mRNA EXPRESSION OF BONE MORPHOGENETIC PROTEIN 7 (*bmp7*) DURING FOLLICULOGENESIS IN ZEBRAFISH

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by

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LIST OF ABBREVIATIONS

ActR	Activin receptor
ALK	activin receptor-like kinase
AMH	anti-Müllerian hormone
ANOVA	analysis of variance
BMP	bone morphogenetic protein
bmp7	bone morphogenetic protein 7
BMPR	bone morphogenetic protein receptor
$CaCl_2$	calcium chloride
cAMP	cyclic adenosine monophosphate
cdc2	cell division control
cDNA	complementary deoxyribonucleic acid
Co-Smad	common Smad
C_T	threshold cycle
DEPC	diethylpyrocarbonate
dH_2O	distilled water
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleoside triphosphate
dpf	day post-fertilization
ef1α	elongation factor 1-alpha
FSHR	follicle-stimulating hormone receptor
GC	guanine-cytosine
GDF	growth differentiation factor
Gi	inhibitory G-protein
GJ	gap junction
GnRH	gonadotropin releasing hormone
GS	glycine-serine
GV	germinal vesicle
GVBD	germinal vesicle breakdown
h2afz	H2A histone family, member Z
hCG	human chorionic gonadotropin
3β-HSD	3β-hydroxysteroid dehydrogenase
17β-HSD	17β-hydroxysteroid dehydrogenase
20β-HSD	20β-hydroxysteroid dehydrogenase
HSD	honestly significantly different
IPTG	isopropyl β-D-thiogalactopyranoside
LB	Luria-Bertani
LCGR	lutropin/choriogonadotropin receptor
LHCGR	choriogonadotropin receptor
LHR	luteinizing hormone receptor
$MgCl_2$	magnesium chloride
MIH	maturation inducing hormone
MIS	Müllerian inhibiting substance
MMLV-RT	Moloney murine leukemia virus – reverse transcriptase
MOPS	4-morpholinepropanesulfonic acid
MPF	maturation promoting factor

mPR	membrane bound progestin receptor
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
nPR	nuclear progestin receptor
oP450arom	P450 aromatase
OMC	oocyte maturational competence
17,20βP	17α,20β-dihydroxy-4-pregnen-3-one
PCR	polymerase chain reaction
P450arom	P450 aromatase
P450c17	17α-hydroxylase/C17-C20 lyase
P450scc	P450 side-chain cleavage
pH	potential hydrogen
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
R-Smad	regulated Smad
RT-PCR	reverse transcription polymerase chain reaction
20β-S	17,20β-21-trihydroxy-4-pregnen-3-one
SEM	standard error of mean
SPSS	Statistical Package for the Social Sciences
StAR	steroidogenic acute regulatory protein
TGF-β	transforming growth factor-β
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

PENGEKSPRESAN mRNA GEN TULANG MORFOGENETIK PROTEIN 7 (*bmp7*) SEMASA FOLIKULOGENESIS DALAM IKAN ZEBRAFISH

ABSTRAK

Peranan keupayaan tentang tulang morfogenetik protein 7 (*bmp7*) dalam ovari kian menarik perhatian. Kajian ini bertujuan untuk mengkaji corak pengekspresan *bmp7a* dan *bmp7b* di dalam ikan zebrafish. Tisu betina dewasa dikorbankan untuk memperolehi otak, mata, insang, jantung, usus, ginjal, ovari, hati dan pundi berenang. Bagi bahagian folikel, lapisan folikel dipisahkan secara mekanikal daripada oosit setelah rawatan renjatan-sejuk. Untuk mengesahkan pengekspresan *bmp7a* dan *bmp7b* analisis semasa peringkat folikel yang berlainan, peringkat pravitelogenik; campuran vitelogenik-awal dan pertengahan; vitelogenik akhir yang belum matang pada siang hari, 0300 h, 0400 h; kematangan (0500 h) dan ovulasi (0600 h) diasingkan daripada ovari yang telah dibedahkan. Dengan menggunakan kaedah real-time PCR separa-kuantitatif, *bmp7a* menunjukkan ekspresi tertinggi dalam ovari manakala *bmp7b* diekspreskan amat tinggi dalam pundi berenang, otak, mata, insang dan ovari. Kedua-dua molekul ini terutamanya diekspreskan di dalam lapisan folikel tetapi bukan di dalam oosit. Ketika folikulogenesis, *bmp7a* dan *bmp7b* diekspreskan amat tinggi pada peringkat pravitelogenik. Pada peringkat seterusnya, *bmp7a* agak malar di sepanjang peringkat perkembangan folikel. Sebaliknya, pengekspresan pada *bmp7b* merosot secara berturutan sehingga ke tahap yang sangat rendah pada ovulasi di sepanjang perkembangan folikel. Secara keseluruhannya, penemuan ini menunjukkan *bmp7b* berkemungkinan penting sebagai faktor pertumbuhan autokrin/parakrin untuk perkembangan folikular pada folikel pravitelogenik dan, sebagai penindas kematangan oosit semasa folikulogenesis.

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ABSTRACT

The potential roles of bone morphogenetic protein 7 (*bmp7*) in ovary have drawn increasing attention. Present study aims to investigate the expression pattern of *bmp7a* and *bmp7b* in zebrafish. Adult female tissues were sacrificed for sampling of brain, eye, gill, heart, intestine, kidney, ovary, liver and swim bladder. For follicle compartments, follicle layers were mechanically separated from oocytes after cold-shock treatment. To verify analysis of *bmp7a* and *bmp7b* expression during different follicle stages, previtellogenic; early- and mid-vitellogenic mixed; full-grown immature at daytime, 0300 h, 0400 h; maturation (0500 h) and ovulation (0600 h) stage were isolated from dissected ovaries. Using semi-quantitative real-time PCR, *bmp7a* was found to be abundantly expressed in ovary whereas *bmp7b* was highly expressed in swim bladder, brain, eye, gill and ovary. Both molecules were predominantly expressed in the follicle layers rather than the oocyte. During folliculogenesis, *bmp7a* and *bmp7b* were expressed highly at previtellogenic stage. Thereafter, *bmp7a* remained rather constant across follicle developmental stages. In contrast, *bmp7b* expression progressively reduced to extremely low level at ovulation across the developing follicles. Overall, this finding indicated the possible importance of *bmp7b* as an autocrine/paracrine growth factor for follicular development at previtellogenic and, as repressor of oocyte maturation during folliculogenesis.

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CHAPTER 1

INTRODUCTION

1.1 Research background

The term bone morphogenetic protein (BMP) or osteogenic protein refers to the active ingredients in crude bone extracts that are capable of inducing new bone at ectopic sites in a rat (Urist *et al*., 1973). In 1988, the first BMPs were purified (Wang *et al*., 1988; Wozney *et al*., 1988). Thereafter, new BMP family genes were identified (Celeste *et al*., 1990; Özkaynak *et al*., 1990; Chang *et al*., 1994). To date, over 20 BMPs have been cloned, making the BMP family the largest subset of transforming growth factor (TGF-β) superfamily (Ye *et al*., 2007). Being an important growth factor, BMP has captured increasing attention in recent years, in regards to their functions in morphogenesis, cell differentiation and proliferation (Hogan, 1996a, 1996b), apoptosis and regulation of ovarian functions (Song *et al*., 1998; Kimura *et al*., 2000; Erickson and Shimasaki, 2003; Shimasaki *et al*., 2004b).

In parallel to the broad spectrum of bone morphogenetic protein 7 (BMP-7) in biological responses, studies on BMP-7 has been shown its importance in organogenesis of kidney, heart, limb, lung, gut, tooth and eye. In mouse, BMP-7 is necessary in developing organ system (Dudley *et al*., 1995). Evidence revealed that deficiency of BMP-7 leads to severe impairment of organs during embryogenesis (Dudley *et al*., 1995; Godin *et al*., 1998; Godin *et al*., 1999) and ischemic adult (Simon *et al*., 1999) in rodent. Analysis on BMP-7-deficient mice embryos also exhibited unilateral hind-limb polydactyly as reported by Dudley *et al*. (1995). Moreover, BMP-7 mutant mice embryos have been shown to reduce the size of kidney (Dudley *et al*., 1995).

