The Bone Morphogenetic Protein (BMP) System in Zebrafish Ovary

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A Thesis Submitted in Partial Fulfillment

of the Requirements for the Degree of

Doctor of Philosophy

in

Biology

The Chinese University of Hong Kong

September 2011

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Abstract of thesis entitled:

The Bone Morphogenetic Protein (BMP) System in Zebrafish Ovary

Submitted by LI Cheuk Wun

for the degree of Doctor of Philosophy

at the Chinese University of Hong Kong

It is well established that gonadotropins regulate ovarian functions and development. However, increasing evidence suggests that local growth factors released from the somatic components of the follicles or oocytes, or both, also form a regulatory network regulating ovarian functions. Bone morphogenetic proteins (BMPs) comprise a large group in the transforming growth factor-β (TGF-β) superfamily, a family that is essential for morphogenesis and organogenesis. Unlike the mammalian models, few studies have been conducted on the BMP family in fish reproduction except BMP15 and GDF9. Using zebrafish as the model, we have characterized the BMP system in female reproduction, in view of the emerging evidence that BMPs take part in reproduction in addition to growth and differentiation.

Spatiotemporal distribution of selected BMP ligands and receptors in the ovary was investigated. With a newly developed method in our laboratory to mechanically separate the follicular layer and oocyte, we were able to localize the BMP ligands and receptors in the two compartments. Localization studies revealed the presence of the BMP ligands *(bmp2a*, *bmp2b*, *bmp4*, *bmp6* and *bmp7a*) in the denuded oocytes while their type II receptors (*bmpr2a* and *bmpr2b*) were expressed exclusively in the follicular layers, indicating a potential paracrine signaling from the oocyte towards the follicular layer by various BMP ligands, as opposed to the traditional belief that the oocyte is passively controlled and nurtured by the follicular layer for its growth and development. The expression of *bmp2a*, *bmp2b*, *bmp4* and *bmp6* increased during the transition from primary growth (PG) to pre-vitellogenic (PV) stage but decreased as folliculogenesis proceeded. On the contrast, both BMP receptors increased in expression during folliculogenesis, reaching the peak levels prior to oocyte maturation.

Using the Chinese hamster ovary (CHO) cells as the bioreactor, we established two stable cell lines that expressed recombinant zebrafish BMP2b (zfBmp2b) and BMP4 (zfBmp4). Both

BMPs could significantly stimulate phosphorylation of Smad1/5/8 in cultured zebrafish follicle cells, and their activities could be diminished by the recombinant extracellular domains of the putative BMP type II receptors (BMPR2a and BMPR2b). With the recombinant cell lines available, we have developed a novel co-culture system involving the recombinant CHO cells producing BMPs and zebrafish follicle cells. Both zfBMP2b and zfBMP4 could regulate the expression of zebrafish gonadotropin receptors (*fshr* and *lhcgr*) and activin-inhibin-follistatin system (*inhbaa, inhbab, inhbb, inha* and *fst*). Briefly, the recombinant CHO cells were pre-plated, followed by co-incubation with zebrafish follicle cells. The CHO cells secreting zfBMP2b and zfBMP4 would mimic the oocyte secreting factors in the zebrafish follicle and their effects on the expression of potential target genes in the follicle cells were investigated.

zfBMP2b and zfBMP4 were found to be one of the long-sought growth factors that differentially up-regulated luteinizing hormone/choriogonadotropin receptor (*lhcgr*) and down-regulated follicle-stimulating hormone receptor (*fshr*). On the other hand, all activin beta subunits (*inhbaa*, *inhbab*, and *inhbb*) were down-regulated while their antagonist, inhibin alpha (*inha*) and their binding protein, follistatin (*fst*) were up-regulated. To further validate the regulatory effects of zfBMPs, siRNA was applied to knock down BMP4 expression in the CHO cells. The up-regulatory effect on follistatin and inhibin alpha by BMP4 was diminished by siRNA transfection. The modulating effects of zfBMP2b and zfBMP4 on both gonadotropin receptors and the activin-inhibin-follistatin system in folliculogenesis highlight the significance of BMP system in zebrafish ovary and provide important insights into the functionality of BMPs as oocyte-derived factors, particularly during oocyte maturation.

摘要

促性腺激素對卵巢功能和發育的調控作用已被廣泛肯定，但亦有一系列的證據顯示濾泡細胞和卵母細胞也會分泌出各類生長因子，形成一個調控網絡調節卵巢功能。骨成型蛋白質 (BMPs) 是轉化生長因數β(TGF-β) 家族中數目最多的成員。骨成型蛋白質家族對於形態發生和器官形成有重要影響。目前只有很少魚類繁殖的研究是關於骨成型蛋白質家族，遠少於其在哺乳類動物的研究。只有骨成型蛋白質15 (BMP15) 和生長和分化因子9 (GDF9) 在魚類繁殖中曾被探討。基於不斷有研究指出BMP除了在生長和分化有作用外，在生殖中亦起了作用，本研究利用斑馬魚作為模型，研究了斑馬魚BMP家族在雌性生殖系統的各種特性。

本研究首先探討了BMP家族的配體和受體的時空分佈。我們實驗室在近年發明了一種新的方式去分開濾泡細胞和卵母細胞。因此，我們可以清晰地掌握BMP配體和受體在卵泡的分佈。研究結果表明， BMP配體包括*bmp2a*, *bmp2b*, *bmp4*, *bmp6* 和*bmp7a*都在卵母細胞表達，而牠們的受體，即*bmpr2a*和*bmpr2b*則只在濾泡細胞中表達，意味著卵母細胞所製造的BMP家族配體可能對濾泡細胞發揮潛在的旁分泌調控作用。這是有別於一般傳統的觀念：卵細胞是受著濾細胞的控制和滋養。*bmp2a*, *bmp2b*, *bmp4*和*bmp6*的表達在卵泡早期發育時有明顯的上升，但隨著卵泡的發育，其mRNA的水平卻逐漸下降。相反地，兩個BMP受體的表達卻顯著地在卵泡發生過程中不斷增加，直至最後卵泡成熟，並且在卵母細胞成熟前達到最高峰。

