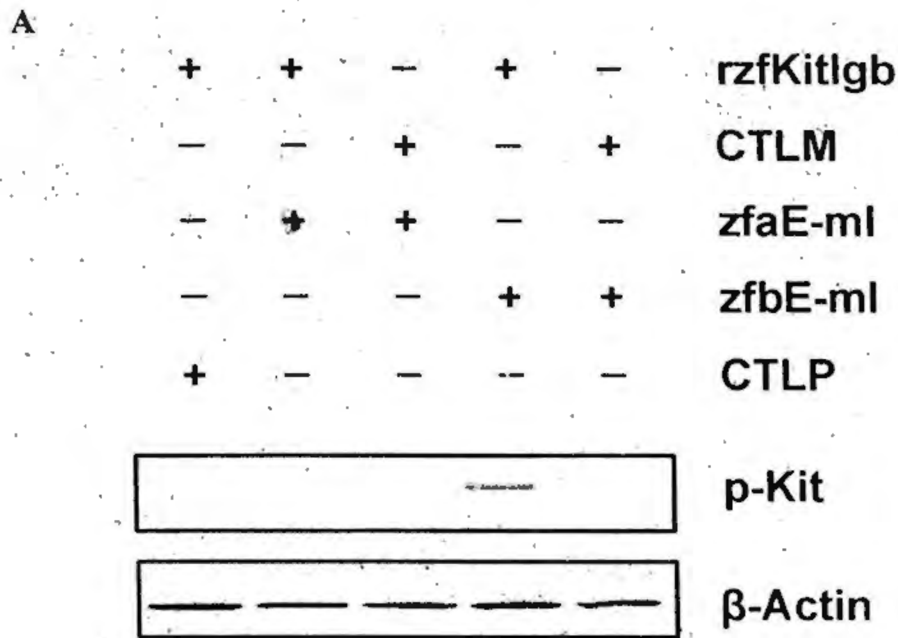


Fig. 3-8 Response of zebrafish chimeras Kita and Kitb to recombinant zebrafish Kitlga. (A) Western blot analysis (10-min treatment with recombinant proteins). (B) The densitometric analysis to signals in (A). CTLM, control medium from CHO cells which was transfected with plasmid pcDNA5/FRT. CTLP, control plasmid, pCMV-Script. zfaE-mI or zfbE-mI, receptor chimera containing extracellular domain of zebrafish Kita or Kitb and intracellular domain of mouse KIT. The graph is the data normalized to the Actin and expressed as the fold change of the first group (mean ± SEM, n=3). Different letters indicate statistical significance ($P < 0.05$).

A



B

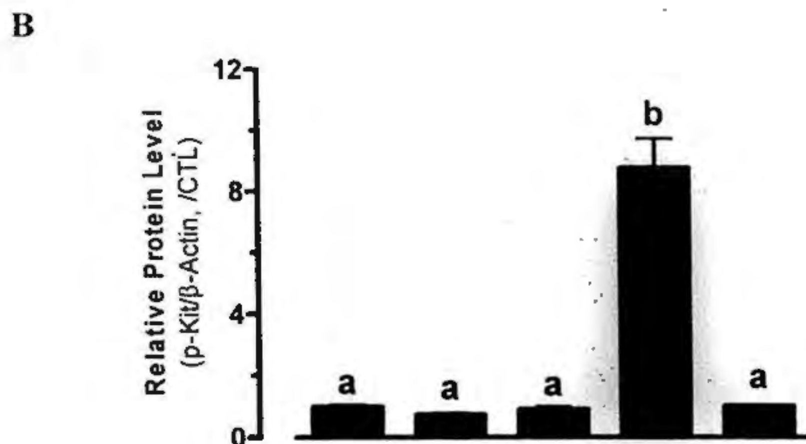
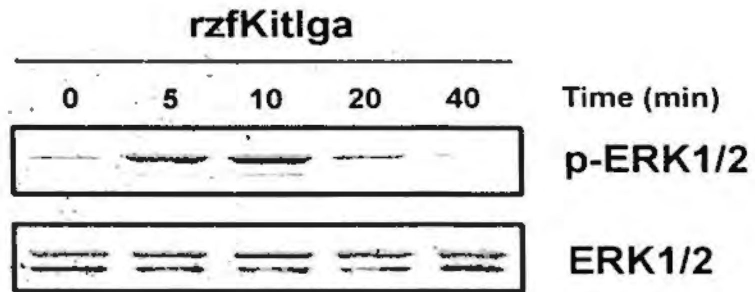
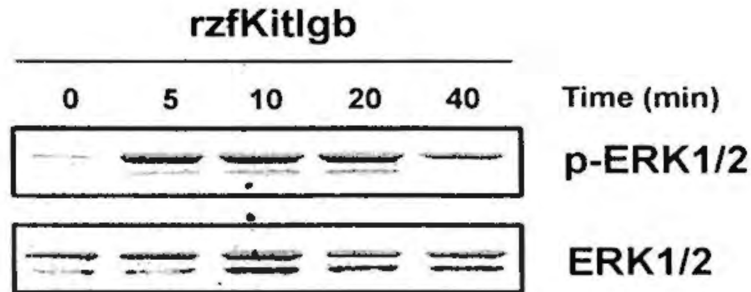
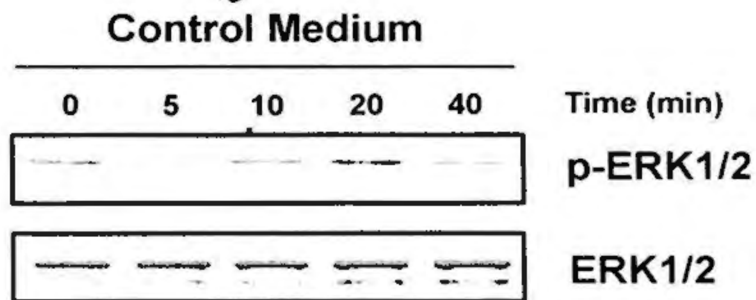


Fig. 3-9 Response of zebrafish chimeras Kita and Kitb to recombinant zebrafish Kitlgb. (A) Western blot analysis (10-min treatment with recombinant proteins). (B) The densitometric analysis to signals in (A). CTLM, control medium from CHO cells which was transfected with plasmid pcDNA5/FRT. CTLP, control plasmid, pCMV-Script. zfaE-mI or zfbE-mI, receptor chimera containing extracellular domain of zebrafish Kita or Kitb and intracellular domain of mouse KIT. The graph is the data normalized to the Actin and expressed as the fold change of the first group (mean ± SEM, n=3). Different letters indicate statistical significance ($P < 0.05$).

A







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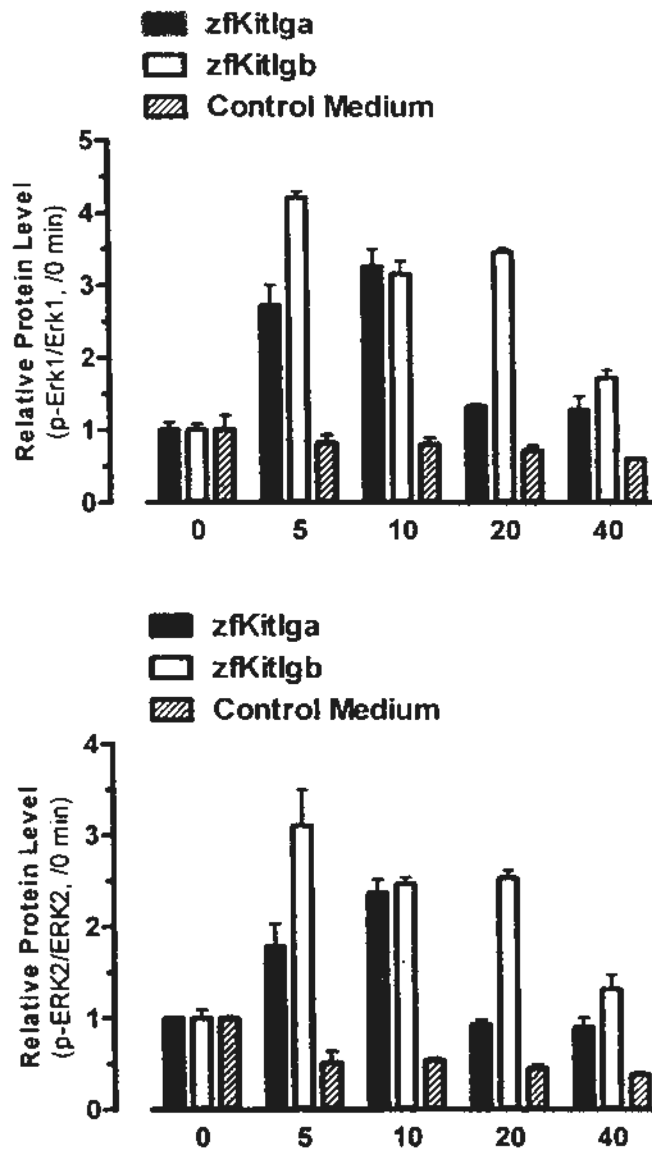


Fig. 3-10 Effects of zfKitlga and zfKitlgb on MAPK phosphorylation in cultured follicle cells. (A) Effects of rzfKitlga (top), rzfKitlgb (middle) and control medium (bottom) on MAPK (10-min treatment with recombinant proteins). (B) The densitometric analysis of signals of Erk1 (upper) and Erk2 (lower) in (A). The black and white columns represent the effects of rzfKitlga and rzfKitlgb, and columns with lines represent effects of control medium (mean ± SEM, n=3).

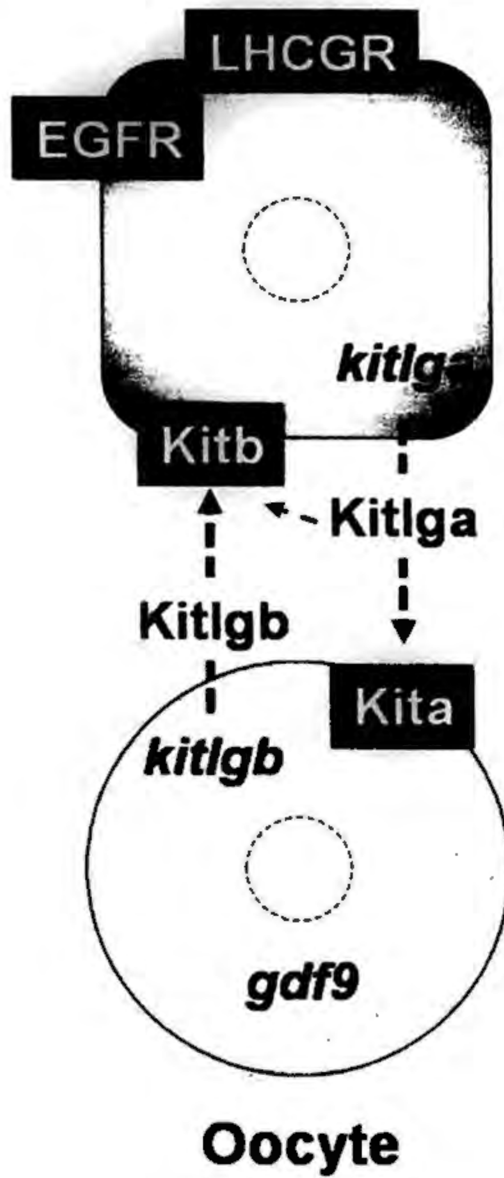


Fig. 3-11 The action modes of Kit ligands and receptors within zebrafish follicle.

Chapter 4

IGF-I Promotes Expression of Kit Ligand A in the Follicle Cells through PI3K-Akt Pathway

4.1 Introduction

It is well known that, as the center of female reproductive system in vertebrates, ovary is subject to multi-factorial regulation. It is not only controlled by extraovarian circulating hormones such as gonadotropins from the pituitary but also a variety of intraovarian paracrine factors including Kit ligand, insulin-like growth factor-I (IGF-I), epidermal growth factor (EGF), activin and steroids (*e.g.*, estradiol), which form a complex communication network in ovarian follicles (95).

Kit ligand is also referred to as stem cell factor (SCF), mast cell growth factor (MGF), and steel factor (SF) which is the product of *Steel* (*Sl*) locus in mice (4-6). The *White Spotting* (*W*) locus encodes its receptor, Kit, which belongs to type III receptor tyrosine kinase (3, 5, 6, 15, 19, 20). In mouse ovary, Kit ligand is derived from the granulosa cells whereas Kit is expressed in the oocyte and theca cells (78, 83). A wide range of *in vivo* and *in vitro* studies have demonstrated that the interaction of Kit ligand with Kit is essential for multiple aspects of oogenesis and folliculogenesis (51, 114, 127-129). In recent years, Kit ligand has attracted increasing attention because a growing body of evidence indicates that Kit ligand plays indispensable roles both in gonadotropin-independent activation of primordial follicles from the quiescent pool (48, 49) and in the maintenance of meiotic arrest before germinal vesicle breakdown (GVBD) (58, 59, 102). Interestingly, Kit ligand has also been demonstrated to be involved in the proliferation of granulosa cells where Kit receptor is absent (53, 113). Up to now, the limited evidence has

demonstrated that the expression of Kit ligand is subject to the regulation of theca cell-derived keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF) (57). On the other hand, the gonadotropin effects on Kit ligand expression have remained intricate under different conditions (37, 57-59). FSH and LH positively in the cultured granulosa cells but LH negatively in the antral follicles regulated Kit ligand expression (57-59). FSH, however, regulated Kit ligand expression in an oocyte stage-dependent manner (37). Despite these findings, the regulation of Kit ligand expression in the ovary remains largely unknown.

In addition to Kit ligand, other growth factors such as IGF-I are also involved in ovarian physiology. The role of IGF-I during folliculogenesis has been extensively studied in mammalian models, and it has been implicated in folliculogenesis and oocyte maturation via various mechanisms (130-132). It has been observed in the IGF-I knockout mice that the follicles are unable to reach the antral stage, leading to sterility due to lack of ovulation (130). Like Kit ligand, a significant positive correlation between IGF-I and granulosa cell proliferation has been reported (133).

IGF-I exerts its effect by activating two major signal transduction pathways: the phosphoinositide 3 - kinase (PI3K) pathway and the mitogen-activated protein kinase (MAPK) pathway (134, 135). IGF-I binds to its receptor, type I IGF receptor, to initiate events resulting in recruitment of PI3K to the inner surface of the plasma membrane where it catalyzes the production of PI $(3,4,5)P_3$ (PIP_3). Binding of Akt to PIP_3 anchors it to the plasma membrane and exposes it to phosphorylation and activation by phosphoinositide-dependent kinase 1 (PDK1) (128, 136, 137). Activated Akt then regulates multiple cellular processes by relocation to different cell fractions. In comparison with activation of Akt, initiation of the MAPK pathway by IGF-I requires activation of Ras, a small GTPase, which in turn activates a cascade of successive protein phosphorylation reactions involving c-Raf, MEK1/2 and finally

ERK1/2 (134, 135).

In mammals, Kit ligand exists as both membrane-associated form (KITL1) and soluble form (KITL2) because of alternative mRNA splicing and proteolytic processing of the protein. In zebrafish, however, two paralogues of Kit ligand, *kitlga* and *kitlgb*, exist as a result of whole genome duplication specific to fish (see Chapter 2). In Chapter 3, we demonstrated that the two isoforms of Kit ligand and Kit exhibited distinct spatial location of expression within the follicle with *kitlga* being expressed in the somatic follicle cells and its preferred receptor *kita* located in the oocyte, similar to the situation in mammals. In contrast, *kitlgb* expression was exclusively restricted to the oocyte whereas its preferred receptor *kitb* was expressed in the surrounding follicle layer. Since the surrounding follicle cells are the targets of numerous endocrine hormones and paracrine factors for regulating folliculogenesis, the location of *kitlga* expression in these cells has led us to speculate that this ligand may be one of the members of the Kit system in the zebrafish follicle that are subject to external regulation. In the present study, we found that the expression of *kitlga* in cultured follicle cells was promoted by IGF-I in a time and dose-dependent manner. Further experiments using pharmacological approach and Western blot analysis revealed that the effect of IGF-I was mediated by PI3K-Akt but not MAPK pathway.