In general, the functional unit within a female reproductive system is the ovarian follicle, which is a single oocyte surrounded by a thin encompassing elements or follicle layer (internal granulosa cells and external theca cells). Unlike mammals, ovarian follicle of

non-mammalian species consist of one layer of granulosa cells only without separated by antrum which is present in mammals. Local factors or paracrine activity, particularly secreted protein of growth factors are responsible for the nurturing and governing of follicular development (Hillier, 2001; Matzuk *et al*., 2002; Ge, 2005; Orisaka *et al*., 2009). It is known that BMP-7 is expressed in theca cells of mammals (Erickson and Shimasaki, 2003; Glister *et al*., 2004). Large of the studies have been ignored whether *bmp-7* will act in paracrine manner via the interaction of thecal-granulosa cells when granulosa cells are appeared in the only layer in non-mammalian during folliculogenesis. The process of ovarian follicle development progression in morphology, physiology and biology is termed as folliculogenesis which involves commencement and completion of meiosis, accumulation of cytoplasmic RNAs, proteins and specific information in driving ovarian development and subsequently to sustain embryo development. Among autocrine/paracrine factors, BMP-7 pronounced significant role in female reproductive system in mammals (Knight and Glister, 2003; Shimasaki *et al*., 2004b; Knight and Glister, 2006).

At present, the role of BMP-7 during folliculogenesis have been partially investigated in rodents (Shimasaki *et al*., 1999; Lee *et al*., 2001; Erickson and Shimasaki, 2003; Miyoshi *et al*., 2007), avian (Onagbesan *et al*., 2003, Onagbesan *et al*., 2009), bovine (Glister *et al*., 2004; Glister *et al*., 2005) and rainbow trout (Lankford and Weber, 2010a, 2010b). *In vivo* injection of BMP-7 into the ovarian bursa of rats clearly showed the role of BMP-7 in promoting follicular growth and development. At the same time, BMP-7 was shown to inhibit ovulation and luteinization (Lee *et al*., 2001). BMP-7 was also observed in the ovary of adult rat by *in situ* hybridization. Transcriptional activity of BMP-7 mRNA increased in parallel with the subsequent follicle growth. However, with initiation of luteolysis, BMP-7 mRNA was barely detectable (Erickson and Shimasaki, 2003).

Further research is needed to further understand the role of BMP-7 particularly in non mammalian species. At present, no experiment has been done to understand the role of BMP-7 in fish except in rainbow trout (Bobe *et al*., 2004; Baron *et al*., 2005; Lankford and Weber, 2010a, 2010b). For zebrafish, a study was done to understand the expression pattern

of newly cloned *bmp-7b* in normal development of vertebrate (Shawi and Serluca, 2008). Indeed, information developed in mammals, for instance rat and bovine ovary may not applicable to lower vertebrates. Therefore, there is a need for discovery of novel or new genomic function. The zebrafish is a useful lower vertebrate model for understanding reproduction due to its prolific spawning activities, presence of various follicle stages at the same time, fast folliculogenesis and easy availability of large number of ovarian follicles at different stages, ease of manipulation and maintenance.

With the above points in mind, a series of experiments were undertaken to analyze the expression of *bmp7a* and *bmp7b* in adult tissues and in the ovarian follicles by using semi-quantitative real-time PCR.

1.2 Objectives

The aims of this study were:

1. To determine the expression profiles of *bmp7a* and *bmp7b* mRNAs in different adult tissues of female zebrafish.
2. To localize spatial distribution of *bmp7a* and *bmp7b* expression in ovarian follicles of female zebrafish.
3. To compare the expression levels of *bmp7a* and *bmp7b* during folliculogenesis at different follicle stages of female zebrafish.

CHAPTER 2

LITERATURE REVIEW

2.1 Ovarian follicle development in teleost

2.1.1 Oogenesis

In general, a follicle consists of an oocyte (germ cells) surrounded by follicle layers (somatic cells compose of granulosa cells and theca cells). There are five stages of oocyte development or oogenesis for producing the egg or female gamete, which are primary growth, cortical alveolus, vitellogenic, maturation and ovulated unfertilized egg. During primary growth stage, cytoplasmic RNA increases abundantly. At the same time, many cytoplasmic organelles and molecules are synthesized at primary growth stage. It is also in this stage that meiotic process is temporarily halted. The transition of primary growth to cortical alveolus stage involves formation and accumulation of yolk and lipid vesicles. Vitellogenesis is the critical stage to increase the oocyte size through yolk protein uptake, a process mediated by vitellogenin. Under stimulation of estrogen (estradiol-17β), vitellogenin is produced in the liver (Jalabert, 2005). Estradiol-17β is actually produced in the follicle layer. Therefore, the production of vitellogenin in the liver is a process controlled by the follicle itself. Primary growth, cortical alveolus and vitellogenesis stages are also considered as oocyte growth phase because at the end of these three stages, the oocyte would have gone through a considerable increase in size. These fully-grown oocytes will be awaited further hormonal signaling before resuming meiosis, a process known as oocyte maturation stage. Lastly ovulation stage is the release of matured oocyte as eggs. Due to the importance of oocyte maturation stage and the focus of this thesis, the specific events during maturation are further provided in subsequent paragraph.

Interaction between oogenesis and folliculogenesis is required for formation of a follicle as displayed in Figure 2.1. Folliculogenesis is a process of the densely-packed follicle layers surrounding an oocyte.

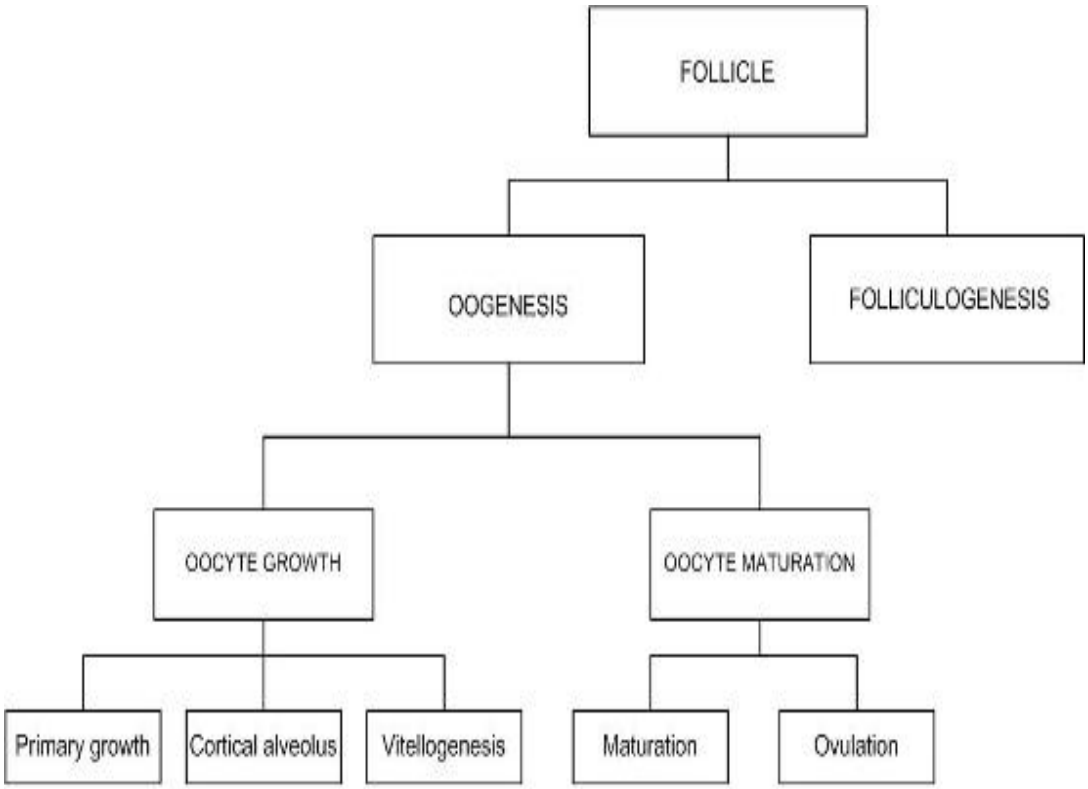

Figure 2.1: Organizational chart showing a follicle formation from primary growth to ovulation which involves oogenesis coupled with folliculogenesis.

2.1.2 Major events during oocyte maturation process

Since it is speculated that *bmp7* may play a role in oocyte maturation or ovulation, a review of the known molecular or cellular mechanisms are provided here. In general, oocyte maturation is under the influences of gonadotropin, maturation inducing hormone (MIH) and maturation promoting factor (MPF) that occurs in sequence from the ovarian somatic cells, the oocyte surface and in oocyte cytoplasm (Nagahama and Yamashita, 2008).