利用中國倉鼠卵巢細胞系 (CHO cells)作生物反應器，我們克隆並表達了*bmp2b*和*bmp4*, 成功建立了兩個能穩定表達*bmp2b*和*bmp4* 的細胞系，隨後我們檢查了其生物學活性。 BMP在斑馬魚的原代培養的濾泡細胞中能激活Smad1/5/8磷酸化，而其活性可以被重組BMP受體的胞外區競爭抑制。有了重組BMP的細胞系，我們發明了一種共培養系統，即重組BMP細胞系和原代濾泡細胞一同培養。重組BMP2b和BMP4都能調控斑馬魚促性腺激素受體，激活素，抑制素和激活素結合蛋白。簡單來說，在共同培養的過程中，我們先將重組中國倉鼠卵巢細胞系傳代，待其貼壁後再加上斑馬魚濾泡細胞，使其共培養。中國倉鼠卵巢細胞系分泌出來的zfBMP2b和zfBMP4則可模擬卵母細胞中釋放出來的BMPs ，並直接作用于共培養的濾泡細胞，從而使得我們可以研究牠們對不同耙基因的表達影響。研究發現， zfBMP2b和zfBMP4 是我們尋找了許久的一種可以激活促黃體素受體表達又能同時抑制促卵泡激素受體表達的生長因子。同時地，zfBMP2b 和zfBMP4 能抑制所有激活素β亞基，並且激活牠們的拮抗劑抑制素α亞基 (inhibin α)和激活素結合蛋白 (follistatin) 。為了深入研究zfBMP的調控作用，我們利用siRNA去敲除zfBMP4在中國倉鼠卵巢細胞系中的表達。siRNA轉化可以部分消除zfBMP4對inhibin α和follistatin的激活作用。 zfBMP2b和zfBMP4在促性腺激

素受體和激活素，抑制素和激活素結合蛋白系統的調控作用中突顯了BMP系統作為在卵母細胞生長因子在斑馬魚卵巢中的重要性，尤其是在卵母細胞成熟過程中。

Acknowledgements

Four years of PhD studies have passed incredibly fast and I cannot believe that it has almost come to an end. There have been many happiness and frustrations during my studies. However, I truly enjoy my research work and the working environment.

First of all, I would like to thank my supervisor Prof. Wei Ge for his supervision during these four years. He always contributes fantastic ideas and precious time to interact with me during my studies. I am inspired by him not only on how to conduct research but also how to pursue a career and an enjoyable life. I would like to thank him for his patience and guidance throughout my studies. He teaches me how to think critically and independently in my study, which is essential in conducting research. Besides, I am grateful to have the opportunity to attend the 16th International Congress of Comparative Endocrinology, 2009 at HKU, as well as the 92nd Annual Meeting & Expo held by the Endocrine Society in 2010 at San Diego, CA. These two international meetings broadened my horizon and allowed me to meet with scientists in the field, which benefits me a lot in my study.

I would also like to thank my co-supervisor, Prof. Kin Ming Kwan, as well as my committee members, Prof. Ming Chiu Fung and Prof. Norman Ying Shiu Woo and my external examiners, Prof. Chun Peng and Prof. Chris Kong Chu Wong for their advice and comments on my research and thesis.

Finally, I would like to thank our lab technician, Rita, for her technical support. Also, I will not be able to complete my project without the help and support of my dear labmates. They are wonderful colleagues as well as cheerful friends, who are always ready to share my happiness and sadness. I would like to thank all of them who always give advice on my project as well as support when I failed in my experiments. Last but not least, I would like to thank my parents and my brother for their love and care throughout all the years.

Time flies and I will be leaving CUHK soon. I am grateful to have the opportunity to join Prof. Ge's lab and have enjoyed so much during these four years of study. I wish you all a productive and fruitful life in the future.

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Symbols and abbreviations

Symbols

alpha	α
beta	β
gamma	γ

Abbreviations

Activin β subunit A	*inhbaa*
Activin β subunit AB	*inhbab*
Activin β subunit B	*inhbb*
Activin receptor-like kinase	ALK
Activin type II receptor	ActR-II
Anti-Mullerian hormone	AMH
Anti-Mullerian hormone type II receptor	AMHR-II
Betacellulin	BTC (*btc*)
BMP and activin membrane bound inhibitor	BAMBI
Bone morphogenetic protein	BMP (*bmp)*
Bone morphogenetic protein type II receptor	BMPR-II
Cartilage-derived morphogenetic protein	CDMP
Chinese hamster ovary	CHO
Connective-tissue growth factor	CTGF
Cyclic adenosine monophosphate	cAMP

Decapentaplegic	Dpp
Early vitellogenic	EV
Elongation factor 1-alpha	*ef1a*
Enzyme-linked immunosorbent assay	ELISA
Epidermal growth factor	EGF (*egf*)
Epidermal growth factor receptor	EGFR (*egfr*)
Erythropoietin	EPO
Escherichia coli	*E. coli*
Factor in the germline alpha	FIGα (*Figα)*
Follistatin	FST (*fst*)
Forkhead box O3A	FOXO3a (*Foxo3a*)
Follicle-stimulating hormone	FSH
Follicle-stimulating hormone receptor	FSHR (*fshr*)
Full-grown	FG
Germinal vesicle breakdown	GVBD
Glycogen synthase kinase 3α	GSK-3α
Glycogen synthase kinase 3β	GSK-3β
Gonadotropin	GTH
Growth differentiation factor 9	GDF9 (*gdf9*)
Heparin-binding epidermal growth factor	HBEGF (*hbegf*)
Human choriogonadotropin	hCG

Inhibin alpha	*inha*
Inhibitors of DNA binding/differentiation	Id
Insulin-like growth factor	IGF
Kit ligand	KL
Luteinizing hormone	LH
Luteinizing hormone receptor	LHCGR (*lhcgr*)
Maturation-inducing hormone	MIH
Maturation-promoting factor	MPF
Mid-vitellogenic	MV
Mullerian inhibiting substance	MIS
Pre-vitellogenic	PV
Primary growth	PG
Primordial germ cells	PGC
Phosphoinositide 3-kinase	PI3K
Phosphatase and tensin homolog	PTEN *(Pten)*
Secreted alkaline phosphatase	SEAP
Sex-determining region Y	SRY
Smad ubiquitination regulatory factor-1	SMURF-1
Solute carrier family 38, member 3	*slc38a3*
Transforming growth factor-α	TGF-α (*tgfa*)
Transforming growth factor-β	TGF-β (*tgfb*)

Transforming growth factor β type II receptor	TβR
Truncated BMP receptor kinase-3	tBRK3
Wingless-type MMTV integration site family, member 4	Wnt4
17α, 20β-dihydroxy-4-pregnen-3-one	DHP
5alpha-dihydrotestosterone	DHT

Chapter 1

General Introduction

1.1 Ovarian development and structure in mammals

Sex determination occurs genetically at conception, depending on whether the zygote has the XY (male) or XX (female) chromosomes. For males, Sry, the Y-chromosome gene, is responsible for the testis formation and development of male sexual characteristics, while the female development was thought to be default (1) although Wnt4 and follistatin were reported to be important in female development (2). In this study, by investigating ovary development, we hope to understand more about female reproduction.