4.2 Materials and Methods

4.2.1 Animals and chemicals

Zebrafish (*Danio rerio*) were obtained from a local tropical fish market and maintained in flow-through aquaria at 28 ± 1°C on a photoperiod of 14L:10D, with lights on at 8:00. The fish was fed twice a day with the commercial tropical fish feed Otohime S1 (Marubeni Nisshin Feed Co., Tokyo, Japan) and once with frozen artemia. All experiments performed were licensed by the Government of the Hong Kong

Special Administrative Region and endorsed by the Animal Experimentation Ethics Committee of The Chinese University of Hong Kong. Unless otherwise indicated, all common chemicals used were purchased from Sigma (St. Louis, MO), USB Corporation (Cleveland, OH), GE Healthcare (Waukesha, WI), or Merck (Whitehouse Station, NJ); enzymes from Promega (Madison, WI); and culture medium from Gibco Invitrogen (Carlsbad, CA). IGF-I, actinomycin D and cycloheximide were purchased from Sigma, LY294002, wortmannin, Akti and PD98059 from Calbiochem (La Jolla, CA). IGF-I was first dissolved in water, cycloheximide in ethanol and actinomycin D, LY294002, wortmannin, Akti and PD98059 in dimethylsulfoxide (DMSO). They were diluted to the desired concentrations with the medium before use. Antibodies for Akt (Cat. 9272), phosphor-Akt (Ser473, Cat. 9271L) were from Cell Signalling Technology (Danvers, MA), and HRP-linked anti-rabbit IgG (Cat. sc-2374) from Santa Cruz (Santa Cruz, CA).

4.2.2 Total RNA isolation and RT

Total RNA was extracted from cultured follicle cells with Tri-Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacture's protocol and our previous study (101). The RT was then performed at 37°C for 2 h in a volume of 10 μl containing 0.5 μg of oligo(dT), 1× M-MLV RT buffer, 0.5 mM each deoxyribonucleotide triphosphate (dNTP), 0.1 mM dithiothreitol, and 100 U of M-MLV RT (Invitrogen, Carlsbad, CA).

4.2.3 Quantification of kitlga mRNA by real-time qPCR

Real-time quantitative PCR was performed to quantify the expression of *kitlga*. The template for the standard curve was prepared by PCR amplification of cDNA fragment with specific primers. After purification with a PCR Purification Kit

(Qiagen, Valencia, CA), the amplified DNA amplicons were quantified with the software Quantity One (Bio-Rad, Hercules, CA) using the Mass Ruler DNA marker (MBI Fermentas, Hanover, MD) as the standard, and the copy numbers of the DNA molecules were calculated before use as templates to construct standard curves in real-time quantitative PCR. All PCR reactions were performed in a total volume of 30 μl containing 10 μl template (RT reaction mix diluted at 1:15), 1× PCR buffer, 0.2 mM each dNTP, 2.5 mM $MgCl_2$, 0.75 U of Taq polymerase, 0.5× EvaGreen (Biotium, Hayward, CA), and 20 nM fluorescein (Bio-Rad) on the iCycler iQ Real-time PCR Detection System (Bio-Rad). The amplification protocol was 30 sec at 94°C, 30 sec at 60°C, and 30 sec at 72°C, with a signal detection period of 7 sec at 80°C. A melt curve analysis was performed at the end of the reaction to check the reaction specificity.

4.2.4 Primary follicle cell culture

The primary follicle cell culture of zebrafish ovary was performed according to our previous report (101). Briefly, the ovaries from about 20 female zebrafish were isolated and dispersed in a 100-mm petri dish containing 60% Leibovitz L-15 medium (Invitrogen). The full-grown follicles were removed by sieving, followed by washing with medium M199 (Invitrogen) for about five times. Afterwards, the follicles were cultured in M199 supplemented with 10% fetal calf serum (Hyclone, Logan, UT, USA) at 28°C in 5% CO_2 for 6 days for the proliferation of follicle cells. Then, the follicle cells were harvested by trypsinization and plated in 24-well plates at the density of about 2.5×10^5 cells/well for 24 h. The cells were then starved with M199 without serum for 24 h before treatment.

4.2.5 Western blotting

The cells were lysed by adding 1× SDS sample buffer (62.5 mM Tris-HCl, pH 6.8 at 25°C, 1% w/v SDS, 10% glycerol, 5% 2-mercaptoethanol, 100 μl per well of 24-well plate). Then the plate was shaken immediately for a few times and the extract from each well transfered to a microcentrifuge tube. All samples were heated to 95–100°C for 5 minutes, cooled on ice and microcentrifuged for 5 minutes. Western blotting was performed according to the manufacture's protocol (Cell Signalling Technology, Danvers, MA). Briefly, samples (about a half for 24-well plate) were loaded and separated in the 12.5% SDS–PAGE gel in 1× running buffer (25 mM Tris base, 0.2 M glycine, 0.1% w/v SDS), followed by blotting to the nitrocellulose membrane (Bio-Rad) using blotting buffer (25 mM Tris base, 0.2 M glycine, 20% methanol). The membrane was incubated in 25 ml blocking buffer (1× TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk) for 1 h at room temperature and then incubated in 5 ml of diluted primary antibody (1:1000 in blocking buffer) at 4°C overnight. The membrane was washed three times for 5 min each with wash buffer (1× TBS, 0.1% Tween-20) and then incubated with HRP-conjugated secondary antibody (1:2000 in wash buffer) for 1 h at room temperature. The membrane was then washed again and equilibrated with the developing solution (Western Blotting Luminol Reagent; Santa Cruz Biotechnology, Santa Cruz, CA). The signals were detected on the Lumi-Imager F1 Workstation (Roche, Mannheim, Germany).

4.2.6 Data analysis

The ratio of expression levels of *kitlga* to that of the internal control *ef1a* was calculated and then expressed as the fold change compared with the control or reference group. All values were expressed as the mean ± SEM, and the data were analyzed by one-way ANOVA, followed by Dunnett test using Prism 5 on Macintosh OS X (GraphPad Software, San Diego, CA).

4.3 Results

4.3.1 IGF-I promotes expression of kitlga

IGF-I was one of the first factors we tested because of its importance in folliculogenesis and oocyte maturation. As shown in Fig. 4-1, treatment of the cultured zebrafish follicle cells with IGF-I significantly increased the expression of *kitlga* in a time-dependent manner and the maximal response was observed at 3-6 h of the treatment. Longer treatment for 12 or 24 h caused a decline of the response. The level of *kitlga* also exhibited a dose-dependent increase in response to IGF-I treatment from 0 to 50 ng/ml for 6 h. The maximal response was observed at 50 ng/ml and the response decreased at higher concentrations of 100 and 200 ng/ml (Fig. 4-2). The effect of IGF-I appeared to be specific because no effect was observed on the expression of the housekeeping gene *ef1a* in both time course and dose response experiments (Fig. 4-1 and 2).

4.3.2 Induction of kitlga mRNA by IGF-I requires ongoing transcription but not new protein synthesis

The above studies demonstrated that the steady-state levels of *kitlga* mRNA increased in response to IGF-I. To further define the mechanism by which IGF-I regulates *kitlga*, the transcriptional inhibitor actinomycin D (AD) and the translational inhibitor cycloheximide (CH) were used to assess the involvement of transcription and translation in IGF-I-induced *kitlga* expression. Treatment of cultured follicle cells with CH alone resulted in a small increase in *kitlga* mRNA level (Fig. 4-3A); however, CH had no effect on the ability of IGF-I to induce *kitlga* mRNA, indicating that new protein synthesis was not required for the IGF-I-induced increase in *kitlga* expression. In contrast, administration of AD alone resulted in a dramatic decrease in *kitlga*

mRNA relative to that of the control (Fig. 4-3B). Furthermore, IGF-I induction of *kitlga* mRNA was completely blocked by treatment with AD, indicating that transcription was required for IGF-I-induced expression of *kitlga*. These studies suggest that the *kitlga* gene is likely a direct downstream target of the IGF-I signal transduction pathway in the ovary.

4.3.3 Level of kitlga mRNA is upregulated by PI3K-Akt pathway

To examine whether PI3K-Akt pathway plays a role in the transcription of *kitlga*, two specific inhibitors of PI3K, LY294002 and wortmannin, were used to treat primary follicle cell culture. The results showed that both LY294002 and wortmannin significantly decreased the level of *kitlga* mRNA in a dose-dependent manner (Fig. 4-4). On the other hand, Akti, a specific inhibitor of Akt, also dramatically blocked transcription of *kitlga* with the maximal effect achieved at the concentration of 5 nM (Fig. 4-5). To further test whether the stimulatory effect of PI3K on the expression of *kitlga* in the zebrafish follicle cells is mediated by Akt, we examined the effects of LY294002 on the expression of *kitlga* in the presence or absence of Akti. As shown in Fig. 4-6, Akti had no synergistic effects on *kitlga* with LY294002 (Fig. 4-6), suggesting that Akt and PI3K may likely work in the same signaling pathway in promoting the expression of *kitlga*.

4.3.4 Specific inhibitors of PI3K-Akt pathway abrogate the effect of IGF-I on Akt phosphorylation and kitlga expression

IGF-I can activate multiple signaling pathways including PI3K-Akt pathway in mammals. In cultured zebrafish follicle cells, IGF-I also induced a significant phosphorylation to Akt. The activation occurred quickly at 15 min of the treatment and lasted up to 1.5 h of the treatment. Afterwards, the response began to decline (Fig.

4-7A). Two specific inhibitors of PI3K, LY294002 and wortmannin, both completely abolished the stimulatory effect of IGF-I on Akt (Fig. 4-7B). To investigate whether the stimulatory effect of IGF-I on the expression of *kitlga* in the zebrafish follicle cells was mediated by PI3K-Akt pathway, we treated the cells with IGF-I in the presence or absence of the above PI3K-Akt pathway inhibitors. As shown in Fig. 4-8 and Fig. 4-9, all inhibitors significantly suppressed or entirely abolished the effects of IGF-I.

4.3.5 MAPK pathway negatively regulates expression of kitlga

As mentioned above, IGF-I can also activate MAPK pathway in mammals. To investigate whether IGF-I-activated MAPK was involved in the regulation of *kitlga* expression in the zebrafish follicle cells, PD98059, a potent inhibitor of MEK1/2, which is an immediate upstream activator of ERK1/2, was used to treat the primary follicle cells. To our surprise, instead of reducing *kitlga* expression as did the PI3K and Akt inhibitors, PD98059 slightly but significantly raised the basal level of *kitlga* expression in a dose-dependent manner (Fig. 4-10). Pretreatment of the follicle cells with PD98059 resulted in an additive increase in IGF-I-induced *kitlga* mRNA expression (Fig. 4-11).

4.4 Discussion

IGF-I enhances the proliferation in a variety of cell types, including granulosa cells (133, 138, 139). It has been demonstrated in mammals that IGF-I receptor is expressed in granulosa cells in all follicles from primary to antral preovulatory stages (140). Interestingly, IGF-I mRNA can also be detected in granulosa cells with the signal most prominent in cumulus granulosa cells of antral follicles (131, 132, 140). Thus, it is likely that IGF-I acts in an autocrine manner to stimulate proliferation of granulosa cells in mammals. In the zebrafish, however, a previous study in our

laboratory showed that IGF-I mRNA could be detected in both the follicle cells and oocytes (Yu and Ge, unpublished data). In this study, we have demonstrated that the distribution pattern of *kitlga* and *kita* mRNAs in the zebrafish follicle is the same as that in mammals and Kitlga in the peripheral follicle layer prefers to activate Kita in the oocyte (see Chapter 3), making *kitlga* a potential target for endocrine and paracrine regulation. The present study demonstrated that IGF-I could be one of the potential factors that control *kitlga* expression in the follicle cells. Although we are not sure about the exact sources of IGF-I that acts on the follicle cells to upregulate *kitlga*, it is possible that IGF-I exerts its effect via autocrine (from granulosa cells), paracrine (from oocytes) and endocrine (from extraovarian tissues) pathways. The increased Kitlga (Kitlg in mammals) in turn may bind to its receptor Kita (Kit in mammals) on the oocyte to regulate oocyte growth and maturation. The stimulatory effect of IGF-I on *kitlga* involved transcription but not translation, suggesting that *kitlga* is likely an immediate downstream gene controlled by IGF-I.

It has been well documented that IGF-I activates two major signal transduction pathways: the phosphoinositide 3- kinase (PI3K) pathway and the mitogen-activated protein kinase (MAPK) pathway (134, 135). Binding of IGF-I to its receptor initiates events leading to recruitment of PI3K to the inner surface of the plasma membrane where it catalyzes the production of phosphatidylinositol-3,4,5-triphosphate (PIP_3). These phospholipids bind to Akt kinase, leading to its relocation to the plasma membrane and phosphorylation by regulatory kinases (128, 136, 137). Akt plays a critical role in controlling cell survival and apoptosis (141-143). In this study, we demonstrated that, in zebrafish ovary, IGF-I increased *kitlga* expression in the somatic follicle cells by activating PI3K-Akt pathway as its effect could be completely abolished by specific PI3K inhibitors, LY294002 and wortmannin, and Akt inhibitor Akti. The exact physiological significance of the increased Kitlga production in

response to IGF-I is entirely unknown at this moment. In mammals, there is evidence that Kit ligand may be involved in promoting the survival of both primordial (52) and pre-antral follicles (48, 53). An experiment using coculture of murine oocytes with Kitl-producing fibroblasts showed that the membrane-associated form of Kit ligand is biologically active in regulating oocyte survival in vitro (102). Further experiments on fetal ovaries demonstrated that the anti-apoptotic effects of IGF-I and Kit ligand could be abolished by either LY294002 or wortmannin (46). Thus, an attracting scenario in the zebrafish ovary is that the binding of IGF-I to its receptor on granulosa cells activates Akt to induce production of Kit ligand, which is released to the oocytes as a survival-promoting signal.

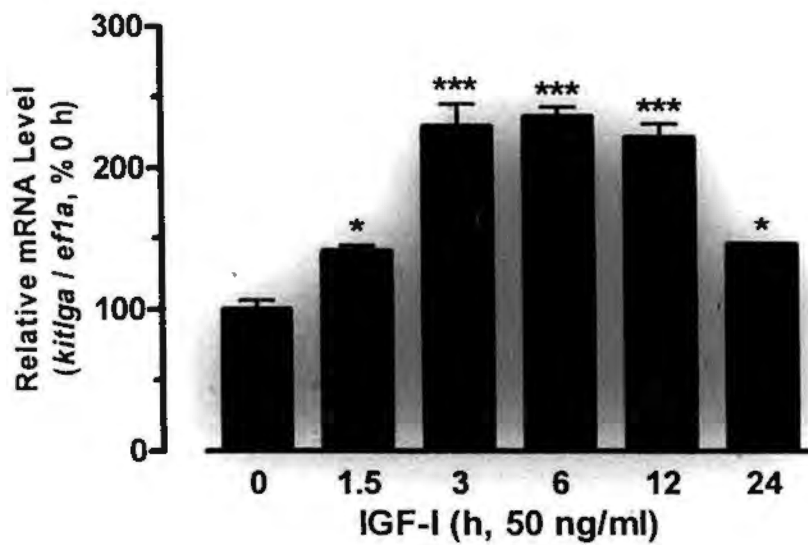
Some recent studies in our laboratory have provided lines of evidence for an important role of IGF-I in the reproduction of female zebrafish. As a major component of the somatotropic axis, IGF-I may most likely serve as an important messenger for cross talks between the growth and reproductive axes. Our recent study using primary pituitary cell culture demonstrated that IGF-I could promote the expression of FSH β subunit (*fshb*) in the zebrafish pituitary cells while suppressing growth hormone (GH) expression (144). In the zebrafish ovary, our preliminary data showed that IGF-I could also enhance the expression of FSH receptor (*fshr*) in cultured follicle cells (Liu and Ge, unpublished data). We have previously reported that *fshr* expression level is very low in the follicles of primary growth (PG) stage but increased dramatically at the pre-vitellogenic (PV) stage when the follicles enter the fast growing vitellogenic stage (117, 122). The regulatory mechanism underlying the increased expression of *fshr* at the time of follicle activation or recruitment is unknown. The stimulatory effect of IGF-I on *fshr* expression in vitro implies a role for IGF-I in initiating the transition from the PG to PV stage, which marks the start of gonadotropin-dependent folliculogenesis and onset of puberty in zebrafish life cycle.