2.1.2.1 Event 1: Initiation of oocyte maturation by LH or hCG

Luteinizing hormone (LH) is produced by the anterior pituitary gland after stimulation of gonadotropin releasing hormone (GnRH). In vertebrates, initial surge of LH marked the start of oocyte maturation. At this time, LH binds to membrane-bound luteinizing hormone receptors (LHR) on thecal to initiate oocyte maturation. LH acts synergistically with follicle stimulating hormone (FSH). LH is considered as steroidogenic hormone during oocyte maturation whereas FSH is vitellogenic hormone for oocyte growth. The binding of LH to LHR was first demonstrated in the salmonid ovary (Salmon *et al*., 1984; Breton *et al*., 1986; Kanamori *et al*., 1987). A series of changes take place near to the LH surge characterized by the dispersal of organelles including mitochondria and cortical granules followed by metaphase progression (de Lesegno *et al*., 2008). Work performed on *Xenopus laevis* (Reynhout *et al*., 1975) and *Cyprinus carpio* (Jalabert *et al*., 1977) elicited that human chorionic gonadotropin (hCG) or pituitary homogenate can be substituted for LH *in vivo* priming. hCG is derived from the urine of pregnant women. Since fish gonadotropin is not easily obtained, mammalian origin hormones have become alternatives in fish. Moreover, hCG is less costly, has a longer half-life than LH and calibrated based on international standard for teleost.

The luteinizing hormone/choriogonadotropin receptor (LHCGR) is also called lutropin/choriogonadotropin receptor (LCGR) or luteinizing hormone receptor (LHR). Hormonal specificity of LH receptors are different amongst species. In African catfish, recombinant cfFSH and cfLH was found to activate follicle stimulating hormone receptor

(FSHR) with a similar biopotency (Bogerd *et al*., 2001; Vischer and Bogerd, 2003; Vischer *et al*., 2003). In zebrafish, LHR is able to interact with follicle stimulating hormone (FSH) and luteinizing hormone (LH)/chorionic gonadotropin (such as hCG) (Kwok *et al*., 2005). A number of LHR in mammals and fish have been cloned including in the catfish (Vischer and Bogerd, 2003), human LHR/hCGR (Minegishi *et al*., 1990), mouse (Gudermann *et al*., 1992), rat (McFarland *et al*., 1989), porcine (Loosfelt *et al*., 1989), bovine (Kawate *et al*., 2002), zebrafish (Kwok *et al*., 2005), Atlantic salmon (Maugars and Schmitz, 2006) and tilapia (Oba *et al*., 2001). LHR can also be found in the ovary and testis as well as in the liver of zebrafish (Kwok *et al*., 2005). LHR and FSHR work at different periods during follicle development. Unlike FSHR, LHR mRNA level starts to increase during vitellogenesis and peak at just prior to oocyte maturation. FSHR is reported to peak at mid-vitellogenic stage and drops significantly at full-grown stage (Kwok *et al*., 2005). Upregulation or downregulation sensitivity of LHR means increase or decrease in number of receptor sites toward its target molecules. It stimulates the ovaries to produce steroids by controlling sensitivity of target cells to LH/hCG. In the ovarian follicle, LHR and FSHR mRNA are expressed in theca cells and granulosa cells in zebrafish (Clelland and Peng, 2009).

In fact, LH secretion is not only controlled by the endocrine system which secretes hormone from the glands into the bloodstream but is also affected by environmental factors. Captivity conditions are different from natural habitat in terms of spawning stimuli such as water quality, stream hydraulics, nutrition, spawning substrate and depth. To induce appropriate endogenous responses, hormonal therapy is required. In aquaculture of cyprinids, injection of a combination of synthetic GnRH analogue with dopamine inhibitory factor is a commonly used method (Kouril *et al*., 1999). As a brain signal, GnRH stimulates secretion of luteinizing hormone from the pituitary gland of vertebrates. Application of dopamine antagonists in aquaculture has shown increment of successful ovulation (Kouril *et al*., 2006, 2007) as dopamine inhibited GnRH-stimulated release of LH. Other future methods including topical gill application (Hill *et al*., 2005) and oral application (Roelants *et al*., 2000; Mikolajczyk *et al*., 2001) will be used for GnRH administration to permit endogenous

LH surge in conservation, sustainable (Kamiński *et al*., 2004) and aquaculture production (Mikolajczyk *et al*., 2004; Berlinsky *et al*., 2005). Besides artificial reproduction, LH secretion also depends on a photoperiod regime to delay or advance teleosts oocyte maturation. Extended photoperiod regimes from 6 to 9 months have been shown to advance spawning in rainbow trout (Bon *et al*., 1997) whereas continuous light regime delayed spawning (Boulier and Billard, 1984). Diet uptake of the broodstock has been reported to affect reproductive performance (Washburn *et al*., 1990). Water temperature (Moreau *et al*., 1991; King and Pankhurst, 2004), salinity (Burg and Garcia-Perez, 1992), pH (Patiño *et al*., 2005) and environmental pollutants (Örn *et al*., 2003; Maack and Segner, 2004) changes might affect gene expression pattern to induce development as well.

2.1.2.2 Event 2: Preparation of oocyte to respond towards steroid (maturation inducing hormone)

In this event, oocytes will acquire the ability to produce MIH and in addition oocytes will acquire the ability to respond to MIH (Figure 2.2). Such event is also termed as gonadotropin-dependent oocyte maturational competence (OMC) stage. During OMC acquisition, connexins and MIH receptor are produced (Patiño *et al*., 2001). Connexins, being a protein in intercellular channels, in turn, aggregate to form gap junctions (GJ). Indeed, homologous (granulosa cell-granulosa cell) and heterologous (granulosa cell-oocyte) GJ contacts increase in parallel with LH or hCG-induced OMC acquisition in teleost ovarian follicle in event 1 oocyte maturation. As heterologous GJ contacts are present in a large fraction, granulosa cell-generate-transduction signals including cAMP, calcium ions are probably moved to oocyte via GJ to modulate or induce OMC (Yamamoto and Yoshizaki, 2008).

Besides, in preparation of oocyte to respond towards MIH, sensitivity of MIH receptors should be increased. Membrane bound progestin receptor (mPR) is the MIH receptor. There are three isoforms of (mPR), mPRα, mPRβ and mPRγ. Both mPRα and mPRβ proteins were detected near the oocyte membrane at full-grown stage of zebrafish