Primordial germ cells (PGCs) are the precursors of gametes. They undergo colonization by gonadal tissues. The germ cells are important for the formation and maintenance of the ovary; otherwise, they degenerate into cord-like structures (3). When the PGCs arrive in the gonads, they start proliferating and are called oogonia, which will undergo mitosis and meiosis. In human, by the 6th week of gestation, there are 10,000 oogonia. The number of oogonia increases to 600,000 at the 8th week of gestation and 6 million by the 20th week of gestation. The rate of oogonial mitosis then declines and ends at the 28th week of gestation, counteracting with the increasing rate of atresia. The result is that the newborn ovary contains 1 million germ cells, of which 3000-4000 remain at puberty, while less than 1% will ovulate (4). It is also believed that post-natal oogenesis can oocur as well (5).

The ovary, as the sexual organ for females, possesses two major functions. First, the ovary is responsible for the production of fertilizable oocytes, which can potentially unite with sperm to form zygotes. Second, the ovary is responsible for the secretion of steroid hormones for preparation of fertilization and pregnancy as well as maintaining secondary sexual characteristics and regulation of sexual behavior (6). The follicle is the functional unit of ovary, which undergoes successive stages of growth and final maturation.

The development of the follicle starts from the primordial follicle stage, of which the follicles first appear in small size, with the oocyte surrounded by pregranulosa cells. The follicles are arrested at diplotene stage (4). Some follicles are activated during the female reproductive life, while some remain quiescent. It is suggested that primordial follicles are activated when inhibitory signals are removed, leading to the initiation of growth (7). The

primordial follicle activation is thought to be gonadotropin-independent, since no follicle-stimulating hormone receptor (FSHR) is expressed in primordial follicles (8). Kit ligand and its receptor Kit (KL-Kit) were reported to be important for primordial follicle growth when FSHR was not expressed (9-11). PI3K signaling, which is the major signaling pathway activated by KL-Kit, has been reported to be functional in primordial follicle activation (12). It was reported that KL activated PI3K signaling pathway in cultured primary oocytes of mouse and rat, inducing the phosphorylation of Akt, Foxo3a, glycogen synthase kinase 3α (GSK-3α) and GSK-3β (13, 14).

Deletion of some other factors in the oocyte also revealed their importance in primordial follicle activation. For example, deletion of PTEN as a tumor suppressor gene in the oocyte in the knockout mice caused premature activation of the primordial follicle pool, implying their suppressive effect in primordial follicle activation (15). Also, the ovaries of *Foxo3a* knockout mice had larger number of growing follicles with the primordial follicles pool depleted, indicating the suppressive role of Foxo3a in primordial follicle activation (16).

During the transition from primordial to primary stage follicles, the flattened granulosa cells become cuboidal. In addition, the oocyte diameter increases, accompanied by zona pellucida acquisition (17). During the transition from primary to preantral and antral follicle stage (also known as secondary follicles), the granulosa cells undergo mitosis, resulting in multiple layers. The oocyte diameter continues to increase. There is also the formation of basal lamina, which is the blood-follicle barrier separating the granulosa cells and theca cells. Zona pellucida and the theca layer also form. The most observable change of this transition stage is the development of fluid-filled spaces within the granulosa cells, which will coalesce to form antral cavity (18). Growth differentiation factor 9 (GDF9) was reported to increase the number of primary and secondary follicles in human and rodents, indicating its importance in follicle growth (19, 20). This is further evidenced by the knockout and mutational animals of GDF9, which suffered from arrested follicle growth (21, 22). Bone morphogenetic protein 15 (BMP15) is another growth factor that regulates this transition. BMP15 stimulates granulosa cell proliferation independent of FSH (23). Other positive regulators during this transition include transforming growth factor-β (TGF-β) from the granulosa cells and theca cells, as well as activins from the granulosa cells (24, 25). On the contrast, anti-Mullerian hormone (AMH) was reported to play an inhibitory role during this transitional process (26).

As the antral follicles grow, they become gonadotropin responsive. Also, their steroidogenic activity is modulated and prevention of premature luteinization is needed in order to select a dominant follicle for maturation (4). Activin A was shown to be a regulator in this process through regulating aromatase activity, estrogen synthesis, luteinizing hormone receptor (LHR) expression and oocyte maturation (27). On the contrast, AMH played a negative role by reducing the responsiveness of antral follicles to FSH, affecting the selection process (28, 29). Also, as the antral follicles grow, inhibin A becomes dominant rather than activin A, thus increasing the effect of LH for the follicles to mature (30).

Ovulation occurs as the follicles mature. During ovulation, the oocyte is released for fertilization. The remaining granulosa cells become luteinized to form corpus luteum, which releases progesterone for maintaining pregnancy. It is the LH surge that leads to ovulation. Furthermore, the EGF-like factors including amphiregulin, epiregulin, betacellulin induce changes in granulosa cells leading to resumption of meiosis in the oocyte and the formation of extracellular matrix, causing cumulus expansion, facilitating the release of oocyte (31).

1.2 Ovarian development and structure in teleosts

Like other vertebrates, pituitary gonadotropins are the major hormones controlling the growth and maturation of the ovary in teleosts, while the ovarian steroid hormones also play an indispensable role. The ovarian follicle of teleosts consists of the oocyte and the surrounding follicle cells, namely the granulosa cells and theca cells. The granulosa cells surround the oocyte, while the outer theca layer also contains connective tissues, capillaries and collagen fibers. The granulosa cells and theca cells are separated by the basement membrane (32).

The fish ovary can be synchronous, in which the ovary contains oocytes at the same stage of development, or it can be asynchronous, in which different stages can be found (32). The zebrafish ovary consists of two lobes. The eggs are ovulated into the ovarian lumen, which then pass through the oviduct and are led to the genital opening. The ovigerous lamellae consist of follicles of different stages that are randomly arranged. The growing oocytes are enclosed within the ovarian follicles and surrounded by the follicle cells (33).

Oocyte development of zebrafish can be divided into five stages, according to their size and morphology. The five stages are namely primary growth stage (Stage I), cortical alveolus stage (Stage II), vitellogenesis stage (Stage III), oocyte maturation stage (Stage IV) and mature

stage (Stage V) (33). Each stage is characterized by its morphology as well as physiological and biochemical events, although we mainly focus on describing the morphology in this chapter.