A recent in vivo study in our laboratory showed that IGF-I might potentially advance the onset of puberty in female zebrafish (Chen and Ge, unpublished data). In addition to increasing *fshr* expression in the ovary, the present study provided evidence that IGF-I might also work by enhancing the production of Kitlga in the somatic follicle layer via PI3K-Akt pathway. The Kitlga in turn may act on the oocyte via its receptor Kita to promote the transition from PG to PV stage. This hypothesis is supported by the evidence in mammals that the Kit system and Akt pathway are both important for mammalian follicle activation or recruitment (48, 128, 145). The blockade or mutation of Kit and Kit ligand and inactivation of Akt could lead to failure of follicle activation (67, 128). During zebrafish life cycle, the IGF-I-induced Kitlga may play an important role in transducing the signals from the somatotropic axis to reproductive axis to initiate female puberty. Given the expression of the IGF-I receptor in the oocytes (140), it cannot be ruled out that IGF-I may also directly act on the oocytes. The IGF-I-induced Kitlga in the granulosa cells may serve as an additional mechanism for amplifying IGF-I signals to the oocyte.

In addition to the classical PI3K-Akt pathway, IGF-I can also activate MAPK pathway (134, 135). It was evidenced in the present study, however, that the MAPK pathway was not likely responsible for the IGF-I-induced *kitlga* expression. Instead of blocking IGF-I effect, the specific MAPK pathway inhibitor, PD98059, caused a significant increase of basal and IGF-I-induced *kitlga* expression. More experiments will be carried out to elucidate differential functions of the PI3K-Akt and MAPK pathways in zebrafish follicle cells in mediating IGF-I signaling. In mammals, Kit ligand exhibits a negative effect on oocyte maturation (58, 59). Consistent with this, our previous study showed that the expression of *kitlga* significantly decreased before oocyte maturation in zebrafish (see Chapter 2). By contrast, IGF-I has been demonstrated to induce germinal vesicle breakdown (GVBD) in oocytes, particularly

in fish (146-149). Given the opposite effects of IGF-I and Kit ligand on oocyte maturation, we tempt to postulate that IGF-I might promote the expression of *kitlga* through PI3K-Akt pathway to activate and stimulate early follicle development including follicle activation, granulosa cell proliferation and oocyte growth. However, IGF-I and other growth factors like EGF could enhance final oocyte maturation by suppressing the expression of *kitlga* through MAPK pathway in later stages of follicle development. Further experiments using different stages of follicles will be performed in the future to test this hypothesis.

In summary, the present study provided evidence that IGF-I was a potent regulatory factor that up-regulated the expression of *kitlga* in zebrafish follicle cells. The stimulation involved transcription but not translation, indicating that the *kitlga* gene is a direct downstream target of IGF-I. The effect of IGF-I on *kitlga* was exerted via PI3K-Akt but not MAPK pathway. In contrast, the MAPK pathway may play a negative role in controlling *kitlga* expression.



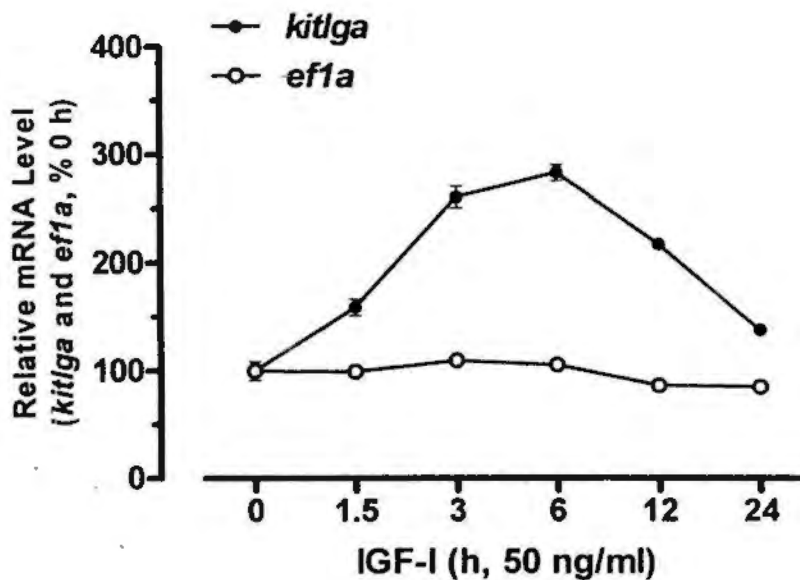
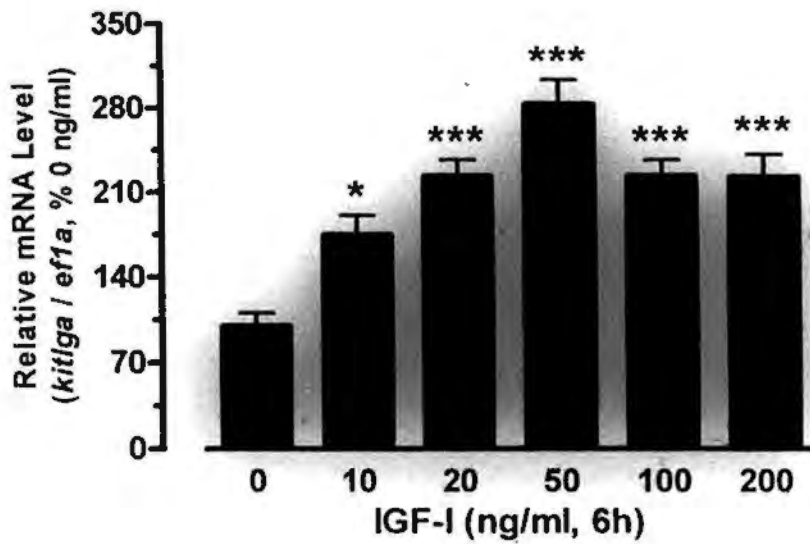


Fig. 4-1 Time course of IGF-I effect (50 ng/ml) on the expression of *kitlga* in cultured zebrafish follicle cells. The upper graph is the data normalized to the housekeeping gene *ef1a* and expressed as the percentage or fold change of the control group (mean ± SEM, n = 4). * $P < 0.05$; *** $P < 0.001$ vs. control. The raw data without normalization to *ef1a* are plotted separately in the lower graph to show that the responses of *kitlga* were not introduced by any changes of house-keeping gene expression.



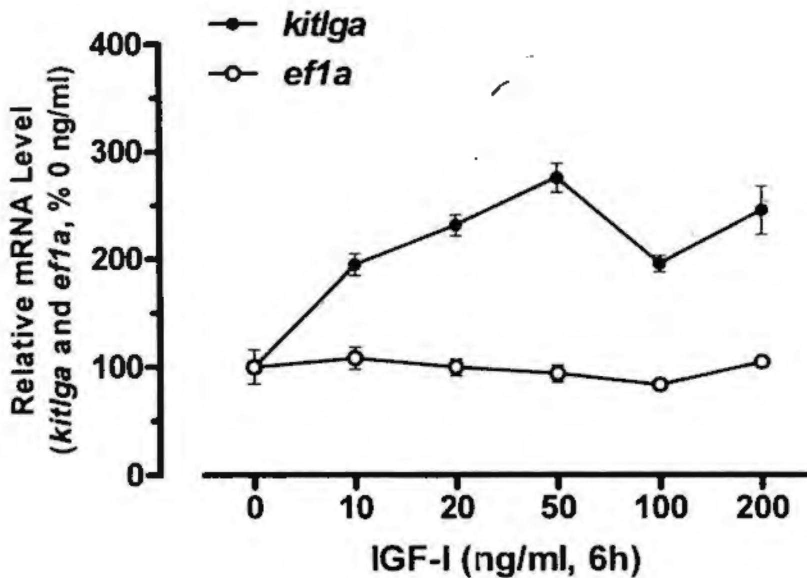
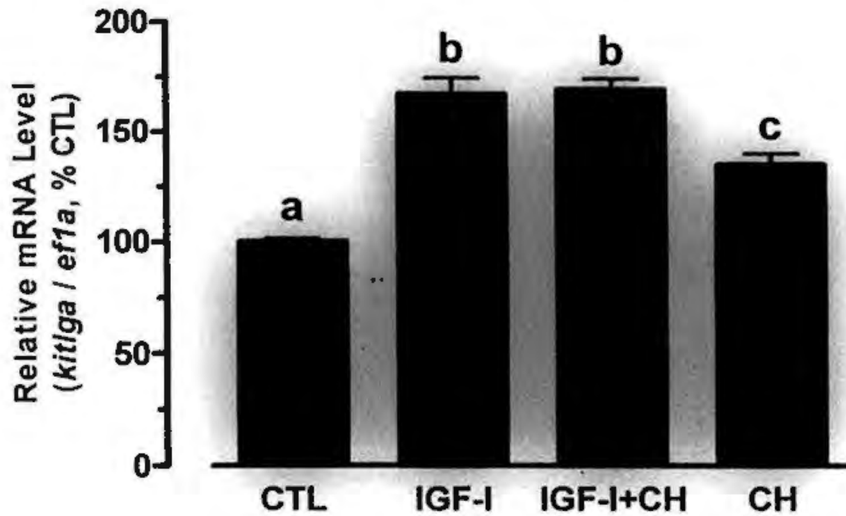


Fig. 4-2 Dose response of IGF-I effect on the expression of *kitlga* in cultured zebrafish follicle cells at 6 h of the treatment. The upper graph is the data normalized to the housekeeping gene *ef1a* and expressed as the percentage or fold change of the control group (mean ± SEM, $n = 4$). * $P < 0.05$; *** $P < 0.001$ vs. control. The raw data without normalization to *ef1a* are plotted separately in the lower graph to show that the responses of *kitlga* were not introduced by any changes of house-keeping gene expression.

A



B

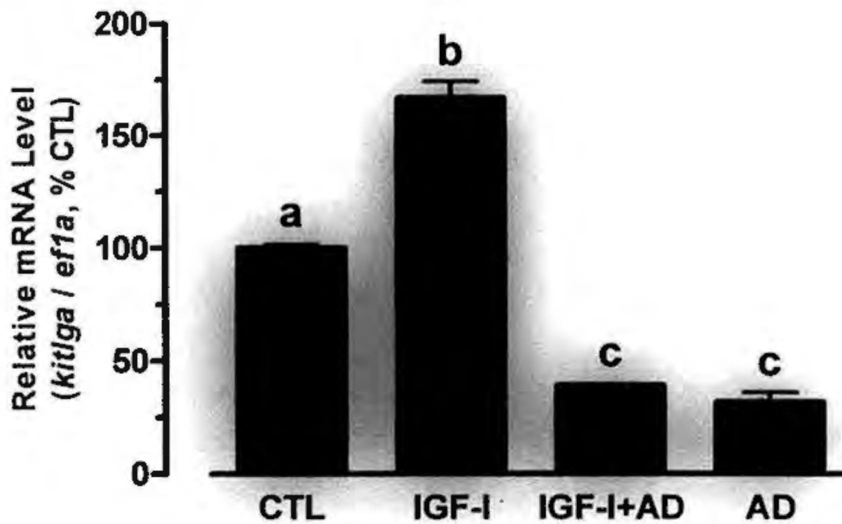
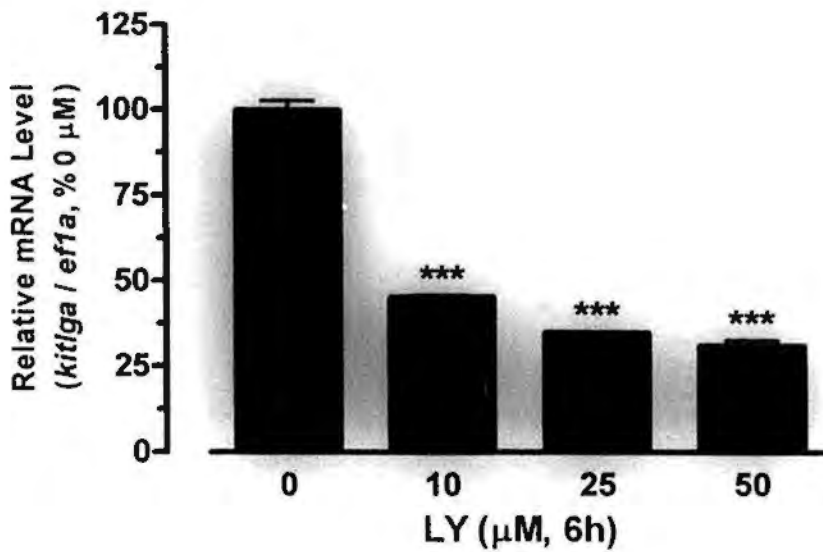


Fig. 4-3 Induction of *kitlga* by IGF-I requires ongoing transcription (B) but not *de novo* protein synthesis (A). Cultured zebrafish follicle cells were pretreated with actinomycin D (AD, 1 μg/ml) or cycloheximide (CH, 1 μg/ml) for 30 min before treatment of IGF-I (50 ng/ml) for 6 h. The graphs are the data normalized to the housekeeping gene *ef1a* and expressed as the percentage or fold change of the control group (mean ± SEM, n = 4). Different letters indicate statistical significance (P < 0.05).

A



B

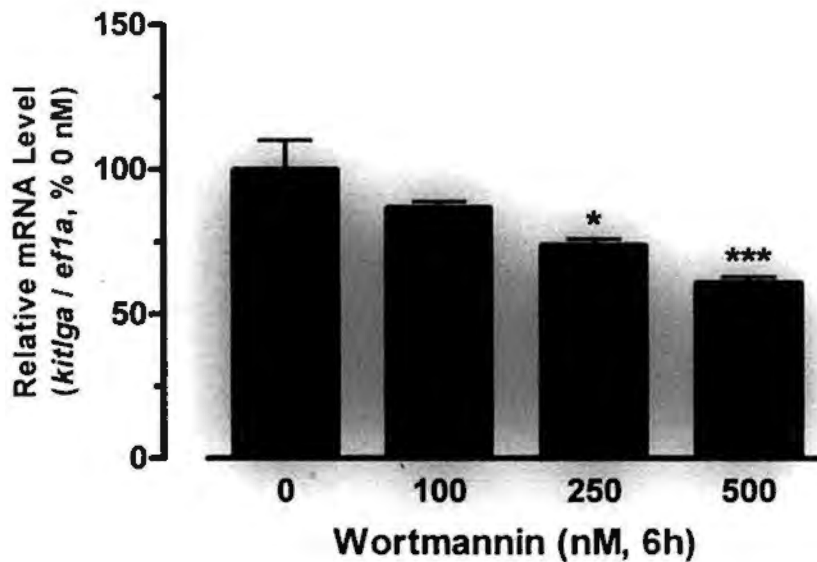


Fig. 4-4 Dose response of LY294002 (A) and wortmannin (B) effects on the expression of *kitlga* in cultured zebrafish follicle cells at 6 h of the treatment. The graphs are the data normalized to the housekeeping gene *ef1a* and expressed as the percentage or fold change of the control group (mean ± SEM, $n = 4$). * $P < 0.05$; *** $P < 0.001$ vs. control.

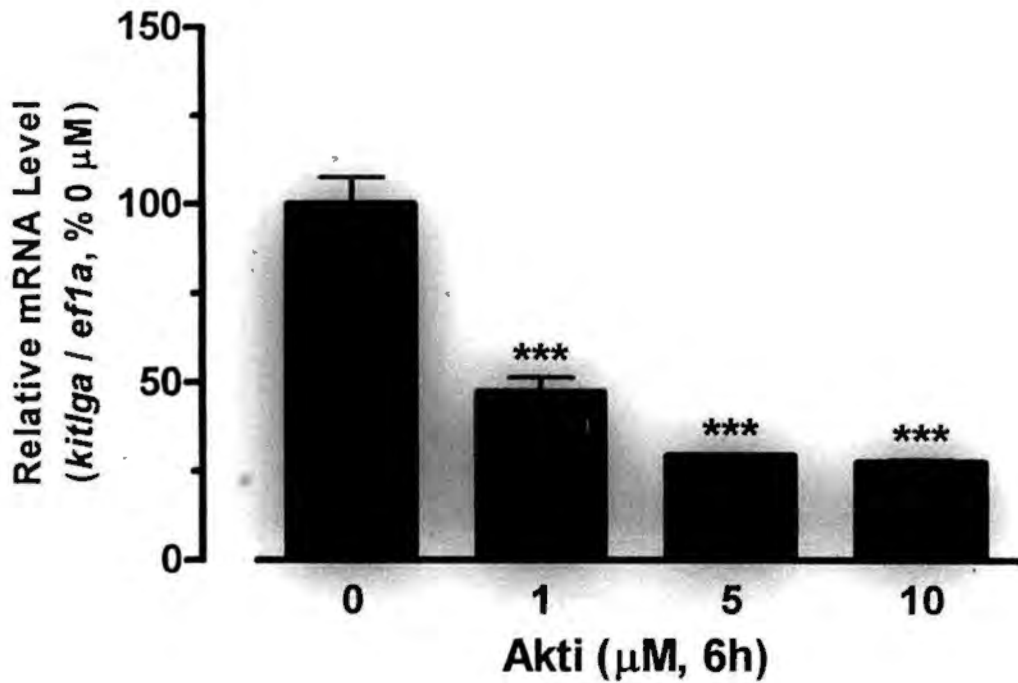


Fig. 4-5 Dose response of Akti effect on the expression of *kitlga* in cultured zebrafish follicle cells at 6 h of the treatment. The graph is the data normalized to the housekeeping gene *ef1a* and expressed as the percentage or fold change of the control group (mean ± SEM, $n = 4$). *** $P < 0.001$ vs. control.