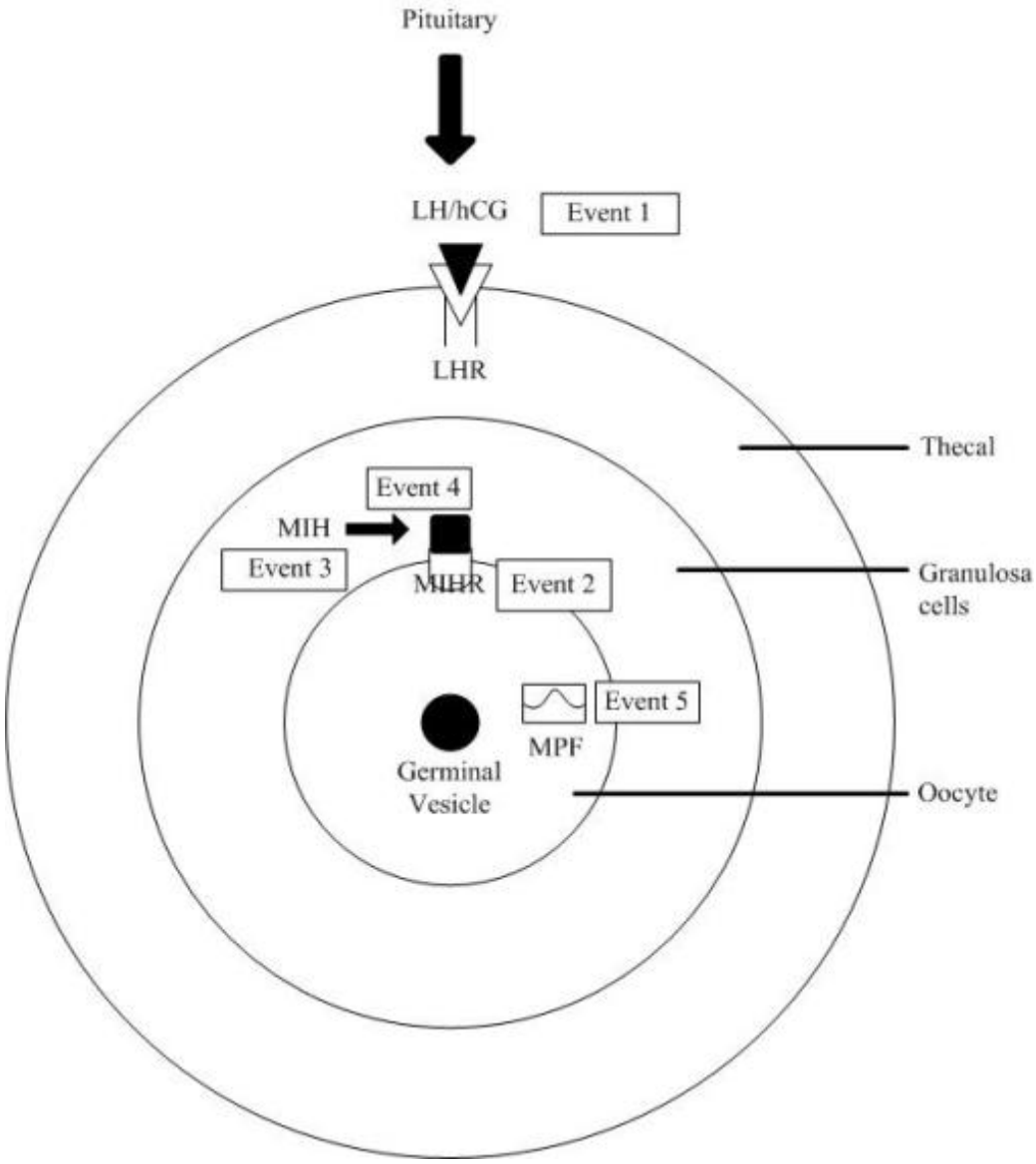

Figure 2.2: Diagram of chronological events during oocyte maturation in ovarian follicle of fish. Event 1, initiation of oocyte maturation by LH/hCG. Event 2, preparation of oocyte to respond to steroid (MIH). Event 3, steroid production. Event 4, binding of MIH to MIH receptor in fish. Event 5, maturation promoting factor (MPF) production and activation which lead to matured germinal vesicle breakdown (GVBD). Event 1 to 3 happen in follicle cells while event 4 to 5 happen in oocyte.

(Hanna and Zhu, 2009). Studies have demonstrated that full-grown immature oocytes (>650 μm) has the highest degree of OMC (Pang and Ge, 2002b; Wu *et al*., 2000), while early stages of oocytes has no response to MIH (Yamashita *et al*., 2000). This indicates that expression of mPR is required in OMC acquisition in mediating MIH-induced oocyte maturation in the following events.

2.1.2.3 Event 3: Steroid production

This event is the actual production of MIH in granulosa cells. MIH is considered as steroidogenic. Production of MIH has been identified to possess dynamic process in steroidogenic pathway to regulate stimulation of LH for 17α,20β-dihydroxy-4-pregnen-3-one (17,20βP) in oocyte maturation. There are two subtypes of MIH in teleost fishes which are species-specific. MIH 17,20βP can be found in salmonids, cyprinids, atherinids and catfish. On the other hand, 17,20β-21-trihydroxy-4-pregnen-3-one (20β-S) is observed in most perciforms and flatfishes (Nagahama and Yamashita, 2008; Clelland and Peng, 2009).

Under stimulation of LH/hCG, 20β-hydroxysteroid dehydrogenase (20β-HSD) transcripts and activity is elevated in the granulosa layer during oocyte maturation (Kazeto *et al*., 2001; Senthilkumaran *et al*., 2002), suggesting its enzymatic activity to initiate maturational event (Senthilkumaran *et al*., 2002) as 17α-hydroxyprogesterone is the immediate precursor of 17,20βP. According to the steroidogenic pathway (Figure 2.3), progesterone is metabolized to 17α-hydroxyprogesterone by 17α-hydroxylase/C17-C20 lyase (P450c17) in the thecal layer. However, previous studies have demonstrated that BMP-7 suppressed progesterone production in mammals (Lee *et al*., 2001; Glister *et al*., 2004; Juengel *et al*., 2006). *In vitro* incubation of full-grown immature follicles demonstrated that 17,20βP accelerated corresponding with the availability of its substrate, 17α-hydroxyprogesterone (Nagahama, 1997). A finding documented that TGF-β1 inhibited MIH-induced oocyte maturation by reduced mRNA level of 20β-HSD in zebrafish (Kohli *et al*., 2005).

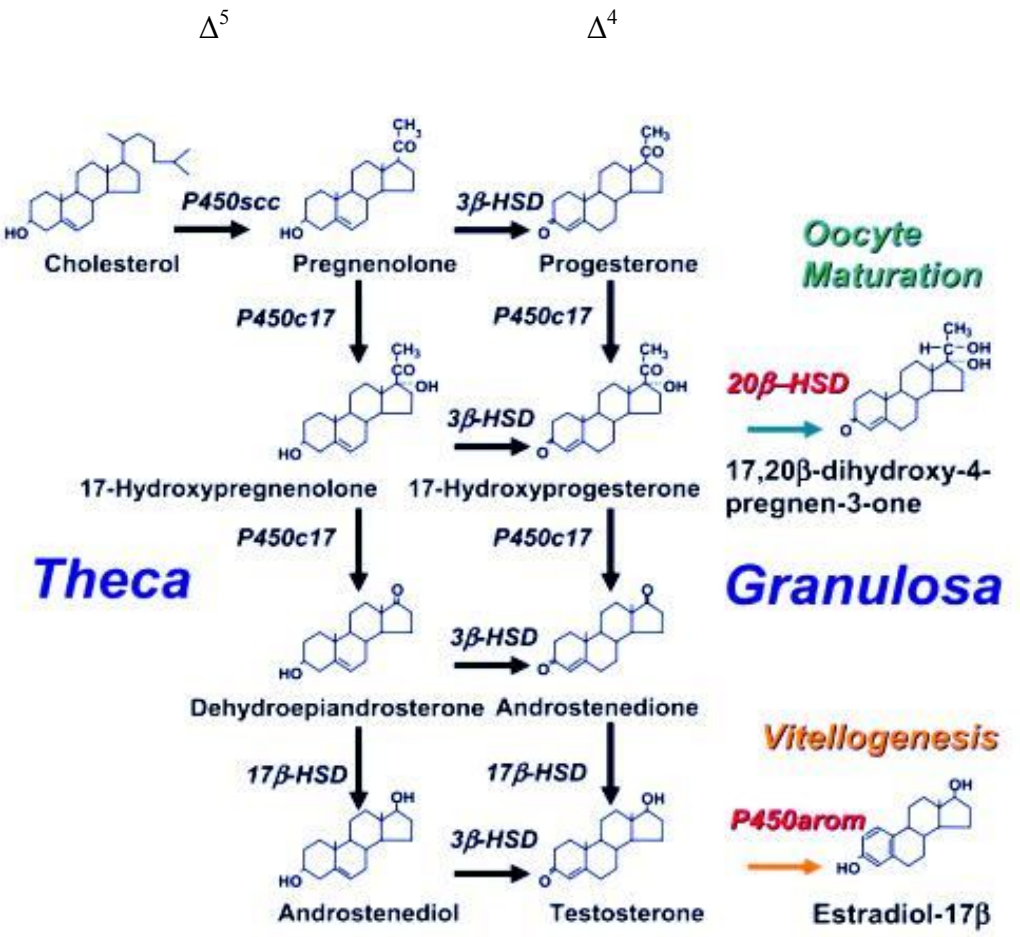

Figure 2.3: Steroidogenic pathway in thecal and granulosa layer of fish ovarian follicles. Δ^5 pathway is responsible for the production of estradiol-17β during oocyte growth (vitellogenesis) while Δ^4 is involved in the production of 17α,20β-dihydroxy-4-pregnen-3-one (17,20βP) during oocyte maturation. Steroidogenic enzymes: P450scc, P450 side-chain cleavage; P450c17, 17α-hydroxylase/C17-C20 lyase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; oP450arom, P450 aromatase; 20β-HSD, 20β-hydroxysteroid dehydrogenase (Nagahama and Yamashita, 2008; Lubzens *et al*., 2010).