During the primary growth (PG) stage (Stage I), the oocyte is arrested in the first meiotic division. The follicles at primary growth stage are around 100 μm in diameter and they appear to be transparent. The primary growth stage can be divided into 2 phases, depending on whether the oocyte is within a nest or within a follicle. In the first phase, namely the pre-follicle phase, the oocytes reside in nests and are separated from the ovarian stroma by a single layer of follicle cells. As the oocytes grow, they are enclosed by pre-follicle cells which separate them into individual follicles. In the second phase, namely the follicle phase, the oocytes leave the nest and become individual follicle. The oocyte is surrounded by a layer of follicle cells lying on the basement membrane. It is then further covered by theca cells, which is a connective tissue compartment (33).

The cortical alveolus stage (Stage II) follows the primary growth stage. This is corresponding to pre-vitellogenic stage (PV) as described in our study. The follicles of size around 250 μm in diameter are classified into pre-vitellogenic stage. During this stage, cortical alveoli appear as yolk vesicles. The follicles increase in size as the cortical alveoli proliferate. The follicles become opaque, versus the transparent appearance at primary growth stage. The vitelline envelope also forms, which is the future chorion. The follicle cells become cuboidal and further divide (33).

After the pre-vitellogenic stage, the follicles enter vitellogenesis (Stage III). Follicles of size around 350 μm to 650 μm in diameter are classified into this stage. Our group further divides this stage into early vitellogenic stage (EV, ~350 μm), mid-vitellogenic stage (MV, ~450 μm), late vitellogenic stage (LV, ~550 μm) and fully-grown stage (FG, ~650 μm), according to their size and morphology. During vitellogenesis, the follicles increase in size. Vitellogenin is sequestered by the oocyte and processed into yolk proteins. The yolk proteins accumulate and lead to the increase in size of the follicles. As vitellogenin accumulates, the cortical alveoli are displaced to the periphery. The follicles are not responsive to endogenous hormones at primary growth and pre-vitellogenic stage. Yet, they become gonadotropin-dependent at the vitellogenic stage, as they approach oocyte maturation. Under the influence of gonadotropins, estrogen is produced by the ovary, which stimulates the synthesis of vitellogenin in the liver. The

vitellogenin is then taken up by the developing oocyte. The accumulation of vitellogenin leads to the growth of the oocyte and provides nutrition needs for the developing embryo (32, 33).

During oocyte maturation (Stage IV, ~0.65-0.75 μm), meiosis is resumed. During final oocyte maturation, the germinal vesicle migrates to the periphery and the nuclear envelope breaks down. Germinal vesicle breakdown (GVBD) is a marker for oocyte maturation, in which the follicles become translucent due to cleavage of yolk proteins and ooplasmic clearing. As the follicles mature, the interior of the follicles become more homogenous as the yolk proteins encounter structural changes (33).

Oocyte maturation is regulated by three major mediators (32). Gonadotropin is the primary mediator of oocyte maturation. The gonadotropins are the primary hormones from pituitary that stimulate growth and trigger oocyte maturation. Follicles mature by injection of gonadotropins preparation or when they are incubated *in vitro* with gonadotropins preparation. Two types of gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), were predominant during vitellogenesis and oocyte maturation respectively. LH is the major hormone that triggers oocyte maturation.

Maturation-inducing hormone (MIH) is the secondary mediator of oocyte maturation. 17α, 20β-dihydroxy-4-pregnen-3-one (DHP) is a naturally occurring maturation-inducing hormone in fish, and it is an effective inducer of GVBD in amago salmon and other teleosts. In a study using salmon, the follicles respond to gonadotropins by producing DHP. Both granulosa cells and theca cells are necessary for the production of DHP. Theca cells produce 17α-hydroxyprogesterone as the steroid precursor, which is metabolized to DHP in the granulosa cells by 20β-HSD, an enzyme that is involved in the conversion of the precursor to DHP (32).

Maturation-promoting factor (MPF) is the tertiary mediator of oocyte maturation. MPF activity was first identified when the cytoplasm from mature fertilized goldfish oocytes was injected into starfish oocytes to induce GVBD. MPF was found in extracts of mature oocyte under the influence of DHP, but not in immature oocytes. MPF is believed to be the cytoplasmic mediator of MIH, mediating the action of MIH that acts on the receptors on the oocyte plasma membrane. Fish MPF consists of cdc2 kinase and cyclin B. Threonine phosphorylation of cdc2 kinase and serine phosphorylation of cyclin B are required for activation of MPF (32).

The final stage of oocyte development is the mature stage (Stage V, ~750 μm). The mature eggs are ovulated into the ovarian lumen and they are fertilizable (33). They are

translucent in appearance. Usually, it takes 10 days for the follicles to grow from primary growth stage to mature stage in zebrafish (34).

1.3 Oocyte control of folliculogenesis

1.3.1 Metabolic cooperativity between the oocyte and granulosa cells

Although it is well established that gonadotropins play vital roles in development and function of vertebrate ovary, bi-directional communication between oocyte and the somatic compartments of the follicle is also needed (35). Granulosa cells are well-known to nurture the oocyte by providing nutrients (36). However, the oocyte also plays an active role in orchestrating the development of the oocyte, for example the formation and activation of primordial follicles. This is supported by the findings that targeted deletion of *Figla*4, a germ cell-specific transcription factor, caused failure of primordial follicle formation (37). Also, oocyte-specific deletion of *Pten* causes premature activation of the primordial follicle pool, demonstrating the importance of oocyte for the development of the follicle (15).

Besides regulating follicle growth, the oocyte is also involved in promoting granulosa cell proliferation, cumulus expansion, as well as orchestrating the rate of ovarian development. The oocyte and the granulosa cells are coupled through gap junctions which allow the delivery of metabolites and amino acid, while the knockout of these gap junctions would disrupt folliculogenesis (38).

There is the presence of metabolic cooperativity between the oocyte and granulosa cells as demonstrated in processes such as glycolysis, amino acid uptake and cholesterol biosynthesis (39). Denuded oocytes cannot utilize glucose as energy for maturation. Nevertheless, the cumulus cell-enclosed oocytes could do so. However, denuded oocytes can mature with pyruvate supply (40). The cumulus cells actually metabolize glucose into pyruvate for the oocyte during glycolysis (41). This is proven by the presence of the transcripts encoding key enzymes in glycolytic pathway in the cumulus cells but not the oocytes (42).