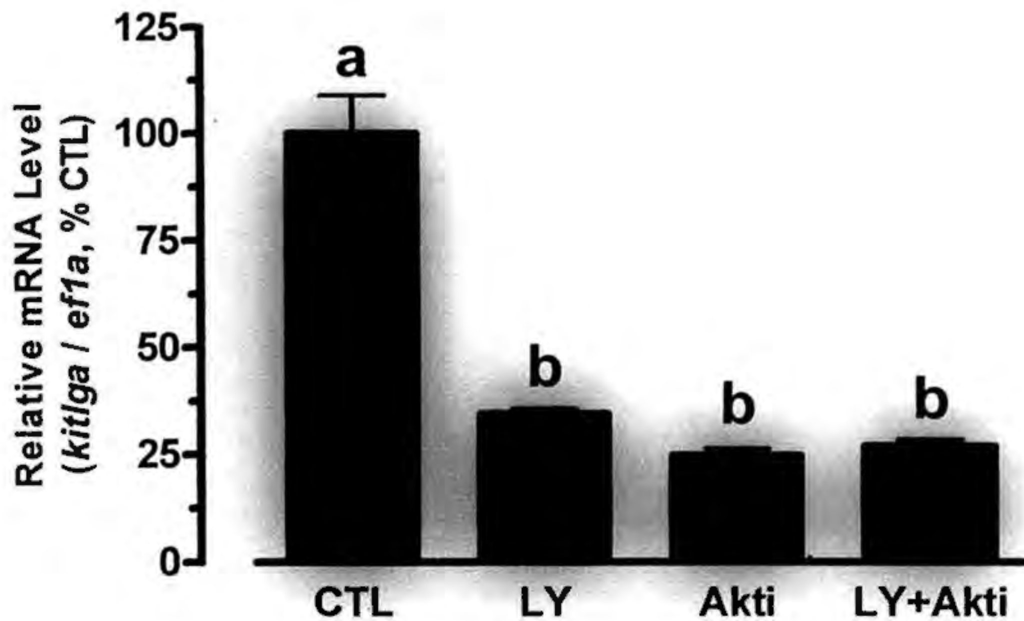
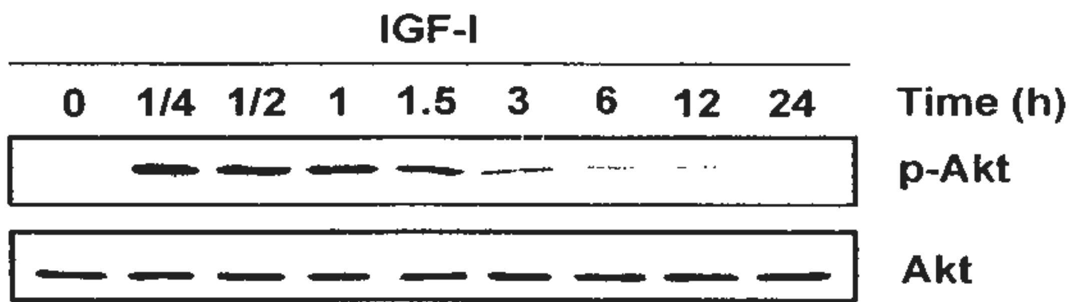


Fig. 4-6 Akt mediates the effect of PI3K on the expression of *kitlga*. Cultured zebrafish follicle cells were pretreated with Akti (10 μM) for 30 min before treatment of LY294002 (50 μM) for 6 h. The graph is the data normalized to the housekeeping gene *ef1a* and expressed as the percentage or fold change of the control group (mean ± SEM, $n = 4$). Different letters indicate statistical significance ($P < 0.05$).



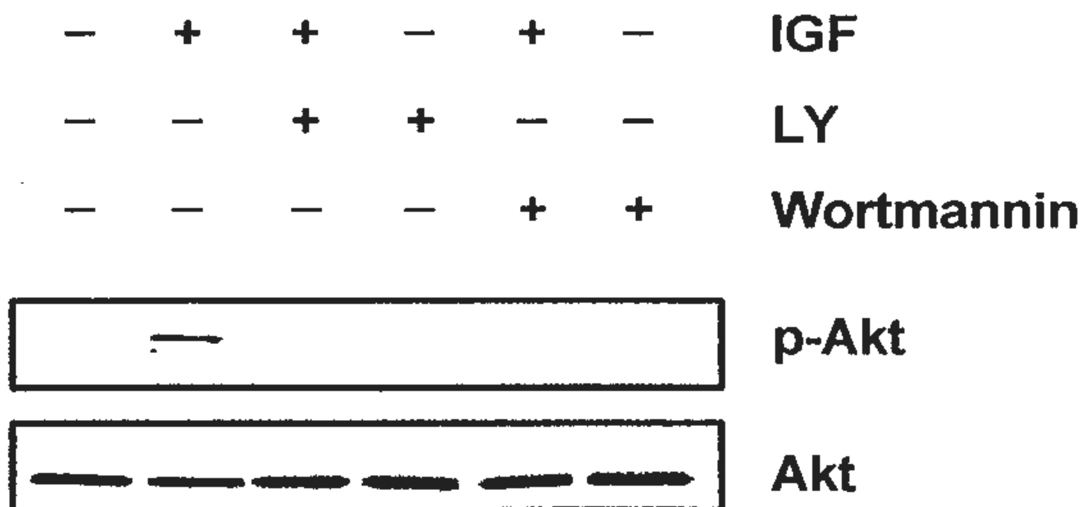
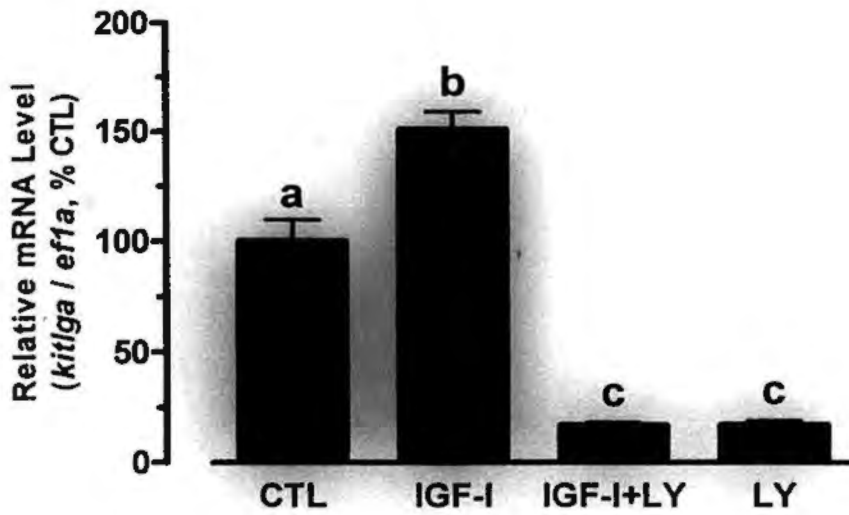


Fig. 4-7 Effect of IGF-I on Akt phosphorylation in zebrafish ovarian follicle cells. (A) Akt was strongly activated by IGF-I. (B) The effect of IGF-I on Akt was completely abolished by specific PI3K inhibitors. Cultured zebrafish follicle cells were pretreated with LY294002 (50 μM) or wortmannin (500 nM) for 30 min before treatment of IGF-I (50 ng/ml) for 15 min.

A



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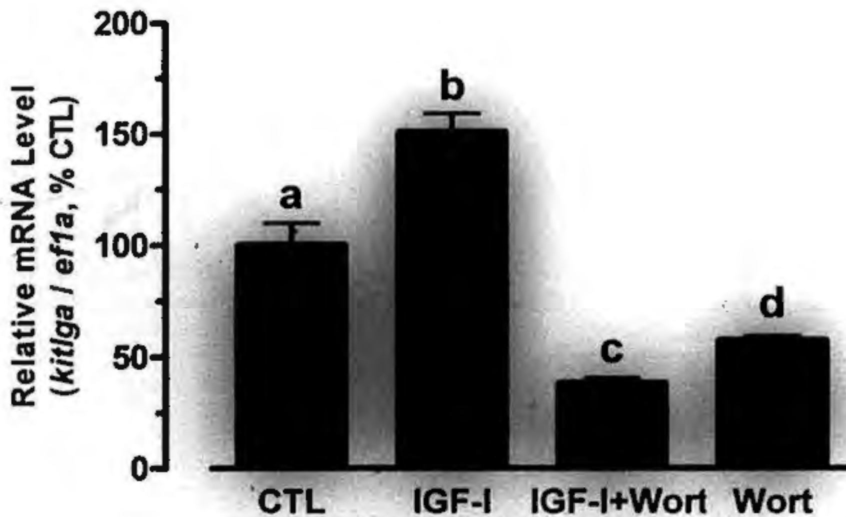


Fig. 4-8 PI3K mediates the effect of IGF-I on the expression of *kitlga*. Cultured zebrafish follicle cells were pretreated with LY294002 (50 μM) or wortmannin (500 nM) for 30 min before treatment with IGF-I (50 ng/ml) for 6 h. The graphs are the data normalized to the housekeeping gene *ef1a* and expressed as the percentage or fold change of the control group (mean ± SEM, n = 4). Different letters indicate statistical significance ($P < 0.05$).

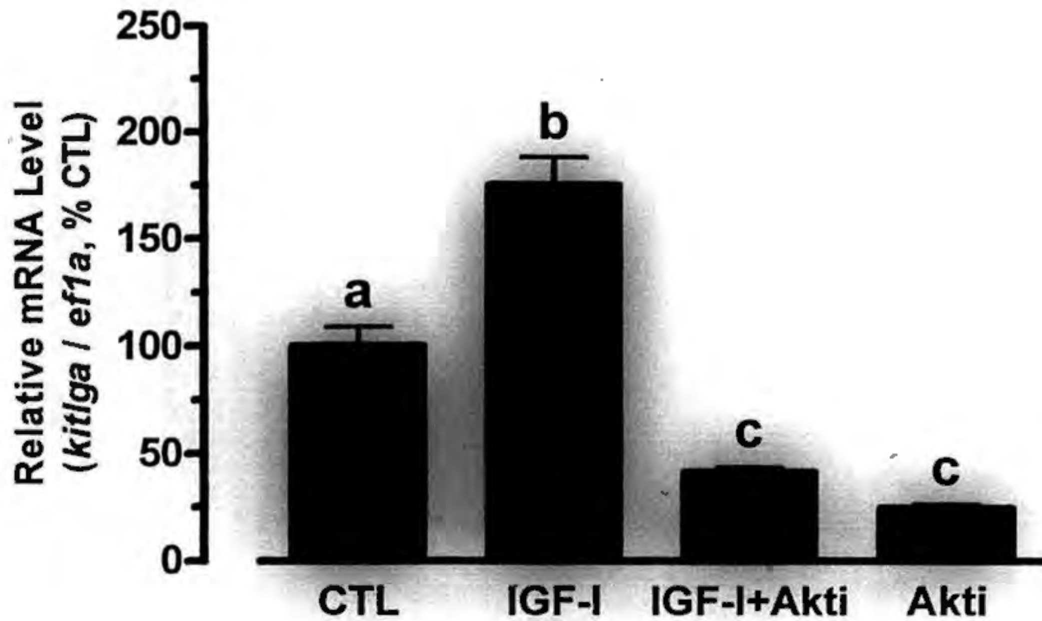


Fig. 4-9 Akt mediates the effect of IGF-I on the expression of *kitlga*. Cultured zebrafish follicle cells were pretreated with Akti (10 μM) for 30 min before treatment of IGF-I (50 ng/ml) for 6 h. The upper graph is the data normalized to the housekeeping gene *ef1a* and expressed as the percentage or fold change of the control group (mean ± SEM, n = 4). Different letters indicate statistical significance (P < 0.05).

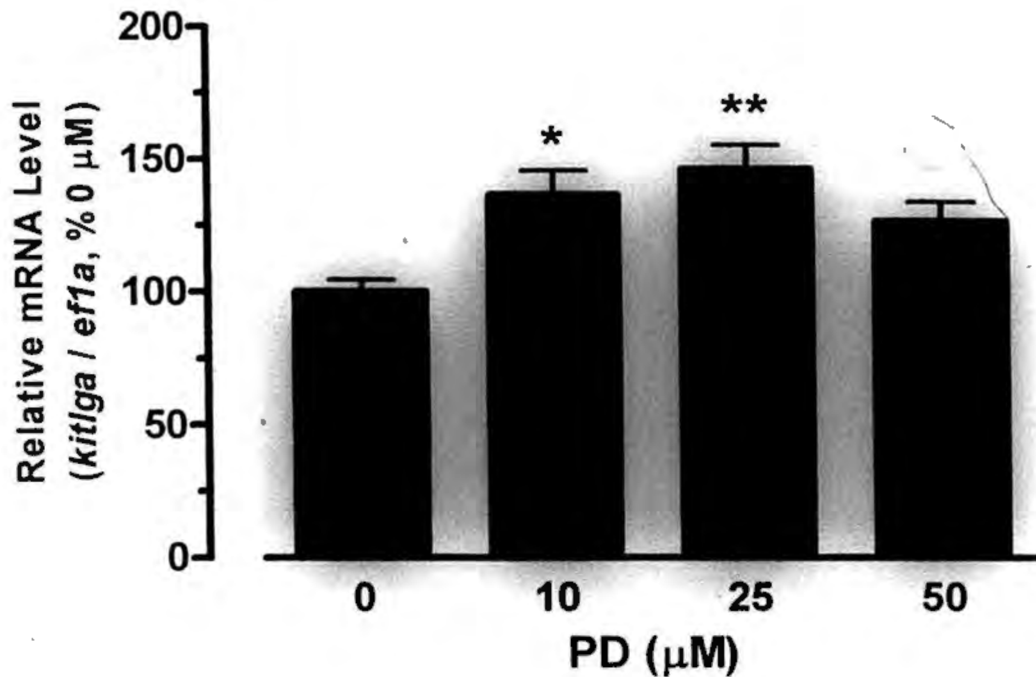


Fig. 4-10 Dose response of PD98059 effect on the expression of *kitlga* in cultured zebrafish follicle cells at 6 h of the treatment. The upper graph is the data normalized to the housekeeping gene *ef1a* and expressed as the percentage or fold change of the control group (mean ± SEM, $n = 4$). * $P < 0.05$; ** $P < 0.01$ vs. control.