2.1.2.4 Event 4: Binding of MIH to MIH receptor in oocyte

The produced MIH will then bind to MIH receptor in the oocyte surface (oolemma). Knockdown experiment on mPRα and mPRβ by morpholino antisense oligonucleotides blocked MIH-induce oocyte maturation (Thomas *et al*., 2004). BMP-15 was discovered as a suppressor of oocyte maturation by inhibiting mPRβ expression. In support of this, another study showed that knockdown of BMP-15 in oocytes by antisense oligonucleotides elevated mPRβ expression (Tan *et al*., 2009a). MIH receptor is coupled to inhibitory G-protein (Gi) in which signals generated from surface receptor mPR is transduced into oocyte cytoplasm.

2.1.2.5 Event 5: Maturation promoting factor (MPF)

MPF is a regulator of cell cycle. Upon formation and activation, MPF reinitiated meiosis in oocytes causing the oocytes to mature into unfertilized egg. Progression of meiosis ceased again at metaphase II until fertilization. Thus, MPF may be a more general factor necessary for GVBD, cell division of both mitosis and meiosis, extrusion of first polar body and subsequently the oocyte became ovulating egg (Nagahama and Yamashita, 2008).

In maturing oocytes, a part of preexisting cell division control 2 (cdc2) is bound to *de novo* synthesized cyclin B to form activated MPF (Yamashita, 2000; Nagahama and Yamashita, 2008). After maturation, matured oocyte is released from the degradation and rupture of the follicle layers. Ovulation can also be promoted by LH and MIH but nuclear progestin receptor (nPR) act as an essential mediator of ovulation. Based on this view, it can be speculated that 17,20βP is not merely the key hormone for the induction of oocyte maturation via membrane receptor but also act via nuclear receptor during ovulation (Nagahama and Yamashita, 2008). Apart from the above mentioned regulators, other factors including growth factors, are also indispensable by cooperation with MIH to accomplish the processes of ovarian development. *Bmp7* may be one of the growth factors act as upstream or downstream of MIH. In order to reveal the expression pattern of *bmp7* during oocyte maturation events, zebrafish is used as the model organism.

2.2 Zebrafish

2.2.1 Taxonomy of *Danio rerio*

The classification of *Danio rerio* is provided below:

- Kingdom : Animalia
- Phylum : Chordata
- Class : Actinopterygii
- Order : Cypriniformes
- Family : Cyprinidae
- Genus : Danio
- Species : *Danio rerio*
- Local name : Ikan zebrafish

2.2.2 General background

Zebrafish is becoming a useful vertebrate organism (Figure 2.4). In aquarium trade, other forms namely long finned and leopard were produced via selective breeding from typical form of short fin zebrafish. However, it has no economic value as food fish due to its small size. It was first identified by the British surgeon Francis Hamilton in 1822. Zebrafish belongs to the class of ray-finned fish which contains most of the species of fish (46 orders). Matured adults are able to grow around 4 to 6 cm in size. There are pairs of well developed barbels in rostral barbels and maxillary barbels. This tropical freshwater fish originates from Ganges region in India, Nepal, Pakistan, Bangladesh and Myanmar. Zebrafish can adapt well in streams, canals, ponds, irrigation channels and rice fields. Optimal habitat condition for this fish range of 24-30°C and pH range of 7.0-8.0. In natural condition, it is omnivorous with preference for zooplankton, insects and phytoplankton.

2.2.3 Zebrafish as a vertebrate model system

In 1970s, a scientist from the University of Oregon, George Streisinger found an alternative for the mouse model system. The vertebrate model organism must be more simple and easy to manipulate genetically. Based on this, the zebrafish became the representative model organism of mutations in his laboratory. Since this pioneering work, the zebrafish has quickly emerged as one of the popular vertebrate models for many aspects

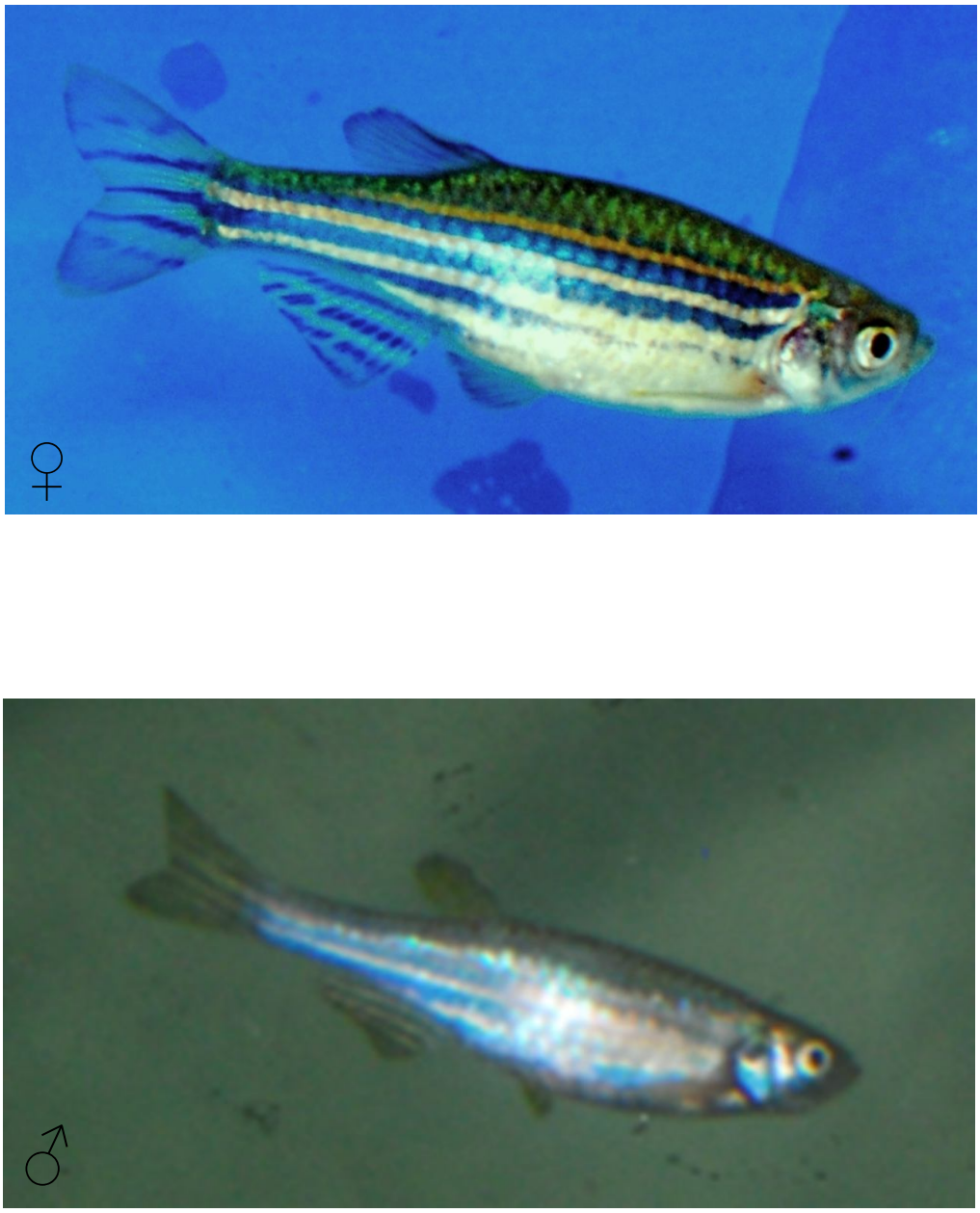

Figure 2.4: Adult female (♀) and male (♂) zebrafish.

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of life science studies including developmental biology, genetics, vertebrate evolution, toxicology, disease, mutation, organ system biology and physiology. This organism is appropriate and relevant to manipulate due to its small body size, less effort and expenses in maintenance, maximize space efficiency, tractable and peaceful disposition.

Due to the above-mentioned virtues, zebrafish has been extensively utilized in embryological research. Zebrafish embryos are optically transparent, fast development from fertilized to hatching. The events from fertilization, hatching and further development take place outside of the maternal body cavity. The special attributes enable classification of embryo development easily and to directly visualize the *ex utero* internal and external anatomical structure (Nüsslein-Volhard *et al*., 2002). The internal anatomy is a minimized version of organ in comparison to complex structure in mammalian.