Another example showing metabolic cooperativity between the oocyte and the granulosa cells is the uptake of amino acids. The mouse oocytes cannot uptake amino acids. In an experiment performed in 1983 by Colonna and Mangia, the cumulus cell-enclosed oocytes or denuded oocytes were cultured in media containing [^{14}C] L-alanine. It was found that radioactivity was higher in the cumulus cell-enclosed oocyte, demonstrating the cumulus cells

took up the L-alanine and transported to the oocyte (43). The transcript of *slc38a3* which encodes an amino acid transporter was highly expressed in cumulus cells but not in the oocyte, providing further evidence that the cumulus cells bear amino acid transporter to deliver amino acids to the oocyte (44).

A third example of metabolic cooperativity between the oocyte and granulosa cells is cholesterol biosynthesis. As the oocyte cannot synthesize cholesterol nor uptake cholesterol, they require the cumulus cells for synthesizing and providing cholesterol to them. The transcripts of the genes involved in cholesterol biosynthesis pathway are expressed in cumulus cells but not the oocytes, giving proof that the cumulus cells as the source of cholesterol for the oocyte (45).

1.3.2 Oocyte control of metabolic activities

Having seen that metabolic cooperativity exists between the oocyte and granulosa cells, the oocyte actually takes control of the metabolic activities in granulosa cells as well. There are two types of granulosa cells, classified according to their proximity to the oocyte. The mural granulosa cells are farther away from the oocyte, while the cumulus cells surround the oocyte. Some genes that are involved in metabolic processes such as glycolysis and amino acid transport have higher expression in cumulus cells (42, 44). However, the oocyte may be influencing their expression and the metabolic process involved. In an experiment with the oocyte removed (OOX, oocytectomy), there was a reduction of transcripts encoding key enzymes involved in glycolysis and amino acid transporter. Glycolytic activity and uptake of radioactive L-alanine were also reduced in OOX cumulus cells. However, when co-cultured with the fully-grown oocytes, the transcript levels of those key genes involved in glycolytic activity and uptake of radiolabeled L-alanine increased, indicating that the oocyte is responsible for promoting these metabolic activities (42, 44). Furthermore, in mouse, the oocyte promotes cholesterol biosynthesis in cumulus cells. There is a higher transcript level of enzymes responsible for cholesterol biosynthesis in the cumulus cells versus the more distant mural granulosa cells. In OOX cumulus cells, there was reduced level of these transcripts encoding enzymes responsible for cholesterol biosynthesis. Co-culturing with fully-grown oocytes restored the transcript levels, suggesting that the oocytes promote the expression of these transcripts for cholesterol biosynthesis through paracrine factors derived from the oocyte (45).

1.3.3 Oocyte factors regulating metabolic processes

In the oocyte, there are various growth factors that could possibly be involved in promoting follicle growth. GDF9 and BMP15 are the most widely studied examples in the mammals. They are both oocyte-derived factors, and were demonstrated to be important in reproductive function in mammals. This is supported by the phenotype of the knockout animals. *Gdf9 -/-* mice are infertile and the granulosa cells were unable to proliferate in primary follicles (21), while *Bmp15 -/-* mice exhibited defects in ovulation and fertilization (46). In the double mutant *Bmp15 -/- Gdf9 +/-* (DM), the cumulus cells underwent abnormal development before LH surge (47). The expression of some transcripts were affected in *Bmp15-/-* and DM cumulus cells. These transcripts were mostly involved in metabolic processes such as glycolysis and sterol biosynthesis (45). As *Bmp15-/-* oocytes cannot promote the expression of these transcripts involved in cholesterol biosynthesis and glycolysis in the OOX cumulus cells, this therefore suggested the importance of BMP15 in regulating these transcripts in the metabolic processes (45).

As a conclusion, the oocyte control of folliculogenesis is based on the fact that oocyte cannot metabolize glucose, carry out cholesterol biosynthesis, nor uptake of amino acids for follicle growth and development. Therefore, the oocyte participates in follicle growth and development by promoting the expression of the transcripts that encode the key enzymes involved in these metabolic processes in the cumulus cells, which then provide the oocyte with their nutritional and energy needs.

1.3.4 Oocyte control of folliculogenesis rate

It was reported that the oocyte controls folliculogenesis by orchestrating the rate of follicular development (48). The mouse oocyte usually takes 12 days to reach the secondary follicle stage, while it takes 18-24 days to become full-grown. Eppig et al. carried out a grafting experiment by isolating the oocytes from secondary follicles on day 12 and reaggregated them with the somatic cells of newborn mouse ovaries. They discovered that it took only 9 days for the follicles to become fully grown, with the presence of antral cavity. The reaggregated follicle exhibited abnormal pattern of granulosa cell differentiation, with LH mRNA expression in mural granulosa cells but not cumulus cells, as usual. Also, cumulus expansion was accelerated and the follicles were able to resume and complete meiosis. More importantly, they were capable of

undergoing fertilization and developing into the blastocyst stage. Therefore, the oocyte not only influences the metabolic processes to support the growth of the follicle, but it also orchestrates the rate of follicle development, leading to the conclusion that the oocyte takes an active role in follicle development, instead of being passively nurtured by the somatic compartment for its growth and development (48).

1.4 BMP family

1.4.1 BMP ligands

1.4.1.1 History of the discovery of BMPs

Bone Morphogenetic Proteins (BMPs) were first identified by Urist in 1965, referring to the decalcified bone matrix that are capable of inducing bone formation when implanted at ectopic sites (49). Nowadays, BMPs are identified as pleiotropic cytokines that can regulate growth, differentiation and apoptosis in various tissues (50). Yet originally, BMPs were found to be involved in morphogenesis of bone and cartilage. In the classical study by Urist in 1965, the bone from different donors (mouse, rat, guinea pig, rabbit, calf, human) underwent different preparations such as by acids, chemical treatment or heat treatment, followed by implanting into various sites (mostly the rectus abdominus) into the host, which sometimes the same or different. The implant was invaded by mesenchymal cells, which later developed into cartilage-forming cells. The bone cells later replaced the cartilage and deposited as bone (49).

The bone consists of three components. First it is the mineral component that is responsible for its structural integrity. Second, it is the collagenous matrix component. Third, it is the growth factor component that contains BMP activity. After mineralization of the bone using acids, this growth factor component can be extracted (51). Sampath and Reddi did an experiment by reconstituting the bone extracts from bovine with the rat bone collagenous matrix, with the endogenous BMP activity removed. They implanted this reconstituted fractions of bone extracts and collagenous matrix into the rat. The implanted area was invaded by undifferentiated cell types, after which the undifferentiated mesenchymal cells differentiated into chondrocytes, which later became mature and calcified. New bone later replaced the cartilage (52). Although it was known that the demineralized extracts could induce bone formation, they were not able to determine which proteins were performing the osteogenic activity (53). Therefore, an alternative approach was undertaken to identify these proteins with potential osteogenic activity (54).