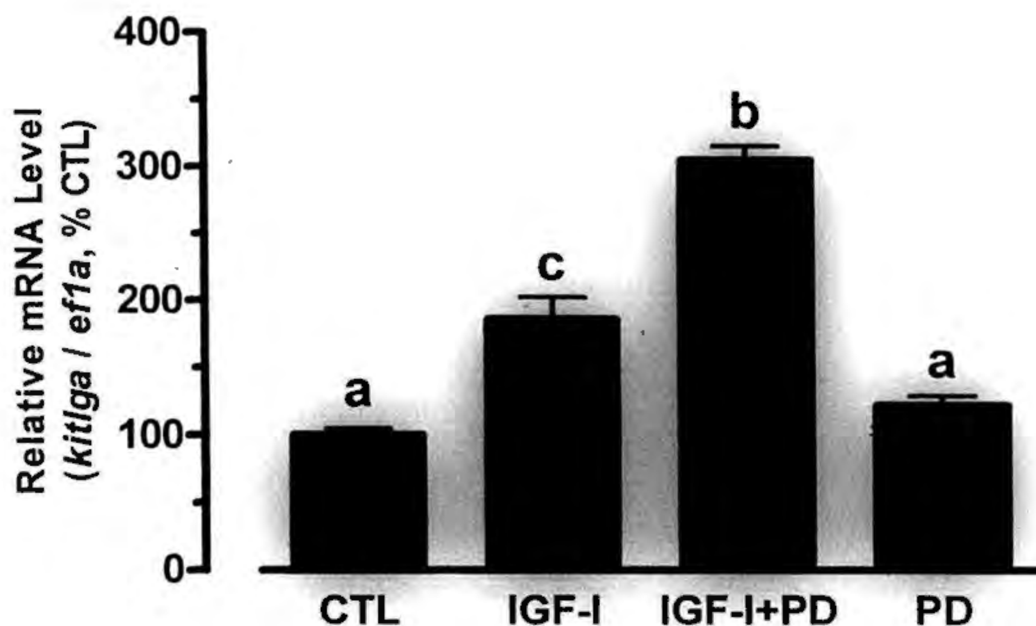


Fig. 4-11 MAPK pathway negatively regulates the expression of *kitlga*. Cultured zebrafish follicle cells were pretreated with PD98059 (25 μM) for 30 min before treatment of IGF-I (50 ng/ml) for 6 h. The upper graph is the data normalized to the housekeeping gene *ef1a* and expressed as the percentage or fold change of the control group (mean ± SEM, n = 4). Different letters indicate statistical significance (P < 0.05).

Chapter 5

Involvement of Cyclic Adenosine 3',5'-Monophosphate in the Differential Regulation of Kit Ligand A Expression in the Follicle Cells

5.1 Introduction

In mammalian ovaries, folliculogenesis begins from the remnant primordial follicles, which consist of the central oocytes arrested at the diplotene stage of the first meiosis and surrounding pregranulosa cells. When follicles leave the resting primordial pool, the oocytes grow and the granulosa cells proliferate to form primary and preantral follicles. Afterwards, the follicles become acutely dependent on gonadotropins from the pituitary for further growth and development until the oocytes resume and complete meiosis and ovulate. The dynamic changes associated with ovarian folliculogenesis are regulated by both endocrine hormones and paracrine factors within the follicle, especially those involved in communications between the oocyte and surrounding follicle layer as evidenced both in mammals (121, 150-153) and non-mammals (95). It is now believed that the Kit system is an important member of the complex regulatory network in the follicle, which is widely involved in various folliculogenic processes including proliferation of granulosa cells (48, 53, 113), growth and survival of oocytes (48, 52, 53, 102) and final oocyte maturation (48, 52, 53, 58, 59, 73, 102). As an important paracrine factor responsible for signaling from the follicle cells to the oocyte, Kit ligand is likely subject to the regulation by various systemic and local factors; however, the information about Kit ligand regulation is very limited.

As Kit ligand is expressed in the follicle cells, it is likely subject to the regulation by pituitary gonadotropins, FSH and LH, which control folliculogenesis by activating

their cognate receptors (FSHR and LHCGR) on the granulosa and theca cells. Both FSH and LH receptors belong to the large superfamily of G protein-coupled receptors and numerous in vivo and in vitro studies have demonstrated that FSHR and LHCGR signal in the follicle cells mainly by activating adenylate cyclase to increase intracellular cAMP levels (154). It has been documented in mammals that FSH can stimulate but LH suppresses the expression of *Kitl* in granulosa cells (37, 59). Given that both FSH and LH rely on cAMP for intracellular signaling, an interesting question is therefore raised: how do FSH and LH differentially regulate *Kitl* expression during folliculogenesis? Up to now, little is known about the underlying mechanism. One possibility is that despite being used as the common second messenger for FSH and LH signaling, cAMP may mobilize different downstream mechanisms in different cells or at different developmental stages, which account for the differential effects of the two hormones.

It has been widely accepted for many years that protein kinase A (PKA) represents the major cAMP effector in various cellular systems (155). However, there has also been evidence that PKA cannot mediate all actions of cAMP such as cAMP-induced mitogenic effects (156, 157). Recent studies have provided increasing evidence for the existence of cAMP-dependent but PKA-independent pathways in different cell types, one of which involves cAMP-activated Epac (including Epac1 and Epac2), a gunanine nucleotide exchange factor (GEF) for the small G protein Rap (158, 159). It has been documented that different cAMP-activated effectors can act synergistically (160), independently (156) and even conversely (161).

Having demonstrated that Kitlga in the zebrafish follicle was expressed exclusively in the follicle cells and its expression was up-regulated by IGF-I, which signals via PI3K-Akt pathway, we were interested in identifying other signaling pathways in the follicle cells and the potential regulatory ligands that may also be

involved in controlling the expression of *kitlga*, which in turn signals the oocyte. To address this question, we examined the cyclic adenosine 3', 5'-monophosphate (cAMP) pathway, which is the major signaling pathway that mediates the actions of gonadotropins in the gonads.

Using pharmacological approaches and Western blot analysis, the present study investigated the roles of cAMP pathways in regulating the expression of *kitlga* in zebrafish ovarian follicle cells in the presence or absence of IGF-I. Our data provided strong evidence that cAMP may differentially control the expression of *kitlga* via different downstream pathways with cAMP-PKA stimulating and cAMP-Epac inhibiting its expression. In addition, experiments also suggested that both gonadotropins and pituitary adenylate cyclase activating polypeptide (PACAP) could be potential ligands that signal through such pathways to regulate *kitlga* expression.

5.2 Materials and Methods

5.2.1 Animals and chemicals

Zebrafish (*Danio rerio*) were obtained from a local tropical fish market and maintained in flow-through aquaria at 28 ± 1°C on a photoperiod of 14L:10D, with lights on at 8:00. The fish was fed twice a day with the commercial tropical fish feed Otohime S1 (Marubeni Nisshin Feed Co., Tokyo, Japan) and once with frozen artemia. All experiments performed were licensed by the Government of the Hong Kong Special Administrative Region and endorsed by the Animal Experimentation Ethics Committee of The Chinese University of Hong Kong. Unless otherwise indicated, all common chemicals used were purchased from Sigma (St. Louis, MO), USB Corporation (Cleveland, OH), GE Healthcare (Waukesha, WI), or Merck (Whitehouse Station, NJ); enzymes from Promega (Madison, WI); and culture medium from Gibco Invitrogen (Carlsbad, CA). hCG, IGF-I and forskolin were purchased from Sigma,

dibutyryl-cAMP (db-cAMP), H89, LY294002 and wortmannin from Calbiochem (La Jolla, CA), and cAMP analogs Sp-6-Phe-cAMPS (6-Phe-cAMP) and Sp-8-pCPT-2'-O-Me-cAMPS (8-CPT-cAMP) were from BIOLOG Life Science Institute (Bremen, Germany). hCG, IGF-I, db-cAMP and 6-Phe-cAMP were first dissolved in water, and forskolin, H89, LY294002, wortmannin and 8-CPT-cAMP in dimethylsulfoxide (DMSO). They were diluted to the desired concentrations with the medium before use. Antibodies for Akt, phosphor-Akt (Ser473) were from Cell Signalling Technology (Danvers, MA), and HRP-linked anti-rabbit IgG from Santa Cruz (Santa Cruz, CA).

5.2.2 Total RNA isolation and RT

Total RNA was extracted from cultured follicle cells with Tri-Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacture's protocol and our previous study (101). The RT was then performed at 37°C for 2 h in a volume of 10 μl containing 0.5 μg of oligo(dT), 1× M-MLV RT buffer, 0.5 mM each deoxyribonucleotide triphosphate (dNTP), 0.1 mM dithiothreitol, and 100 U of M-MLV RT (Invitrogen, Carlsbad, CA).

5.2.3 Quantification of kitlga mRNA by real-time qPCR

Real-time quantitative PCR was performed to quantify the expression of *kitlga*. The template for the standard curve was prepared by PCR amplification of cDNA fragment with specific primers. After purification with a PCR Purification Kit (Qiagen, Valencia, CA), the amplified DNA amplicons were quantified with the software Quantity One (Bio-Rad, Hercules, CA) using the Mass Ruler DNA marker (MBI Fermentas, Hanover, MD) as the standard, and the copy numbers of the DNA molecules were calculated before use as templates to construct standard curves in

real-time quantitative PCR. All PCR reactions were performed in a total volume of 30 μl containing 10 μl template (RT reaction mix diluted at 1:15), 1× PCR buffer, 0.2 mM each dNTP, 2.5 mM $MgCl_2$, 0.75 U of Taq polymerase, 0.5× EvaGreen (Biotium, Hayward, CA), and 20 nM fluorescein (Bio-Rad) on the iCycler iQ Real-time PCR Detection System (Bio-Rad). The amplification protocol was 30 sec at 94°C, 30 sec at 60°C, and 30 sec at 72°C, with a signal detection period of 7 sec at 80°C. A melt curve analysis was performed at the end of the reaction to check the reaction specificity.

5.2.4 Primary follicle cell culture

The primary follicle cell culture of zebrafish ovary was performed according to our previous report (101). Briefly, the ovaries from about 20 female zebrafish were isolated and dispersed in a 100-mm petri dish containing 60% Leibovitz L-15 medium (Invitrogen). The full-grown follicles were removed by sieving, and the follicles of earlier stages were washed five times with medium M199 (Invitrogen). Afterwards, the follicles were cultured in M199 supplemented with 10% fetal calf serum (Hyclone, Logan, UT) at 28 °C in 5% CO_2 for 6 days for the follicle cells to proliferate. Then, the follicle cells were harvested by trypsinization and plated in 24-well plates at a density of about 2.5×10^5 cells per well for 24 h. The cells were starved with M199 without serum for 24 h before treatment.

5.2.5 Western blotting

The cells were lysed by adding 1× SDS sample buffer (62.5 mM Tris-HCl, pH 6.8 at 25°C, 1% w/v SDS, 10% glycerol, 5% 2-mercaptoethanol, 100 μl per well of 24-well plate). Then the plate was shaken immediately for a few times and the extract from each well transfered to a microcentrifuge tube. All samples were heated to

95–100°C for 5 minutes, cooled on ice and microcentrifuged for 5 minutes. Western blotting was performed according to the manufacture's protocol (Cell Signalling Technology, Danvers, MA). Briefly, samples (about a half for 24-well plate) were loaded and separated in the 12.5% SDS–PAGE gel in 1× running buffer (25 mM Tris base, 0.2 M glycine, 0.1% w/v SDS), followed by blotting to the nitrocellulose membrane (Bio-Rad) using blotting buffer (25 mM Tris base, 0.2 M glycine, 20% methanol). The membrane was incubated in 25 ml blocking buffer (1× TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk) for 1 h at room temperature and then incubated in 5 ml of diluted primary antibody (1:1000 in blocking buffer) at 4°C overnight. The membrane was washed three times for 5 min each with wash buffer (1× TBS, 0.1% Tween-20) and then incubated with HRP-conjugated secondary antibody (1:2000 in wash buffer) for 1 h at room temperature. The membrane was then washed again and equilibrated with the developing solution (Western Blotting Luminol Reagent; Santa Cruz Biotechnology, Santa Cruz, CA). The signals were detected on the Lumi-Imager F1 Workstation (Roche, Mannheim, Germany).

5.2.6 Data analysis

The ratio of expression levels of *kitlga* to that of the internal control *ef1a* was calculated and then expressed as the fold change compared with the control or reference group. All values were expressed as the mean ± SEM, and the data were analyzed by one-way ANOVA, followed by Dunnett test using Prism 5 on Macintosh OS X (GraphPad Software, San Diego, CA).

5.3 Results

5.3.1 cAMP is involved in regulating kitlga expression in zebrafish follicle cells

We have characterized PI3K and Akt as the mediators of IGF-I-induced *kitlga*

expression (Chapter 4). To provide evidence for roles of cAMP pathway in the regulation of *kitlga* expression, we first tested the effect of dibutyryl cAMP (db-cAMP), a membrane-permeable analog of cAMP, in cultured zebrafish ovarian follicle cells. As shown in Fig. 5-1, treatment of the follicle cells with db-cAMP for 6 h significantly suppressed *kitlga* expression in a dose-dependent manner with the maximal inhibition achieved at the concentration of 1.5 mM. In agreement with the effect of db-cAMP, treatment of the cells with foskolin, a potent activator of adenylate cyclase, also dose-dependently inhibited the expression of *kitlga* (Fig. 5-2).

5.3.2 PKA mediates a stimulatory effect on kitlga expression

As the major intracellular mediator of cAMP signaling, PKA is responsible for most of cAMP activities in many cells. To examine if PKA was involved in cAMP-inhibited *kitlga* expression in zebrafish follicle cells, we examined *kitlga* expression in the presence of H89, a specific PKA inhibitor, in cultured zebrafish follicle cells. Surprisingly, H89 significantly reduced basal *kitlga* mRNA level in a dose-dependent manner (Fig. 5-3A). Furthermore, when the cells were treated with H89 together with forskolin, the level of *kitlga* mRNA was even lower than those in the cells treated with H89 or forskolin alone (Fig. 5-3B). These results suggested that instead of increasing *kitlga* expression level as would be expected for mediating the inhibitory effect of cAMP, PKA might exert a stimulatory impact on *kitlga* expression. To confirm this, we then tested the effect of 6-Phe-cAMP, a cAMP analog that specifically activates PKA. As shown in Fig. 5-4, treatment of the follicle cells with 6-Phe-cAMP significantly increased the level of *kitlga* mRNA in a clear dose-dependent manner with the maximal effect observed at 50 μM.

5.3.3 cAMP inhibition of kitlga expression is likely mediated by Epac

Having demonstrated that PKA was not responsible for cAMP-induced *kitlga* suppression, we then turned our attention to Epac, another potential cAMP mediator in cells. In contrast to PKA, the activation of Epac with 8-CPT-cAMP, a specific activator of Epac, significantly suppressed *kitlga* expression (Fig. 5-5).

5.3.4 Cross-talk between cAMP-PKA and IGF-I/PI3K/Akt pathways in stimulating kitlga expression

We have previously demonstrated that IGF-I stimulated *kitlga* expression in zebrafish follicle cells via PI3K-Akt pathway (Chapter 4). To investigate whether the cAMP-PKA pathway interacts with the IGF-I/PI3K-Akt pathway in stimulating *kitlga* expression, we carried out a series of experiments. As shown in Fig. 5-6A, forskolin significantly reduced basal *kitlga* mRNA level and its presence also completely abolished IGF-I-stimulated *kitlga* expression. However, although H89 also reduced basal level as described above, it did not affect the responsiveness of the cells to IGF-I, which could increase *kitlga* expression in the absence or presence of H89 (Fig. 5-6B). On the other hand, when we blocked the PI3K-Akt pathway and PKA simultaneously with LY249002 and H89, there was a further decrease in *kitlga* expression although it was not statistically significant compared to the effect of LY249200 or H89 alone (Fig. 5-7).

To understand the role of Akt in PKA stimulation of *kitlga* expression, we performed a Western blot analysis for Akt phosphorylation in response to IGF-I (positive control) and PKA inhibition by H89. As expected, IGF-I significantly enhanced Akt phosphorylation in zebrafish follicle cells, and its effect could be abolished by LY249002 and wortmannin. H89 alone exhibited no effect on Akt phosphorylation; however, it significantly enhanced IGF-I stimulated Akt phosphorylation (Fig. 5-8).

5.3.5 Gonadotropins and PACAP are potential ligands that mimic the effect of cAMP on kitlga expression in the follicle cells

Given that LH and PACAP primarily signal through cAMP in ovarian follicles, especially in late folliculogenesis (162-165), we attempted to find out whether they could be the ligands that mobilize the cAMP pathways to regulate *kitlga* expression. As an analog of LH in the zebrafish, hCG dose-dependently reduced both basal and IGF-I-stimulated *kitlga* expression although its effect on the basal level was not statistically significant (Fig. 5-9). Similarly, PACAP also suppressed IGF-I-stimulated *kitlga* expression despite a lack of effect on the basal level (Fig. 5-10).