2.2.4 Zebrafish as model for reproduction

Small scale or even large scale experiment are amenable on zebrafish breeding (Brand *et al*., 2002). Breeding can be conducted with ease which merely involves small, light and portable breeding equipments. Each breeding experiment offers a large amount of matured and ovulated samples and the samples can be obtained all year round as zebrafish is a highly prolific breeder. It also provides an array of follicles at various stages ranging from previtellogenic to vitellogenic. Apart from that, the breeding technique and skill are simple and convenient in that no hormone injection is needed, without much time consuming to obtain maturation and ovulation as well as the timing of these samples can be controlled and predicted precisely.

There are many of established *in vitro* bioassays regarding oocyte maturation under stimulation of exogenous hormone in zebrafish (Selman *et al*., 1994; Pang and Ge, 2002b; Seki *et al*., 2008). In addition, incubation of particular size of follicles is considered as fast development *in vitro*. Accordingly, it could examine the events of induction process of oocyte maturation (Patiño *et al*., 2001) whether *bmp7* exerts its role in either upstream or downstream or in both of steroid. This approach will be useful for investigating the

transcriptional activities of *bmp7a* and *bmp7b* and to determine the correlation with other growth factors. Further, the finding could contribute to the fundamental aspects of growth factors particularly BMP system in governing and participating in signaling pathway of oocyte maturation in teleost fish reproduction. Therefore, detailed molecular analysis will be useful to disclose BMP system more thoroughly.

2.3 Reproduction

2.3.1 Reproduction of zebrafish

Basically, the female possesses a larger whitish belly with silver and blue-black horizontal stripes. In contrast, the male can be distinguished from female by several features, torpedo shaped as well as yellowish and blue-black stripes. An adult zebrafish completes its life cycle in about 4-5 months. Zebrafish displays an ovuliparous reproductive mode, in that its eggs are externally inseminated. Fertilized eggs develop without parental care with their nutrients supply from its own egg yolk. Once the egg envelope or chorion is broken, hatching occurs.

Zebrafish ovaries undergo asynchronous development. The ovary of the zebrafish is remarkably dynamic which contain follicles at various stages and no dominant populations in a lump of ovary. An asynchronous fish breeds in a small group and eggs are released batch by batch. This is also known as batch spawner. In fact, zebrafish belongs to daily spawner (Selman *et al.*, 1993). Normally, the spawning of the zebrafish takes place at dawn or the first few hours of daylight comes in regardless of natural (Spence *et al*., 2006) or captivity condition (Selman *et al*., 1993). Female and male of zebrafish that are kept separately, when pair up to breed at 10-day intervals will produce maximum embryo viability.

The demersal eggs are transparent and non-adhesive. Upon fertilization, the development of embryo will persist in being transparent instead of turning to opaque. Hatching occur within 2 to 3 days post-fertilization (dpf). Free swimming larvae are typically formed from 5 to 7 dpf (Lawrence, 2007). In this regard, reproduction of teleosts is

influenced by hormone and growth factors regulation, environmental influences including photoperiod, diets, pollutants and husbandry practices (Devlin and Nagahama, 2002; Bobe and Labbé, 2010).

2.3.2 Gonadogenesis of zebrafish ovary

During gonadal development, teleosts display diverse morphology. Generally, fish belong to the gonochoristic group, whereby undifferentiated primordial germ cells grow into ovaries or testes in sexually matured individuals. In contrast, hermaphrodite fish undergo sex change. In zebrafish, some of their ovaries during early life switched into testes throughout sexual differentiation during ontogenesis which are regarded as juvenile protogynous hermaphrodite fish.

In developing teleosts, endocrine and molecular controls are the key aspects in determining oogenesis and folliculogenesis in addition to environmental influences (Brooks *et al*., 1997) as discussed earlier in event 1 during the initiation of oocyte maturation by LH. A matured adult zebrafish has bilobed ovary of the cystovarian type whereby paired primordial join during ovarian formation and encompass a portion of the coelomic cavity. Ovulated eggs are located at the ovarian lumen and shifted to a connected short oviduct as well as a genital opening posterior to anus. The ovarian wall is composed of a thin epithelium on smooth muscle which is coated with a connective tissue compartment that protrudes as longitudinal folds or ovigerous lamellae into the ovarian cavity. Various stages of follicles, atretic follicles and postovulatory follicles are distributed on ovigerous lamellae. Oogonia and the earliest oocytes can be found at the ovarian stroma. Ovarian follicle contains growing follicle-enclosed oocytes (Selman *et al*., 1993; Clelland and Peng, 2009).

2.3.3 Developmental stages of follicle in zebrafish

Information provided by growth and development has contributed to fisheries biology and teleosts reproduction. It is pertinent to mention that teleost reproduction is a complicated aspect and involve dynamic process. In spite of that, the overall teleost fish

share the basic pattern of growth. The developmental events of zebrafish oocytes can be classified into five common stages according to morphological (Figure 2.5) and physiological event. In general, the five stages are: (i) stage I, primary growth, (ii) stage II, cortical alveolus stage, (iii) stage III, vitellogenic stage, (iv) stage IV, maturation stage and (v) stage V, mature eggs or ovulation stage. The descriptions in Table 2.1 are stages in oocyte development of zebrafish studied by Selman *et al*. (1993). This staging system in zebrafish has mapped a useful foundation for future advanced researches (Kwok *et al*., 2005; Clelland *et al*., 2007; Hanna and Zhu, 2009) including *bmp7* study. Given the advantages of zebrafish, a series of experiment were conducted on *bmp7* expression in adult tissues, follicle compartments and follicle developmental stage.

2.4 TGF-β superfamily-BMP

The transforming growth factor-β (TGF-β) superfamily consists of multifunctional growth factors with over 40 members which includes TGF-β (TGF-β1, TGF-β2 and TGF-β3), anti-Müllerian hormone (AMH), two inhibins (A and B), three activins (A, B and AB), some 20 bone morphogenetic proteins (BMP-1 to BMP-20) and at least nine growth differentiation factors (GDF-1 to GDF-9) (Massagué and Wotton, 2000). TGF-β superfamily members are structurally related extracellular, secreted growth factors that function as local regulators via autocrine/paracrine signaling in a variety of species ranging from worms, fish to mammals. It is widely known that TGF-β contributes to molecular regulation of reproductive processes (Ingman and Robertson, 2002; Knight and Glister, 2006).

BMP has been shown as a disulfide-linked dimeric protein. There are seven cysteine residues in each monomer for the majority of BMP proteins which are conserved among the members (Guimond *et al*., 2010). In this way, BMPs share the identical three-dimensional structures to other members in TGF-β superfamily. Similar to other members of TGF-β superfamily, BMP are synthesized as large precursor proteins that are comprised of an amino-terminal signal sequence, a pro-domain and a mature domain (C-terminal regions). The amino-terminal signal is likely to direct the precursor to the secretory pathway. The pro-