Bovine bone proteins that had bone inducing activity were isolated. The mixture of polypeptides was digested with trypsin. Wozney et al. deduced the amino acid sequence from the highly purified preparation of BMPs obtained from bovine bones. After that, probes were designed which hybridized to a recombinant from a bovine genome library. This recombinant was then used as a probe to screen the human cDNA encoding the protein. BMP1, BMP2A, BMP2B and BMP3 were identified in this way. The cDNA inserts were cloned into vector and expressed in COS cells and CHO cells. The recombinant proteins were proven to induce cartilage formation. BMP1 through BMP7 were all identified (54, 55).

Wozney et al. asked the questions whether the recombinant BMP proteins that they produced would be therapeutically useful in humans. Therefore, they collaborated with others and performed some experiments to demonstrate the induction of bone in the segmental defect model. Together with Yasko et al. (1991), they created a femoral defect in the rat and implanted recombinant human BMP2 (rhBMP2) in the defect. With the defect treated with rhBMP2, bone formation was observed with high dose of rhBMP2, demonstrating the bone inducing activity of rhBMP2 (51).

In the second study, Wozney et al. collaborated with Gerhart et al. A defect was created in the sheep femur and BMP2 was implanted in the defect. Biomechanical studies showed that the BMP2-treated bone resulted in large amount of bone formation, again demonstrating the bone-inducing activity of BMP2 (51).

In the third study, Wozney et al. collaborated with Toriumi et al (56), created defect in the dog mandibles. When treating with BMP2, the defect was filled with bone after 3 months, identifying the ability of BMP2 to induce bone formation.

Other BMP family members were later identified and many of them were identified by homology-based cDNA cloning. As a result, not all of them were proven to have osteogenic activity. In addition, they were given different names such as osteogenic protein, growth and differentiation factor (GDF) and cartilage-derived morphogenetic protein (CDMP). Table 1.1 listed some alternative names for the BMP family members (57).

Zebrafish *bmp2a*, *bmp2b* and *bmp4* were first cloned by Martinez-Barbera in 1997 (58). The deduced amino acid sequences showed high homology to the mammalian counterparts. In addition, their mRNA expression was detected in zebrafish embryos by *in situ* hybridization, opening the door to investigate more of the roles of BMPs in zebrafish development. Up to date,

there are 16 zebrafish BMPs being identified. A phylogenetic analysis has been performed to compare the sequences of the zebrafish BMPs with their mammalian counterparts, human and mouse. It was observed that individual BMP ligands have high homology among themselves in the three species (Fig. 1.1).

BMPs are morphogens, as indicated by the name, "bone morphogenetic protein". BMPs exert different effects on cells depending on the concentration gradient. The same BMP might affect cells in different ways, for example, stimulating proliferation or promoting differentiation, depending on the concentration. For example, BMP4 as a morphogen can pattern the *Xenopus* epidermal cells into different fates at high and low concentration (59). It was also demonstrated that a gradient of BMP activity specified the dorsal-ventral fate in early *Xenopus* embryos, although the gradient was not caused by simple diffusion of BMP4 but the inhibitory binding proteins that act on a uniform level of BMP4 to specify dorsal-ventral patterning (60).

1.4.1.2 Structure

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor-β (TGF-β) superfamily, which consists of more than 35 members, including the BMPs, TGF-βs, activins/inhibins, Mullerian inhibing substances (MIS) and some others. BMP family is the largest among the TGF-β superfamily, which consists of around 20 members (61). Different from TGF-β and the activin family which have nine conserved cysteine residues, BMPs are characterized by seven conserved cysteine residues, which form a specialized structure called a cystine knot (62, 63). One of the conserved cysteine residues forms a disulfide bridge to form dimer (64, 65). It is believed that BMPs form homodimers and heterodimers. Heterodimers were shown to be more active both *in vivo* and *in vitro* (66) . GDF9 and BMP15 lack the cysteine residue which is for disulfide bridge formation (67, 68). Yet, it was demonstrated that GDF9 and BMP15 form homodimers that are non-covalently linked and also heterodimers when co-expressed (69). Therefore, it is not necessary to form disulfide bridge in order for the BMP subunits to dimerize.

BMP family members share structural homology with others, with the exception of BMP1, which is a metalloprotease that can cleave procollagen I, II, III into monomers. BMP1 can also cleave chordin, which is a BMP binding protein, in order to antagonize BMP activities (70).

BMPs undergo post-translational processing in order to become biologically active. They are first translated as preproproteins, which consist of the signal peptide, prodomain and mature domain. The proprotein dimerizes upon removal of the signal peptide. The proteolyic enzymes then cleave the proprotein at RXXR site to generate the mature dimer protein (71) (Fig. 1.2).

1.4.1.3 Function

BMPs are multifunctional cytokines that participate in cell proliferation, differentiation and apoptosis in various tissues (72). BMPs have wide range of functions in different species, such as mesoderm formation and patterning. During gastrulation in *Xenopus*, BMP4 as a ventralizing factor promotes ventral mesoderm cell fate in the marginal zone (73). BMP4 also participates in neural patterning by inducing the formation of epidermal cells versus neural tissues at the ectoderm (59). In terms of skeletal development, BMPs promote multipotent stem cells to become chondrogenic or osteogenic, versus myogenic or adipogenic (74, 75). It was also suggested that BMPs promote growth of cells that are committed to chondrogenic or osteogenic lineage, while at the same time promoting the differentiation of cells that are committed to other lineages (76). In mouse, BMP2, BMP4 and BMP7 have different expression patterns in the mesenchyme of the limb, suggesting their potential roles in limb patterning (77). BMPs are also involved in kidney development. BMP5 was demonstrated to have a role in ureter development (78) while BMP7 was important for nephrogenesis (79). Besides, BMPs participate in tooth development by regulating cell proliferation, apoptosis, epithelial-mesenchymal interactions and differentiation in different stages of tooth development (80). In addition, with *bmp4*, *bmp5* and *bmp7* detected in embryonic lung, a potential role of BMPs in lung development is implicated (78).