5.4 Discussion

As the major intracellular second messenger for pituitary gonadotropins, cAMP regulates a number of cellular processes in the ovary, such as proliferation and differentiation of granulosa cells, oocyte growth, maturation and ovulation (166-168). In the present study, we demonstrated by using cAMP analog or adenylate cyclase activator that cAMP evidently suppressed the expression of Kit ligand A in zebrafish ovarian follicle cells. In agreement with this, hCG and PACAP, which both use cAMP as the major signaling second messenger, also suppressed IGF-I-induced *kitlga* expression in zebrafish follicle cells, suggesting that they might be signaling through the cAMP pathway. The physiological relevance of hCG and PACAP-induced suppression of IGF-I-stimulated *kitlga* is unknown at this moment. Since both hCG and PACAP have been reported to enhance final oocyte maturation in the zebrafish (99, 169), one possibility is that the down-regulation of *kitlga* in the follicle cells may be part of the mechanisms by which hCG and PACAP promote the maturation. It has been reported in mammals that oocyte maturation (GVBD) is accompanied by a shift

of KIT ligand expression in the granulosa cells from membrane-bound to soluble form and a loss of expression of both forms of KIT ligand in the cumulus cells, and that the presence of KIT ligand delayed GVBD or resumption of meiosis (59). It can be hypothesized that in the zebrafish the preovulatory LH surge may increase cAMP level in the follicle cells to suppress IGF-I-induced expression of *kitlga*, leading to the resumption of meiosis. PACAP, as a local ovarian factor, may amplify the inhibitory effects of pituitary-derived LH on *kitlga* as we have previously reported that PACAP expression in zebrafish follicle cells is highly responsive to hCG (169).

In addition to the suppression of oocyte maturation, it has been demonstrated in mammals that KIT ligand enhances the proliferation of granulosa cells (48, 53, 113) and promotes oocyte growth (50, 51, 102, 170). During the transition of the follicle from primordial to antral stage in mice, however, there is a cessation of KIT ligand expression in the cumulus granulosa cells (35, 78). Oocytes are generally thought to complete their growth phase near the transition and the granulosa cells will exit from the cell cycle when follicles progress to preovulatory stage (167, 171). Thus, an alternative hypothesis is that the expression of KIT ligand is suppressed by elevated cAMP induced by LH, which leads to the cessation of oocyte growth and granulosa cell proliferation prior to final maturation and ovulation.

We have demonstrated that IGF-I stimulates the expression of *kitlga* through PI3K-Akt pathway and blockade of PI3K and Akt activities by LY249002 and Akt inhibitors decreases both basal and IGF-I-induced expression of *kitlga* (Chapter 4). To investigate if the cAMP-induced suppression of *kitlga* expression involves protein kinas A (PKA), we examined the effect of H89, a potent PKA inhibitor, on basal and forskolin-inhibited *kitlga* expression. To our surprise, instead of reversing the suppressive effect of forskolin, H89 also reduced *kitlga* expression alone and the suppression became even stronger in combination with forskolin. This strongly

suggests that instead of mediating cAMP-induced suppression of *kitlga* expression, PKA might even stimulate its expression. This idea was supported by the evidence that 6-Phe-cAMP (a specific PKA activator) alone significantly increased *kitlga* expression. Paradoxically, although PKA might directly stimulate *kitlga* expression, it seemed to suppress IGF-I-stimulated Akt activity as shown by the enhanced Akt phosphorylation in the presence of H89. We have therefore suggested that Akt may play a key role in the regulation of *kitlga* expression. Given that hCG and PACAP alone had littlte effect on *kitlga* expression but significantly suppressed IGF-I-induced *kitlga* expression, we hypothesize that hCG and PACAP may exert their effects through the cAMP pathway, which in turn influences the PI3K-Akt pathway. Forskolin completely blocked the effects of IGF-I, further supporting a cross-talk between the two pathways. Interestingly, the classical cAMP mediator PKA exhibited a stimulatory effect since its inhibitor, H89, suppressed and its activator, Sp-6-Phe-cAMPS, promoted *kitlga* expression. However, when IGF-I and H89 were applied together, the IGF-I-induced phosphorylation level of Akt further increased significantly, suggesting that cAMP may block the activation of Akt via PKA in the follicle cells. In many other cell types, cAMP has been reported to inhibit Akt activity. In transiently transfected COS cells, cAMP pathway negatively regulates PDK1 by inhibiting its translocation to the plasma membrane and inhibits the lipid kinase activity of PI3K to decrease the levels of phosphatidylinositol 3,4,5-triphosphate (PIP_3) in vivo, which are required for the membrane localization of PDK1 (172). In PCCL3 thyroid follicular cells, cAMP inhibits Akt activity via phosphorylation of Rap1b by PKA (173). Thus, it is possible that PKA stimulates *kitlga* expression in zebrafish follicle cells through a more potent pathway to compensate its negative effects on Akt, which increases *kitlga* expression. Also, as the basal Akt activity is very low as demonstrated by western blot, its inhibition by PKA in the absence of IGF-I might be

neglectable.

It is worth noting that cAMP had a net negative effect on *kitlga* expression, but the net effect of PKA was positive to *kitlga* expression despite its inhibition of Akt phosphorylation. It is therefore possible that cAMP may exert its inhibitory action through a PKA-independent pathway. In recent years, Epac has been demonstrated to be another cAMP-activated protein (158, 159). Our study showed that Epac down-regulated the expression of *kitlga*. As a guanine exchange factor, Epac is able to activate Rap1, which in turn modulates other pathways (174). There is evidence that cAMP-Epac-Rap1 pathway negatively regulates Akt activity by increasing dephosphorylation of Akt, which is mediated by PP2A, an Akt phosphatase (160). Epac-activated Rap1 also activates MAPK by binding Raf, which is an upstream kinase of MEK1/2. MAPK pathway has been found to suppress the expression of Kit ligand in the follicle cells (Chapter 4). Thus, both Akt dephosphorylation and MAPK activation caused by cAMP-Epac-Rap1 pathway may be responsible for diminishing the expression of *kitlga* in zebrafish follicle cells. More studies will be carried out to test this hypothesis.

In mammals, folliculogenesis is dependent on FSH and LH after follicles progress beyond preantral stage. Interestingly, FSH promotes massive growth and proliferation of granulosa cells in the preantral follicle by regulation of genes involved in cell cycle and stimulates androgen aromatization via activation of the cAMP-signaling cascade (167, 171). Conversely, through activation of seemingly identical cAMP signaling in the mature follicle, LH promotes the exit of granulosa cells from the cell cycle and the suppression of aromatase expression and androgen aromatization (167, 171). It has been postulated that their diverse biological activities may be exerted by different cell context at specialized developmental stage. The present study provided evidence for multiple signaling mechanisms downstream of

cAMP. It is likely that different signal transduction pathways or components have spatial and temporal variation in abundance or activity during folliculogenesis, leading to different responses of the cells to gonadotropins at different developmental stages. In mammals, FSH and dibutyryl cyclic AMP have been demonstrated to promote the expression of *Kitl* in granulosa cells from preantral follicles (37, 50). hCG, however, decreases the expression of *Kitl* in cumulus cells of preovulatory follicles (59). Therefore, an interesting hypothesis is that the relative abundance of PKA and Epac at different stages may determine cAMP-mediated modulation of Kit ligand expression in granulosa cells, regardless of the derivation of cAMP either from FSH or LH. Further studies are required to elucidate the underlying mechanism for involvement of cAMP in Kit ligand expression by different mediators.

In summary, cAMP is involved in regulating the expression of *kitlga* in zebrafish follicle cells. Two cAMP-activated effectors, PKA and Epac, have reverse effects. PKA promotes but Epac inhibits the expression of *kitlga*, which was identified by the respective activator. The effect of forskolin and H89 on IGF-I-induced expression of *kitlga* suggests a cross-talk between the two signaling pathways. Both hCG and PACAP inhibit IGF-I-induced *kitlga* expression, indicating that they may have negative regulation through cAMP signaling pathways in the full-grown follicles. A schematic outline of the signaling pathways analyzed in Chapter 4 and 5 is summarized in Fig. 5-11.

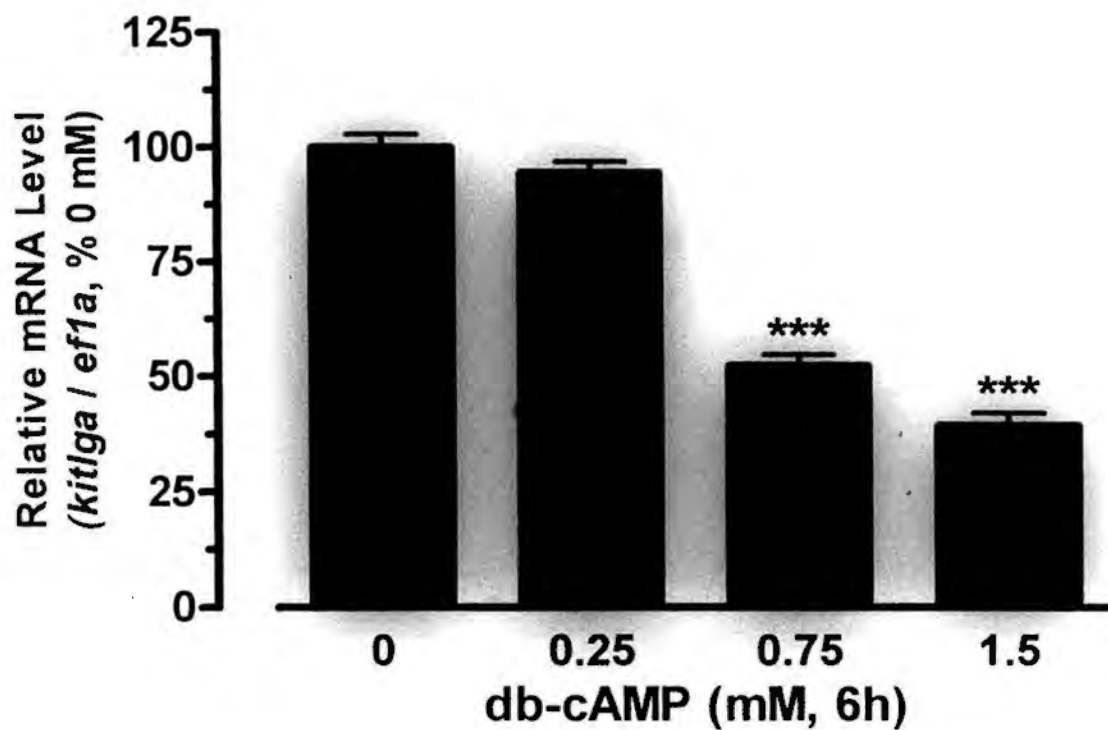


Fig. 5-1 Dose response of db-cAMP effect on the expression of *kitlga* in cultured zebrafish follicle cells at 6 h of the treatment. The graph is the data normalized to the housekeeping gene *ef1a* and expressed as the percentage or fold change of the control group (mean ± SEM, $n = 4$). *** $P < 0.001$ vs. control.

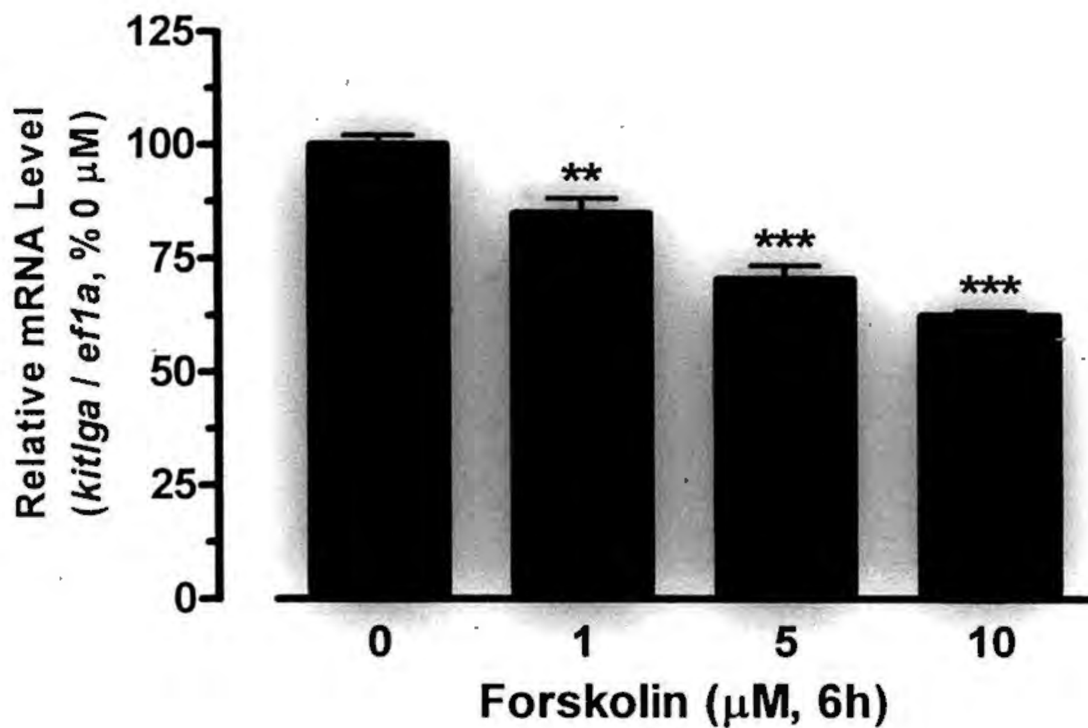
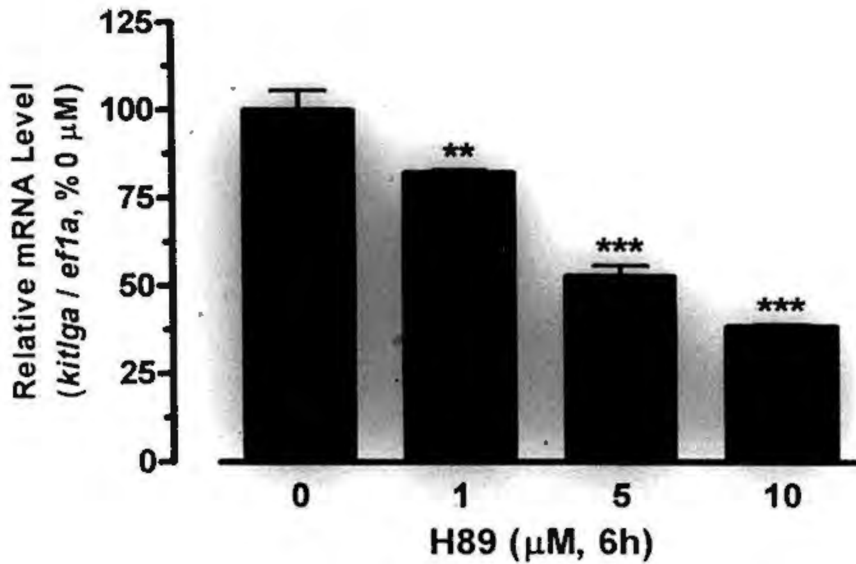


Fig.5-2 Dose response of forskolin effect on the expression of *kitlga* in cultured zebrafish follicle cells at 6 h of the treatment. The graph is the data normalized to the housekeeping gene *ef1a* and expressed as the percentage or fold change of the control group (mean ± SEM, $n = 4$). ** $P < 0.01$; *** $P < 0.001$ vs. control.

A



B

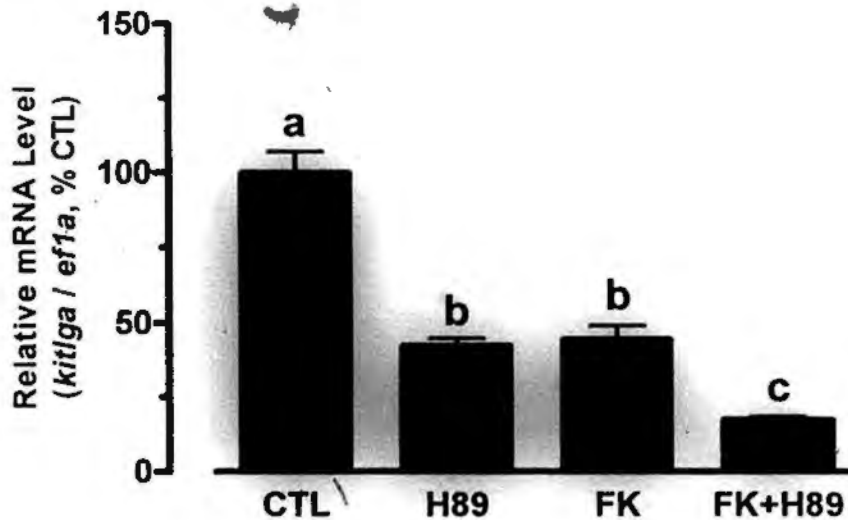


Fig. 5-3 Effects of PKA-dependent and independent pathways on *kitlga* expression. (A) Dose response of H89 effect on the expression of *kitlga* in cultured zebrafish follicle cells at 6 h of the treatment. ** $P < 0.01$; *** $P < 0.001$ vs. control. (B) Effect of forskolin on *kitlga* expression in the presence of H89. Cultured zebrafish follicle cells were treated with H89 (10 μM) or forskolin (10 μM) or both for 6 h. Different letters indicate statistical significance ($P < 0.05$). The graph is the data normalized to the housekeeping gene *ef1a* and expressed as the percentage or fold change of the control group (mean ± SEM, $n = 4$).