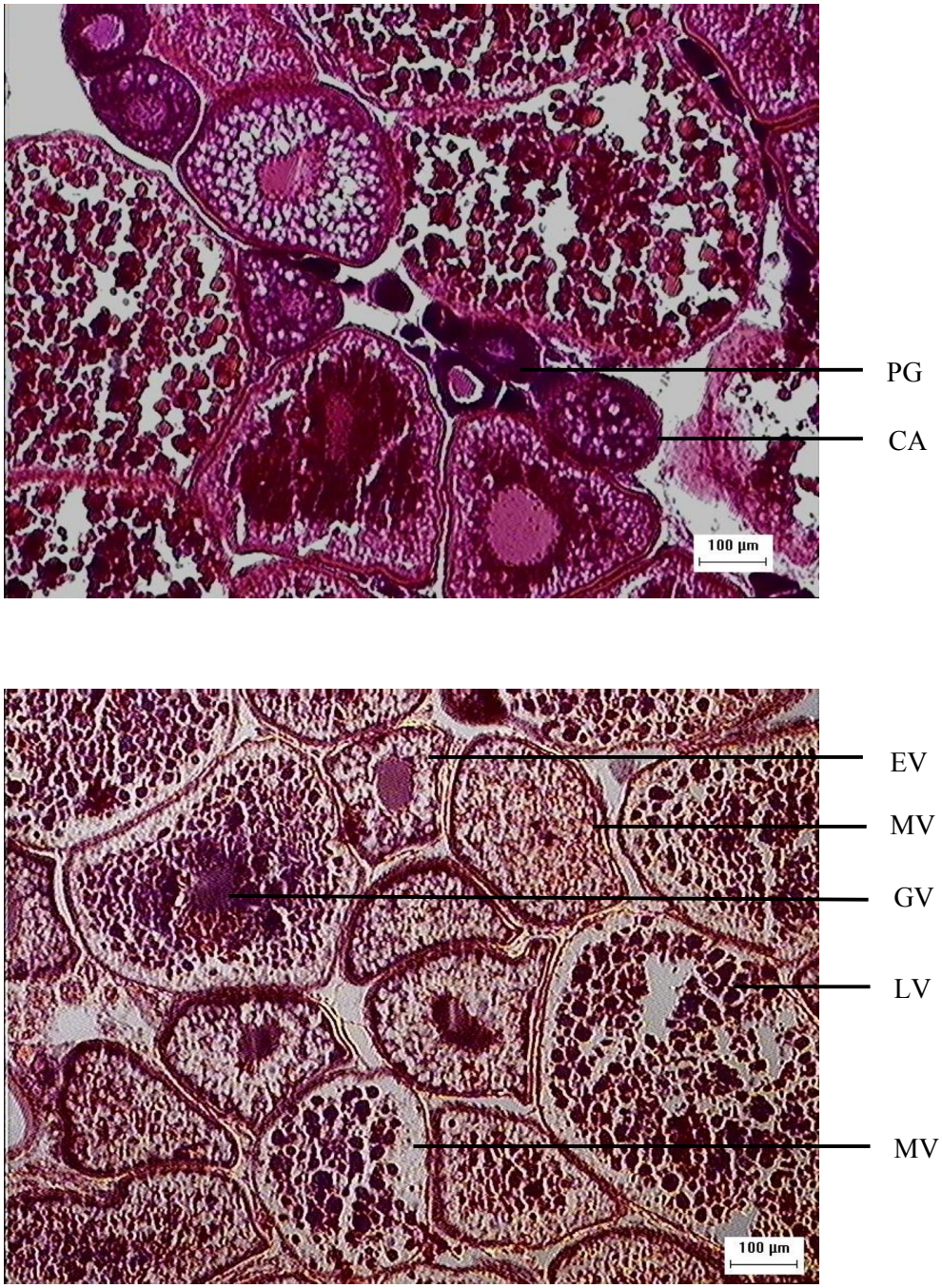

Figure 2.5: Morphological of follicle stages in zebrafish by histological examination. PG, primary growth; CA, cortical alveolus; EV, early-vitellogenic; MV, mid-vitellogenic; LV, full-grown; GV, centrally located germinal vesicle.

Table 2.1: Stages of oocyte development in the zebrafish (Selman *et al*., 1993).

Developmental stage	Morphological and Physiological Event
Stage I (Primary growth)	Stage IA (pre-follicle phase): oocyte diameter = 7-20 µm Pre-follicle cells form a boundary between small oocytes in nests and ovarian stroma as well as ovarian follicles in which oocytes share the same cell borders. Once oocytes commence its growth, they become entirely protected by a sheath of pre-follicle cells. Stage IB (follicle phase): follicle diameter = 20-140 µm Newly formed follicles are transparent with noticeable nucleus contained in oocytes. The functional unit of ovarian consists of oocyte enclosed by squamous follicle cells, vascularized thecal and a thin surface epithelium. Vitelline envelope (zona radiata) is just starting to develop in the perivitelline space.
Stage II (Cortical alveolus)	Follicle diameter = 140-340 µm Cortical alveoli (yolk vesicles) exhibit in peripheral zone of the ooplasm. Oocytes become opaque and germinal vesicles are not visible. Presence of tripartite vitelline envelope (zona radiata externa, zona radiata interna 1 and zona radiata interna 2). Cuboidal follicle cells which are external to the oocyte proliferate and specialized theca cells appear.
Stage III (Vitellogenesis)	Follicle diameter = 340-690 µm Follicle opacity and size increase. Hepatic-derived vitellogenin are processed into yolk proteins and accumulation of yolk bodies (Selman and Wallace, 1982). Vitelline envelope gets thinner, the specialized theca cells become numerous and larger. Somatic cells are still cuboidal. Small vitellogenic follicles (<520 µm) showed no response when incubated with exogenous hormone (Wu *et al*., 2000). Competency of follicle increased with the follicle size (Selman *et al*., 1993; Wu *et al*., 2000; Pang and Ge, 2002b).
Stage IV (Maturation)	Follicle diameter = 690-730 µm Germinal vesicle moves towards the oocyte peripheral zone, the nuclear envelope breakdown (GVBD), the first meiotic division takes place and subsequently arrest at second meiotic metaphase. Follicle is translucent. Somatic cells retract from oocyte prior to ovulation occur. Dissected maturation follicle usually attached with young follicles.
Stage V (Mature egg/ Ovulation)	Egg diameter = 730-750 µm Translucent eggs are expelled into ovarian cavity leaving behind follicle layers. Ovulated eggs are covered by tripartite vitelline envelope. Upon immersion in medium, chorion immediately expanded and detached from oocyte surface of dissected egg. Ready to be fertilized.

domain may enable folding, dimerization and controlling of TGF-β superfamily members (Sopory *et al*., 2006). Carboxyl-terminal domain contains signaling molecules that form a cysteine knot structure (Böttner *et al*., 2000; Alvarez *et al*., 2009).

2.4.1 BMP pathway

The activation of cellular signaling involves interaction of ligand-receptor. A ligand interaction between BMP and the suitable receptor is necessary in order to activate a series of signaling molecules. TGF-β superfamily members employ a general mechanism to signalize target gene in nucleus. In mammals, there are five type-II receptors designated activin receptor (ActR-IIA, ActR-IIB), BMP type-II receptor (BMPR-II), Müllerian inhibiting substance/anti-Müllerian hormone type-II receptor (MIS/AMHR-II) and TGF-β type-II receptor (TGFβR2); and seven type-I receptors namely activin receptor-like kinase (ALK)-1 to -7 (Shimasaki *et al*., 2004b). BMP-7 strongly binds to BMPR-II, ActR-IIA and ActR-IIB for type-II receptor; and ALK-2 (ActR-IA) and/or ALK-6 (BMPR-IB) for type-I receptor depending on cell type. Upon BMP ligand binding to the specific type II receptor complex, the specific type I receptor is recruited into a heteromeric complex. The type II receptor phosphorylates the type I receptor within intracellular glycine-serine-rich (GS) domain (Kaivo-oja *et al*., 2006). The activated type I receptor in turn, phosphorylates appropriate cytoplasmic substrate of signaling protein called receptor-regulated Smads (R-Smads). TGF-β/Activin favours Smad 2 and 3 whereas BMP-Smad transduces signal via Smad 1, 5 and 8. In certain cell types, Smad 1 and 5 have been found in signal transduction of TGF-β pathway (Oh *et al*., 2000; Bakkebø *et al*., 2010). Subsequently, this phosphorylation enables R-Smad association with Co-Smad (Smad 4). As a result, the activated R-Smad-Co-Smad complex is accumulated in the nucleus to promote or repress target genes. Another group of Smads, I-Smads (Smad 6 and 7) inhibit BMP-Smad pathway at different levels (Kaivo-oja *et al*., 2006). Figure 2.6 depicts the ligand-receptor signal transduction pathway for BMP-Smad pathway relates to TGF-β/Activin pathway.