In the zebrafish, BMPs have been widely studied in developmental area. For example, *bmp2a* and *bmp2b* were identified to be important in fin development (81). *bmp6* is involved in fin regeneration (82). *bmp2b* is involved in morphogenesis of the semi-circular canals (83), while *bmp4* helps establishing left-right asymmetry (84). *bmp7* participates in dorsal-ventral patterning (85, 86), while the new homolog, *bmp7b,* plays a significant role in organ development (87).

1.4.2 BMP receptors

There are two major types of membrane-bound receptors in the TGF-β superfamily, namely the type I and type II receptors. The TGF-β receptors contain the extracellular binding domain, transmembrane domain and intracellular domain which has the serine/threonine kinase regions. The difference between type I and type II receptor is that the type I receptor has a characteristic GS domain (SGSGS), which is important for signal transduction (88). The first receptor that was cloned in the TGF-β superfamily was an activin type II receptor, ActR-II (89). Following the cloning of ActR-II, other mammalian type II receptors were cloned, including ActR-IIB, AMHR-II, BMPR-II and TβR-II (90-94). The type I receptors, also called activin receptor-like kinase (ALK) 1-7 were also subsequently cloned (95-99). A novel type I receptor, ALK8, was also cloned (100). BMPR-II is specific for BMP ligands, including BMP2 (101), BMP4 (101, 102), BMP6 (103), BMP7 (93, 101), BMP15 (104) and GDF9 (105). Yet, some BMP ligands may bind to activin receptors. In contrast to BMPR-II which specifically binds BMP ligands but not the activins, ActR-II and ActR-IIB can bind BMP6 (103), BMP7 (106), GDF5 (107) in addition to activins. For type I receptors, ALK2 (ActR-IA), ALK3 (BMPR-IA) and ALK6 (BMPR-IB) have been designated as BMP type I receptors (107-110). BMP7, BMP2 and GDF5 have been shown to bind ActR-I (ALK2) as well as BMPR-IA and BMPR-IB. Although a particular BMP ligand may bind different type I receptors in different cell types, there are still some preferences for the BMP ligands to bind to particular BMP type I receptors. For example, BMP2, BMP4 and GDF5 preferentially bind ALK3 or ALK6 (107, 108, 111), while BMP6 and BMP7 mostly bind ALK2 or ALK6 (103, 106, 108, 110, 111).

It is well established that for TGF-β and activins, the ligands bind to type II receptors first, which then recruit type I receptors, since the ligands do not have affinity for type I receptor but type II only (90). However, the situation is different for the BMPs. BMPs have little affinity for BMPR-II but more for type I receptors. Yet, the affinity of BMPs for BMPR-II is increased if BMPR-II is over-expressed with type I receptor (102, 108, 109). Therefore, the two types of receptors may act together to form a complex to bind BMP ligands. However, it was also proposed that BMP ligands bind to type I receptors first, and then recruit type II receptors (104, 112). This proposed model is in contrast to that for TGF-β and activins, which bind to type II receptors first, then recruit type I.

Disruption of the BMP receptors causes blockade of BMP signaling and thus affects the development processes. In *Xenopus*, BMP4 induces ventral mesoderm formation (73).

However, the truncated form of BMPR-IA could block BMP4 signaling, resulting in dorsal mesoderm formation (113, 114). In chicken, BMPs mediate apoptosis in the interdigital regions of the chicken feet. However, the dominant negative form of BMPR-IB could block BMP signaling, causing the formation of webbed feet as well as the transformation of scales to feathers (115).

In mouse embryos, BMPR-IA has ubiquitous expression (except in the liver) while BMPR-IB has a more restricted expression (116). This may reflect their differential roles in organogenesis. Yet, both of them were observed in developing cartilage and bone, indicating that they have potential roles in bone morphogenesis (117).

1.4.3 Smads

Smads are intracellular molecules that participate in BMP signaling. Smads were first identified in *Drosophila*. The BMP-2/4 homologue, Decapentaplegic (Dpp) binds to type II receptor, Punt and type I receptor, Thick veins and Saxophone (118). Mother against dpp (*Mad*) was identified in a genetic screen as enhancer for *dpp* (119, 120). *Mad* mutants were found to have similar phenotype as *dpp* mutants (120). Besides, *Mad* could partially rescue the eye phenotype of *dpp*, suggesting *Mad* acted downstream of *dpp* (121). In *C. elegans*, *sma-2*, *sma-3* and *sma-4* were identified as they had similar phenotypes as *daf-4* mutants (122), which encode serine/threonine kinase. In addition, *daf-4* was unable to rescue *sma-2* mutation, indicating that Sma molecules acted downstream of *daf-4*. *sma-2*, *sma-3* and *sma-4* were structurally similar to *Mad* in *Drosophila* (122). *Xenopus* Xmad 1-4 and mammals MADR1 and DPC4 were also found to be homologous to Sma and Mad (123-126). For easy nomenclature, the name "Smad" was suggested for the vertebrate homologue of Mad and Sma (118).

Smad molecules are around 50-55 kDa. They have conserved N-terminal and C-terminal domains, named MH1 and MH2, which are linked by a spacer sequence that is rich in serine, threonine and proline (88). It is believed that MH1 and MH2 domains make contact with each other while in inactive form, and they open up upon activation by the receptors (118). In BMP signal transduction, it is proposed that type II receptor phosphorylates type I at the GS domains, which is rich in glycine and serine (96, 127). The phosphorylated type I receptor then transphosphorylates the Smad molecules. The Smad molecules that are phosphorylated by type I receptor are called R-Smads (receptor-activated Smads). Smad 1, 2, 3, 5 and 8 are R-Smads.

Activins and TGF-β use Smad 2 and 3 as R-Smads while Smad 1, 5 and 8 are specific for BMPs (128, 129). The common partner for R-Smads, Smad 4, is known as Co-Smad. Smad 4 interacts with Smad1/5/8 to form a complex, which translocates into the nucleus and interacts with other transcription factors to regulate gene expression (130). In addition, there are inhibitory Smads, Smad 6 and 7, that can inhibit BMP signaling pathway by inhibiting the phosphorylation of Smad1/5/8 (131, 132).

1.5 Regulation of BMP action

There are two types of regulation of BMP action, namely extracellular and intracellular. In extracellular regulation, BMP ligands are prevented from access to the receptors by BMP binding proteins, while in intracellular regulation, inhibition of intracellular signaling molecules are involved.