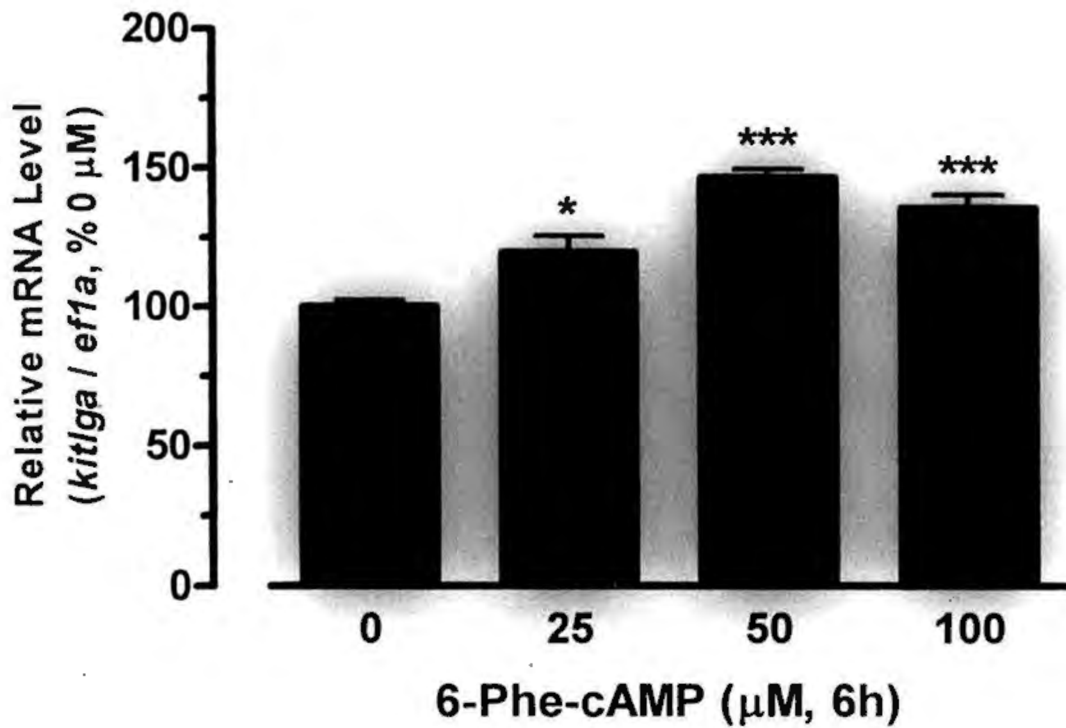


Fig. 5-4 Dose response of 6-Phe-cAMP effect on the expression of *kitlga* in cultured zebrafish follicle cells at 6 h of the treatment. The graph is the data normalized to the housekeeping gene *ef1a* and expressed as the percentage or fold change of the control group (mean ± SEM, $n = 4$). * $P < 0.05$; *** $P < 0.001$ vs. control.

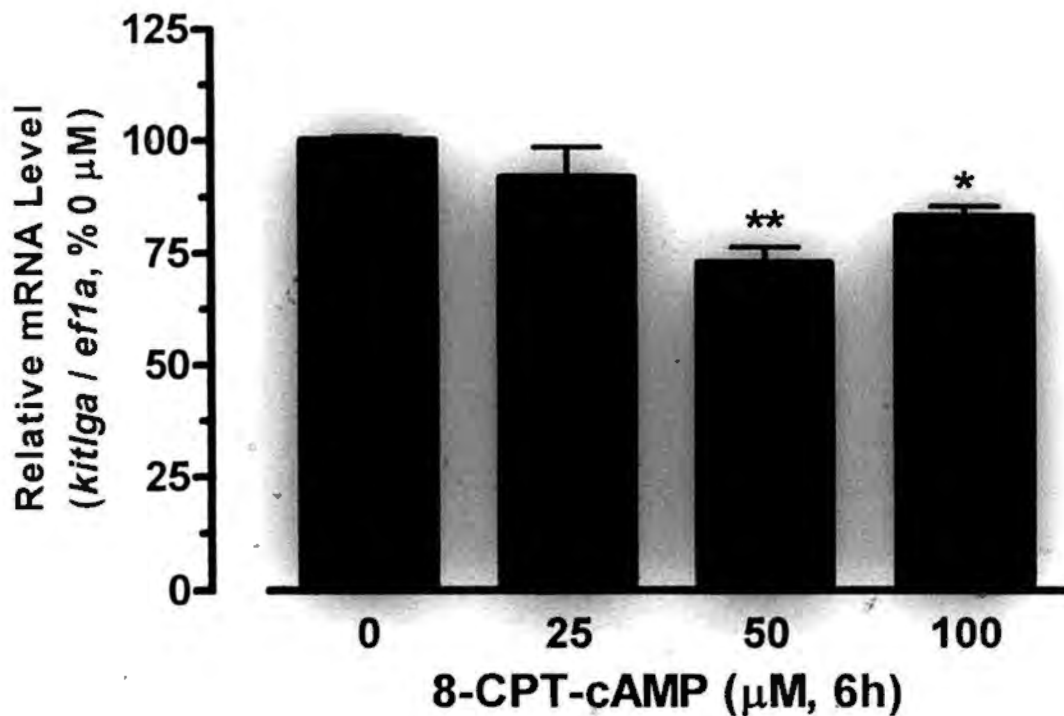
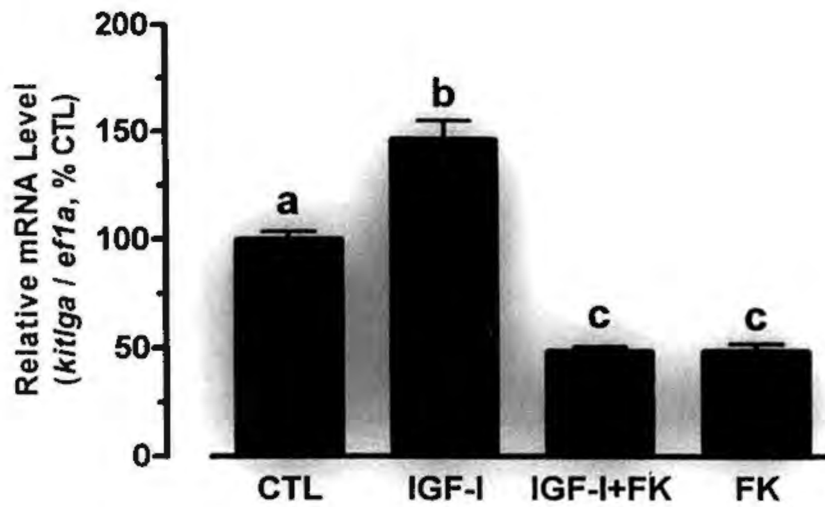


Fig. 5-5 Dose response of 8-CPT-cAMP effect on the expression of *kitlga* in cultured zebrafish follicle cells at 6 h of the treatment. The graph is the data normalized to the housekeeping gene *ef1a* and expressed as the percentage or fold change of the control group (mean ± SEM, $n = 4$). * $P < 0.05$; ** $P < 0.01$ vs. control.

A



B

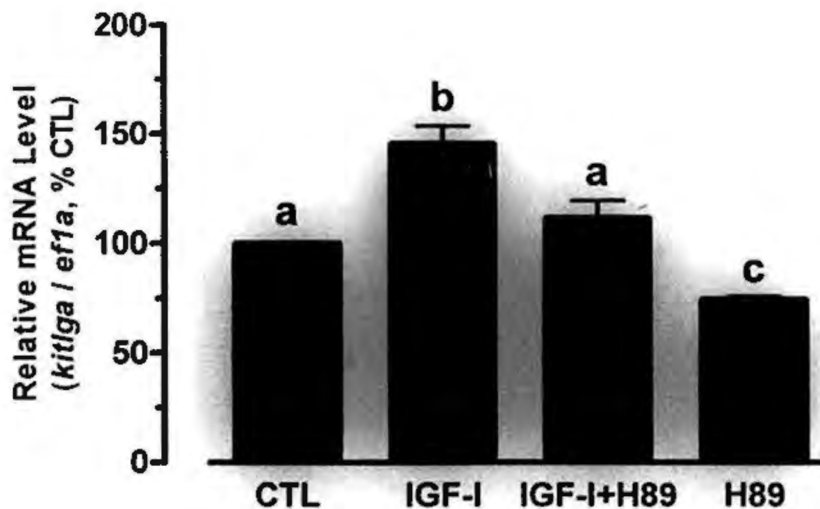


Fig. 5-6 Effects of cAMP (A) and PKA (B) on IGF-I-induced *kitlga* expression. Cultured zebrafish follicle cells were pretreated with forskolin (10 μM) or H89 (10 μM) for 30 min before treatment of IGF-I (50 ng/ml) for 6 h. The graph is the data normalized to the housekeeping gene *ef1a* and expressed as the percentage or fold change of the control group (mean ± SEM, $n = 4$). Different letters indicate statistical significance ($P < 0.05$).

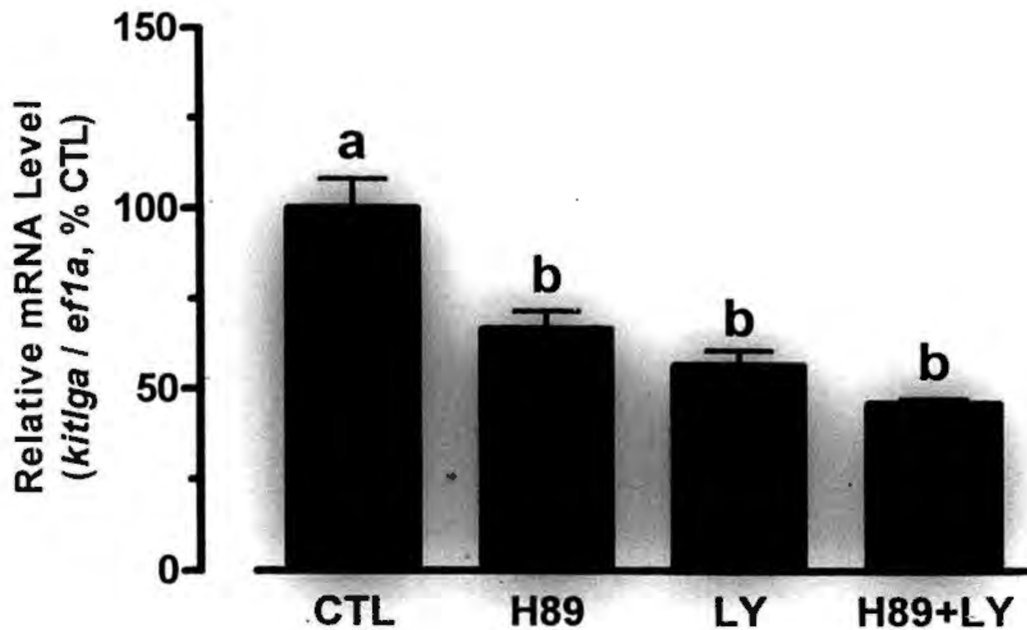


Fig. 5-7 Interactive effects of PKA and PI3K-Akt pathway on *kitlga* expression. Cultured zebrafish follicle cells were treated with H89 (10 μM) or LY294002 (50 μM) or both for 6 h. The graph is the data normalized to the housekeeping gene *ef1a* and expressed as the percentage or fold change of the control group (mean ± SEM, $n = 4$). Different letters indicate statistical significance ($P < 0.05$).

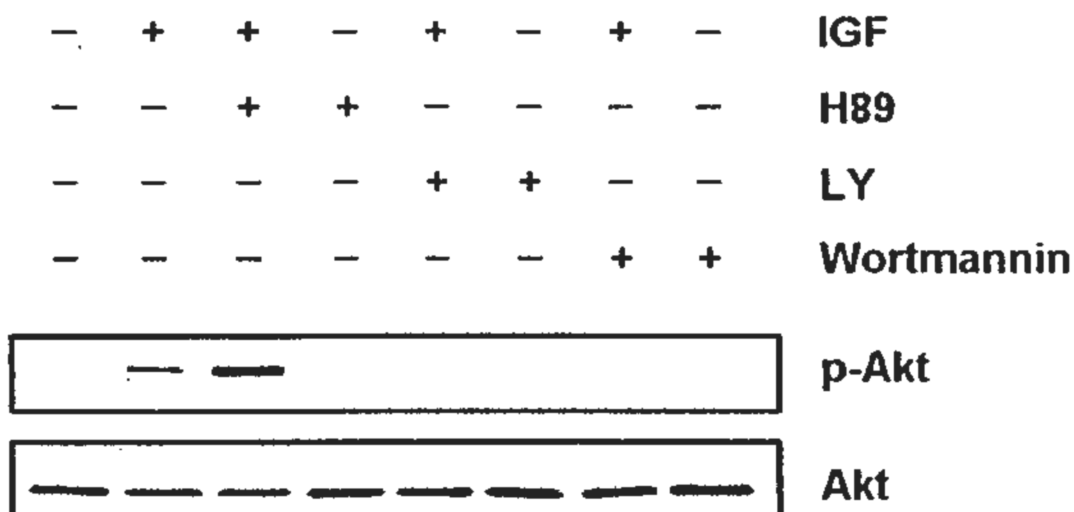


Fig. 5-8 Interactive effects of PKA and IGF-I on Akt phosphorylation. Cultured zebrafish follicle cells were pretreated with H89 (10 μM), LY294002 (50 μM) or wortmannin (500 nM) for 30 min before treatment of IGF-I (50 ng/ml) for 30 min. Proteins were extracted at the end of the treatment for Western blot analysis.

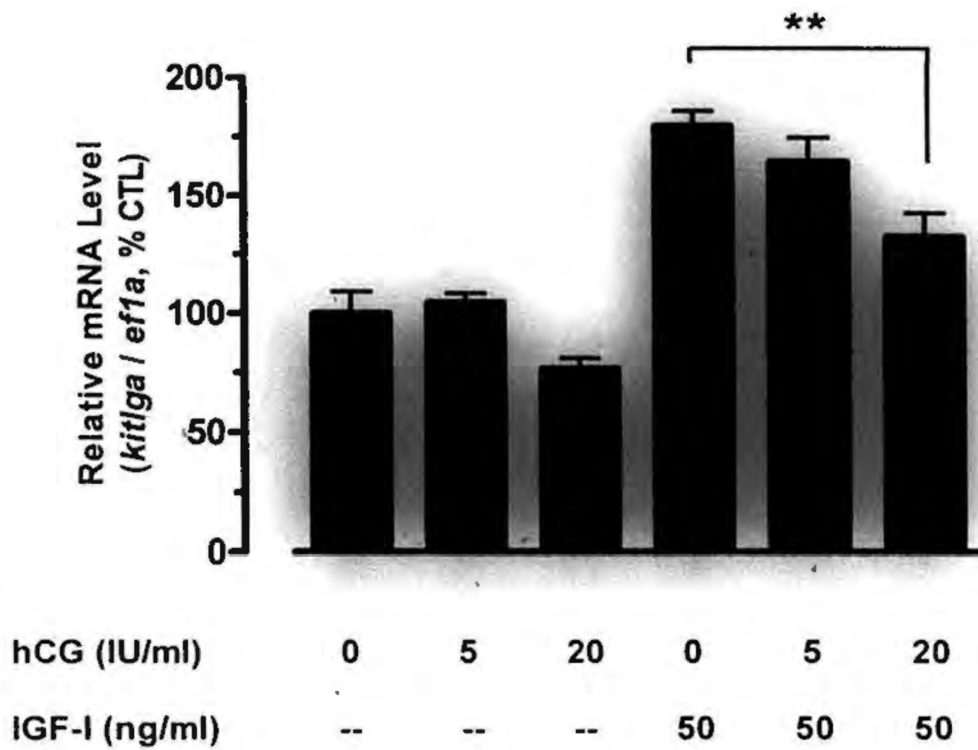


Fig. 5-9 Dose response of hCG effects (6 h) on basal and IGF-I-regulated expression of *kitlga* in cultured follicle cells. The graph is the data normalized to the housekeeping gene *ef1a* and expressed as the percentage or fold change of the control group (mean ± SEM, $n = 4$). ** $P < 0.01$ vs. IGF-I-alone group.