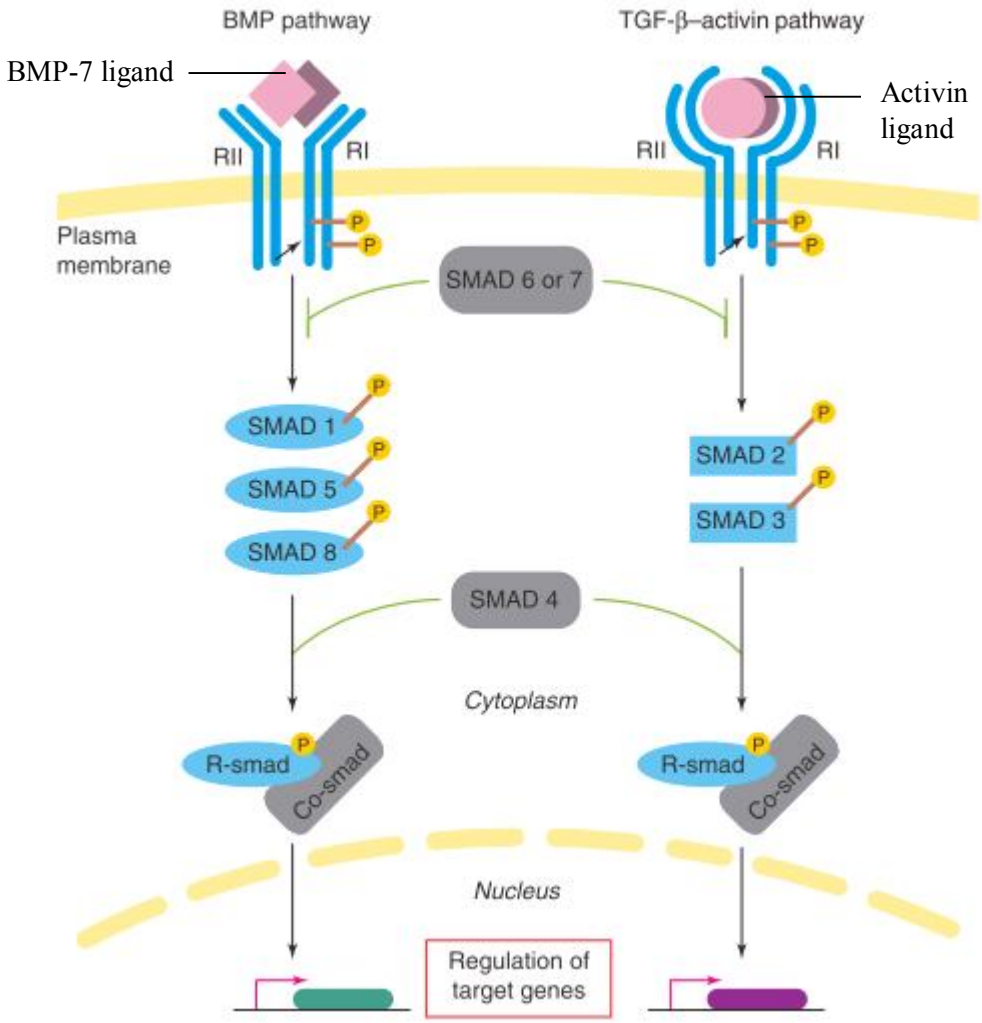

Figure 2.6: The crosstalk of BMP-Smad pathway with TGF-β/Activin pathway. Ligand-receptor binding leads to phosphorylation of the type I receptor by the type II receptor which in turn, phosphorylates a cascade of Smads action. R-Smad may not be shared among TGF-β superfamily members. R-Smad for TGF/Activin pathway is Smad 2 and 3 while BMP-Smad pathway transmits signal via Smad 1, 5 and 8. TGF-β superfamily members share the same Co-Smad, Smad 4 when assembly with R-Smad which permits Smad complex translocates to the nucleus to regulate the target genes of TGF-β superfamily members depending on whether coactivator or corepressor is present. Additional regulation involves inhibitory Smads (Smad-6 and 7) which can attenuate TGF-β/Activin and BMP-Smad signaling (Lin *et al*., 2003).

CHAPTER 3

MATERIALS AND METHODS

3.1 Fish maintenance

The zebrafish used for the current study were obtained from population maintained at the Aquatic Research Complex, Universiti Sains Malaysia on 14L:10D with lights on at 0600 h. Female and male zebrafish were kept in separate tanks. Breeding activity was carried out once a week to ensure active reproduction cycle. The fish were fed twice daily with frozen bloodworms and supplemented with newly hatched brine shrimp.

3.2 Sampling procedure

3.2.1 Sampling of zebrafish adult tissues and ovarian follicles

To verify *bmp7a* and *bmp7b* expression profiles on adult females, tissue samples including brain, eye, gill, heart, intestine, kidney, ovary, liver and swim-bladder were excised. Only healthy fish without diseases and abnormalities were used for experimental sampling. The behavior of fish must be normal whereby they are not showing sign of stress or isolating themselves from other fish. The fish display active swimming movement and show healthy appetite towards food. The external body of the fish is bright and shiny in coloration without any wounds and the fins do not clamp together.

Before follicle sampling, fish were pair up for mating in the afternoon using breeding containers. The breeding container consists of a 1-litre acrylic box and a barrier to separate the pair of fish. Follicle sampling were conducted at different time points for full-grown immature (0300 h and 0400 h), maturation (0500 h) and ovulation stage (0600 h). During daytime, ovaries from 10-15 sexually mature gravid female zebrafish were dissected and pooled for each single replicate. The ovaries removed were immersed in petri dish containing 60% medium Leibovitz L-15 (Sigma-Aldrich). The follicles of varied stages were manually isolated with the aid of fine forceps and divided into five groups according to Selman *et al*. (1993): previtellogenic stage (PV, ≤0.3 mm); mixed of early- and mid-vitellogenic stage (EV

and MV, 0.35-0.55 mm); full-grown immature stage (LV, 0.6-0.7 mm); maturation stage (>0.7 mm) and ovulation stage (>0.7 mm). Total RNA was extracted with Tri-Reagent® (Molecular Research Centre, USA). The experiment was repeated four times.

3.2.2 Isolation of follicular layers and oocytes

Teleost follicle including zebrafish contains an oocyte which is encompassed in a follicle layer namely granulosa and theca cells. In order to separate the follicle layers from oocytes, cold-shock treatment was applied (Liu and Ge, 2007) with some modifications. Gravid female zebrafish were freshly sacrificed. Ovaries were removed and immediately pretreated in 60% medium Leibovitz L-15 on ice (4°C) for 45 min. With the aid of fine forceps, follicle layer of full-grown immature follicles at daytime were carefully peeled off under stereomicroscope (Olympus MVX10, Japan). This was followed by total RNA extraction of isolated and pooled follicle layers or denuded oocytes with 500 µl Tri-Reagent® respectively.

3.3 Gene expression studies

3.3.1 Total RNA isolation

Various tissues and isolated follicles weighing 50-100 mg were homogenized in 1 ml of TRI Reagent® according to the protocol described by the manufacturer (Molecular Research Centre, USA). Tissue samples were homogenized and stored for 5 min at room temperature for complete dissociation of nucleoprotein complexes. The homogenate was then centrifuged at 12,000 g for 10 min at 4°C cold centrifuge to remove the insoluble material. After that, 0.2 ml chloroform was added to the transferred clear supernatant in another sterile 1.5 ml microcentrifuge tube and shaken vigorously for 15 s. The mixture was stored at room temperature for 15-20 min and centrifuged at 12,000 g for 15 min in 4°C, in order to separate the clear upper aqueous phase from the intermediate phase, and the lower red, phenol-chloroform phase. After centrifugation, the aqueous phase was transferred to another sterile 1.5 ml microcentrifuge tube and precipitated by adding 0.5 ml of isopropanol

(Amresco, Ohio). The tube was then stored at -20°C overnight, followed by centrifugation at 12,000 g for 10 min at 4°C. The supernatant was decanted and the RNA pellet was rinsed with 75% ethanol to wash away the salts and centrifuged at 7,500 g for 5 min at 4°C to sediment the pellet. Subsequently, the ethanol was decanted carefully and the RNA pellet was air-dried for 10-15 min, resuspended in sterile DEPC-treated water. Finally, the isolated total RNA was incubated for 15 min at 55°C. The sample was stored at -80°C for further analysis.

3.3.2 Quantification of total RNA

The isolated total RNA concentration and purity was measured by spectroscopy at wavelength absorbance of 260 nm and 280 nm using SmartSpec^{TM} Plus Spectrophotometer (Bio-Rad, USA). Samples with A_{260}/A_{280} ratio between 1.8 and 2.0 were used for the following analysis.

The integrity of isolated total RNA sample was verified by electrophoresis on 1% of denaturing formaldehyde-agarose gel electrophoresis with modification (Maniatis *et al*., 1982). Formaldehyde-agarose gel was prepared by melting 1 g of agarose (Promega, USA) in 72 ml of sterile distilled water. After cooling to approximately 60°C, 18 ml of 12.3 M (37%) formaldehyde (Sigma, USA) and 10 ml of 10X MOPS buffer were added, which resulted to the final concentration of 2.2 M and 1X, respectively. Sample for loading were prepared by mixing 2 to 3 µg of each RNA sample with 1 µl of ethidium bromide (0.5 mg/ml) (Invitrogen, USA), heated to 65°C for 10 min and chilled on ice for 2 min. Subsequently, 5X RNA loading buffer was added to the RNA samples before they were gel analyzed at 70 V for 75 min in the running buffer using 1X MOPS buffer and 2.2 M formaldehyde. The gel was then visualized using GeneSnap software on Gene Genius Bio Imaging System (Syngene, UK).