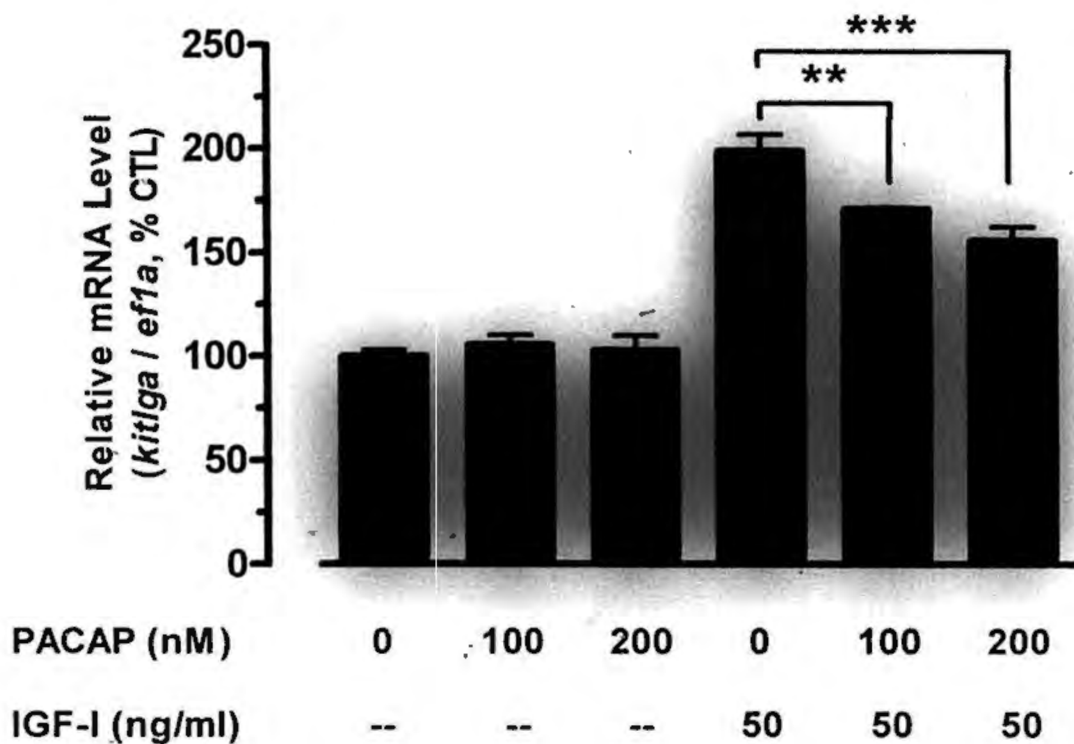


Fig. 5-10 Dose response of PACAP effects (6 h) on basal and IGF-I-regulated expression of *kitlga* in cultured follicle cells. The graph is the data normalized to the housekeeping gene *ef1a* and expressed as the percentage or fold change of the control group (mean ± SEM, $n = 4$). ** $P < 0.01$; *** $P < 0.001$ vs. IGF-I-alone group.

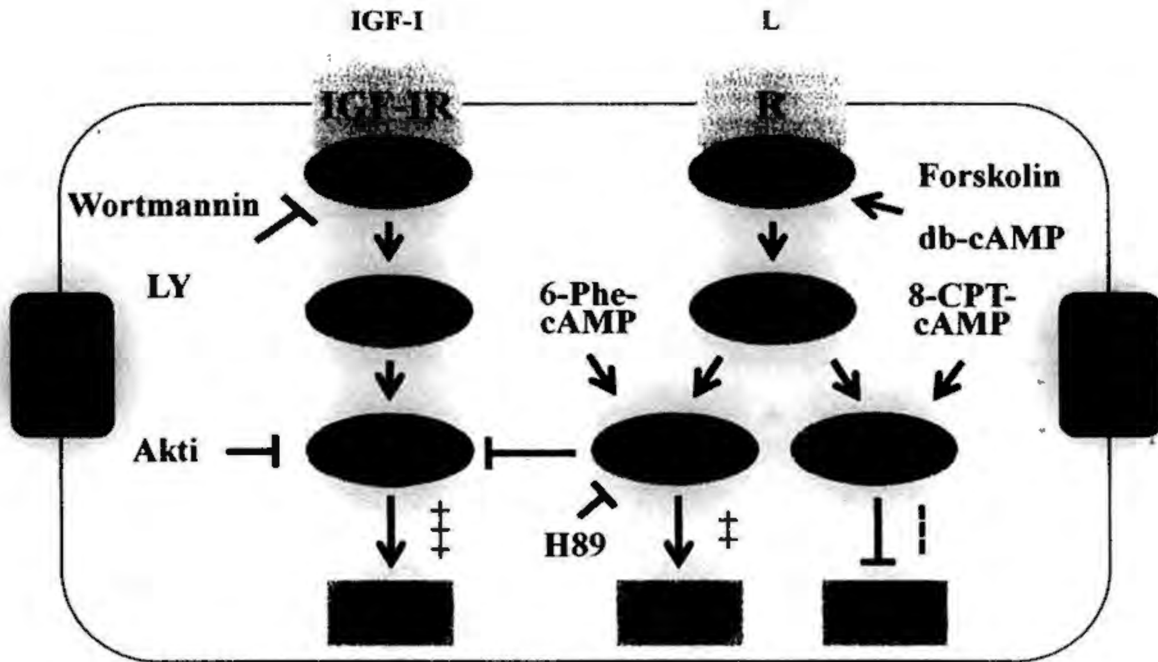


Fig. 5-11 A summary of signaling pathways analyzed in Chapter 4 and 5. IGF-I promotes the expression of *kitlga* through PI3K-Akt pathway. cAMP is produced to decrease through Epac but increase through PKA *kitlga* expression in cultured follicle cells. Plus and minus indicate up-regulation and down-regulation of *kitlga* expression, respectively.

Chapter 6

General Discussion

Based on our studies on the Kit system, we speculate that the Kit system may be extensively involved in the recruitment, growth and final maturation of follicles in the zebrafish ovary.

Different from mammals, there are four members belonging to Kit system in zebrafish. It includes two ligands, *kitlga* and *kitlgb*, and two receptors, *kita* and *kitb* which are likely derived from the third genome duplication by phylogenetic and synteny analyses (Chapter 2). After genome duplication, the duplicated genes might have different fates: nonfunctionalization, subfunctionalization, and neofunctionalization (88-90, 93). The expression profiles of Kit system members during folliculogenesis, particularly in the periovulatory period, have implied the functional divergence of the duplicated genes. The analysis of distribution of the Kit system members in the ovary revealed that *kitlga* and *kitb* are exclusively expressed in the follicle layer while *kitlgb* and *kita* only in the oocyte. It is therefore suggested that the duplicated genes in Kit system may be neofunctionalized. Furthermore, we further analyzed the binding specificity between ligands and receptors both in mammalian cell lines and in zebrafish primary follicle cells. The results suggested that two opposite paracrine pathways are formed in the ovary. One is between Kitlga and Kita, which may represent a signaling from the follicle cells to the oocyte. Another is between Kitlgb and Kitb, which may represent the regulation in an opposite direction. In comparison with the situation in mammals, *Kitl* in the granulosa cells and *Kit* in the oocyte and theca cells, neofunctionalization may most likely have occurred for Kitlgb-Kitb.

Follicle activation or recruitment from the remnant pool in female reproductive cycle has been an interesting and intricate issue in research for a long time. It has been well documented that many local and systemic growth factors are responsible for this process including insulin, IGF-I and PDGF. Recently, increasing evidence from studies in mammalian models also points to Kit ligand as another candidate involved in the process (48). In the zebrafish ovary, our studies also support a role for Kit system in the early stage of folliculogenesis. Firstly, the expression of *kitlga* significantly increases in the transition from primary to secondary growth (PG-to-PV transition) (Chapter 2), which implies a potential function for Kitlga in this special period. Secondly, the expression of *kitlga* is strongly and directly promoted by IGF-I, an important component of the somatotrophic axis (Chapter 4).

The effects of signals from the somatotropic axis on the reproductive axis have been reported in many species including fish (144). A preliminary study in our laboratory recently showed that IGF-I could advance the onset of puberty in female zebrafish (Chen and Ge, unpublished data). We have recently reported that IGF-I has stimulatory effects on the expression of FSH β subunit (*fshb*) in the pituitary cells of zebrafish (144), which may partly account for its role in initiating puberty. FSH has been demonstrated to play important roles in initiating sexual maturation and reproductive cycle. The present study suggests that in addition to the pituitary, IGF-I may also directly act in the ovary and one of its potential effects is to increase the expression of Kit ligand A (*kitlga*) in the follicle cells whose specific receptor Kita is located in the oocyte. Therefore, the Kit system may likely serve as a bridge between the somatic follicle cells and the oocyte for IGF-I to signal the oocyte for follicle activation and recruitment. Considering the temporal expression profile of *kitlga* in folliculogenesis, its up-regulation by IGF-I, and studies in mammals, we hypothesize that at the onset of zebrafish puberty when the body growth accelerates, IGF-I may

exert a direct influence in the ovary by enhancing the expression of *kitlga* in the follicle cells, leading to the initiation of sexual maturation. Afterwards, IGF-I further promotes the reproductive cycle by increasing the output of FSH from pituitary and the expression of FSH receptor in the ovary (131, 144, 175). Therefore, the Kit system may play a vital role in mediating the signals from the somatotropic axis to reproductive axis.

Following the activation, the follicles are driven into fast secondary growth stage, which is termed vitellogenesis in teleosts. Many physiological events occur in this process, including theca cell recruitment and proliferation, granulosa cell proliferation, steroidogenesis, and oocyte growth, survival, meiotic arrest and final maturation. According to the reports in mammals, Kit system seems to be involved in all these events (127). Our findings showed that Kit system might also be associated with these events in the zebrafish ovary although our results are far from conclusive. As discussed above, Kitlga may play a role in folliculogenesis partly as a mediator or amplifier of IGF-I action. This is supported by some reports in mammals. For example, both IGF-I and KITL are involved in promoting proliferation of granulosa cells in mammals (113, 133). Considering the relatively high expression of *kitlga* in growing stages (including PV, EV and MV stages), we tempt to speculate that zebrafish *kitlga* may be also associated with the intial phase of follicle activation and growth, including oocyte growth and proliferation of granulosa cells. This issue will be further addressed in future studies.

In addition to mediating and amplifying the effects of IGF-I, the Kit system in the zebrafish ovary may also have other functions, one of which is the maintenance of oocyte meiotic arrest. In our previous study, we have demonstrated that the follicles of MV stage could also undergo GVBD in vitro if they were pre-treated with hCG or activin followed by treatment with DHP (101). In mammals, oocytes from antral

follicles also resume meiosis spontaneously in vitro (176). So the question is why these oocytes remain meiotic arrest in vivo although they are meiotically competent. The spontaneous maturation of oocytes in vitro suggests that there may be certain factors in the ovary that function as inhibitors of oocyte maturation before oocytes complete all events necessary to further development. We found that full-grown follicles incubated in vitro had much higher level of *kitlga* mRNA than that of follicles experiencing GVBD and that the expression of *kitlga* was down-regulated just before GVBD in vivo (Chapter 2). Furthermore, our preliminary data showed that full-grown follicles remained meiotically arrested when incubated with recombinant zebrafish Kitlga (data not shown). In mammals, when the oocytes in antral follicles undergo GVBD, Kit ligand mRNA and protein also dropped and disappeared from the cumulus cells surrounding the oocyte (59). Therefore, Kitlga may be a good candidate for the maintenance of oocyte meiotic arrest across vertebrates. Interestingly, we previously found that follistatin, an activin-binding protein, also suppressed GVBD and that its expression dropped significantly in full-grown stage prior to oocyte maturation (98). Different from activin whose subunits are exclusively expressed in the follicle layer, follistatin is mostly expressed in the oocyte as we recently reported (122). We thus speculate that the oocyte-derived follistatin may act as a barrier surrounding the oocyte to inhibit the action of activin until final maturation. The interesting questions that arise from these observations are what maintains the high expression level of follistatin in the oocytes before full-grown stage and what leads to the sharp decline of its expression prior to final maturation. Considering the functional relevance of Kitlga and follistatin to the final oocyte maturation, we wonder whether Kitlga plays a role in regulating follistatin expression in the oocyte by binding to Kita on the oocyte. If yes, the down-regulation of *kitlga* in full-grown follicles might serve to weaken the "protective role" of follistatin to oocytes, resulting

in LH-induced resumption of meiosis by activin. Thus, part of the future work will focus on the relationship between Kitlga from the follicle cells and follistatin in the oocyte.

Another characteristic of the Kit system in the zebrafish is the dual regulation of *kitlga* expression by cAMP. As demonstrated in Chapter 5, cAMP positively regulated *kitlga* expression by binding to PKA but negatively by binding to Epac, which prompt us to hypothesize that the ratio of PKA to Epac in follicle cells may determine the effects of gonadotropins on the expression of *kitlga*, leading to its differential response to gonadotropins at different stages of folliculogenesis. In mammals, FSH stimulates *Kitl* expression (37) and both of FSH and KITL promote the proliferation of granulosa cells in the growing follicles. In contrast, LH promotes the exit of granulosa cells from cell cycle, which is accompanied by the suppression of *Kitl* expression in the preovulatory follicles. Although both FSH and LH signal through cAMP in the follicle cells, the downstream pathways mobilized may be responsible for the differential functions of FSH and LH during folliculogenesis. We will further address this issue by examining the change of relative abundance of PKA and Epac in follicles throughout folliculogenesis.

In addition to *kitlga*, other members of Kit system in the zebrafish ovary also demonstrate distinct expression profiles in the periovulatory stages (Chapter 2). Particularly, the expression of *kitb* dramatically increases after entry into full-grown stage. We therefore have two questions: why does *kitb* increase expression at this stage and what triggers such an increase? Considering the localization of *kitb* in the follicle layer (Chapter 3), we studied its regulation in cultured follicle cells. We found the expression of *kitb* was significantly suppressed by GF109203x, a specific inhibitor of PKC (data not shown). Our future studies will focus on identification of regulatory factors that may signal through PKC in the late stages of folliculogenesis.

Our future work will concentrate on the following questions. First, how is the Kit system, particularly Kitlga, involved in the PG-to-PV transition and is it a predominant factor in this process? Second, considering the dramatic changes in expression, we want to determine what function the Kit system has during the periovulatory period, particularly in oocyte maturation, ovulation and fertilization. Third, in addition to the regulation of *kitlga*, we will further examine the regulation of other Kit system members.

In summary, we studied the expression, regulation and function of the Kit system in the zebrafish ovary. In Chapter 2, we proved the existence of two isoforms of Kit (*kita* and *kitb*) and Kit ligands (*kitlga* and *kitlgb*) in the follicle and provided evidence for their functional divergence during folliculogenesis. Based on these findings, we further studied the spatial localization of Kit system in the follicle and the interaction between ligands and receptors. We found *kitlga* and *kitb* were localized in follicle cells while *kitlgb* and *kita* in oocytes. Kitlga preferred to activate Kita rather than Kitb. In contrast, Kitlgb significantly activated Kitb without any effects on Kita. These observations showed that as part of the complex communication network in the follicle, the Kit system may play important roles in mediating bidirectional signaling from follicle cells to oocyte via Kitlga/Kita and from oocyte to follicle cells via Kitlgb/Kitb. Finally, as the first step to study the regulation of Kit system, we found that the expression of *kitlga* was subject to up-regulation by IGF-I through Akt. On the other hand, cAMP seemed to have dual effects on the expression of *kitlga* through different mediators. It promoted *kitlga* expression through PKA but suppressed it via Epac. In all, we have systematically investigated the Kit system in the zebrafish ovary on its expression, regulation, and function, which represents a significant contribution to our understanding of the regulatory network in the ovary that underlies the control of folliculogenesis in vertebrates.

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