Follistatin is an activin-binding protein. However, it was found that follistatin could also regulate the activities of BMP4, BMP7, BMP4/7 heterodimers and BMP15 (106, 133, 134). Mice over-expressing follistatin show defects in reproduction. For example, female mice over-expressing follistatin have small ovaries as a result of folliculogenesis (135). This could be the result of action of follistatin in inhibiting BMPs. Besides follistatin, there are also other BMP binding proteins responsible for extracellular regulation. Connective-tissue growth factor (CTGF) is another BMP-binding protein that binds BMP4 and TGF-β. Yet, CTGF exerts different actions on them. CTGF inhibits the activity of BMP4, while enhancing that of TGF-β (136). Noggin is also a binding protein that exerts inhibitory actions on BMP2, 4, 7, 14 and GDF5 (137-140). Chordin, another binding protein, binds BMP2 and BMP4 with high affinity, while binding BMP7 with low affinity (141, 142). Noggin and chordin are important regulators in development. Inhibin belongs to TGF-β superfamily. It binds to β-glycan, which is a membrane-bound binding protein, which then associates with activin receptor, sequestering them and thus blocking activin signaling (143). It was found that inhibin A can inhibit BMP signaling by sequestering ActR-II, ActR-IIB and also BMPR-II (144).

Besides extracellular regulation by the BMP binding proteins, intracellular regulation is also performed by the intracellular Smad proteins, the inhibitory Smads, including Smad 6 and 7. It was found that Smad 6 can form complex with Smad 1, competing with Smad 4 to form the complex to be translocated into the nucleus (145). BMP and activin membrane bound inhibitor

(BAMBI) resembles type I receptor in structure, though lacking the intracellular serine/threonine kinase domain. BAMBI competes with type I receptor for ligand binding and thus inhibits the signaling of BMP ligands as well as other members in the TGF-β superfamily (146). SMURF-1 (Smad ubiquitination regulatory factor-1) targets Smad 1 and Smad 5 for ubiquitination and leads to proteasome degradation, thus inhibiting BMP signaling. SMURF-1 is specific for BMP signaling based on its specificity in targeting Smad 1 and Smad 5 only (147).

1.6 BMP family in reproduction

1.6.1 Reproductive function of BMP family in mammals

In addition to bone formation, BMPs have been demonstrated by numerous studies to be pleiotropic factors that play important roles in growth, differentiation and apoptosis in various tissues. For example, in *Xenopus*, BMP4 functions as a ventralizing factor, patterning the development of the embryo (73); and it promotes epidermal fate of the tissue versus neural fate (148). BMPs are also involved in limb patterning. Expression of BMP2, BMP4 and BMP7 occurs in the forelimbs at different stages of mouse embryonic development (77). In recent years, the physiological importance of BMPs in reproduction has been demonstrated (71). A functional BMP system in the ovary was demonstrated using primary cultures of rat granulosa cells, supported by the finding that both BMP4 and BMP7 could increase FSH-induced estrogen production and decrease FSH-induced progesterone production (149). In addition, BMP4 and BMP7 have been shown to promote early folliculogenesis in the rat and mouse model respectively (57, 150). A study revealed the expression of BMP15 in rat granulosa cells exerting stimulatory actions on granulosa cell proliferation and inhibitory actions on progesterone production (151). Using cultured sheep granulosa cells, it was demonstrated that BMP2 could increase FSH-induced estradiol and inhibin A production (152), while using cultured rat granulosa cells, it was demonstrated that BMP6 could inhibit FSH-induced progesterone synthesis (153). BMP15 was identified as an oocyte factor and it was found that BMP15 could stimulate granulosa cell proliferation as well as inhibiting FSHR expression (23, 154). Thus, numerous studies have reported the functional roles of BMPs in mammalian reproductive system, emphasizing the importance of BMPs in reproduction.

Knockout and mutational studies provide convincing evidence of BMPs in reproduction. For example, the Inverdale strain of sheep was found to carry a single point mutation in the

BMP15 gene. Both the heterozygous and homozygous displayed aberrant reproductive phenotypes, with the heterozygous Invedale female mutants having increased ovulation rates and precocious follicle maturation, while the homozygous Inverdale female experienced infertility because of arrested follicle development at primary follicle stage (155-157). Besides the naturally occurring mutations, knockout animals also provide important hints on the role of the BMP family in reproduction. GDF9 knockout mice were created, with the observation that homozygous females being infertile as a result of arrested folliculogenesis at the primary stage (21). Further characterization of the GDF9 knockout homozygous females revealed the importance of GDF9 in regulating granulosa cell proliferation, differentiation of follicle cells and oocyte development (158, 159).

Mutations in the BMP receptors also disrupted BMP signaling in the ovary and led to defects in reproductive function. For example, mutation of *bmpr1b* ewes were associated with increased ovulation (160). In addition, null knockout of *bmpr1b* in mice resulted in defects in cumulus expansion (161). The demonstrated functional roles of BMPs and the knockout/mutational studies revealed the important roles that BMPs play in mammalian reproduction.

1.6.2 Reproductive function of BMP family in teleosts

Although quite a number of studies suggested the importance of BMPs in mammalian reproduction, relatively fewer studies have been performed on teleosts. A real-time PCR-based gene expression survey was performed in the rainbow trout to investigate the expression profiles of some target genes, including BMPs, during maturation competence acquisition or oocyte maturation. *bmp7* increased in expression in maturing females and those females that acquired high competence to mature, while *bmp4* expression increased later at the time of oocyte maturation, suggesting potential role of BMPs in the control of oocyte maturation in the rainbow trout (162). A similar study was performed later in the rainbow trout, focusing on selected target genes during gonadal differentiation and early gametogenesis. *bmp7* was found to be among the group of genes with the highest relative expression in the ovary during gonadal differentiation and at the beginning of gametogenesis, while *bmp4* showed a similar expression profile in testis and ovary (163). Recently, the spatiotemporal expression of *bmp4* and *bmp7* has been investigated in the rainbow trout (164). In this study, Lankford et al. looked at the expression

pattern of *bmp4* and *bmp7* during folliculogenesis. They found that *bmp4* and *bmp7* peaked at pre-vitellogenic stage and steadily decreased in the following stages. This implied a potential role of *bmp4* and *bmp7* in promoting early folliculogenesis. In another study, *gdf9* and *bmp15* cDNA were cloned in the European sea bass and had their expression examined in the ovarian reproductive cycle (165). It was found that *gdf9* and *bmp15* had high level of expression during primary oocyte growth and pre-vitellogenic stage, followed by a decline during vitellogenesis and maturation. This might suggest an important role of *gdf9* and *bmp15* in early primary oocyte growth.

In zebrafish, only *gdf9* and *bmp15* in the BMP family have been characterized. Zebrafish BMP15 cDNA has been cloned. However, in contrast to its oocyte-specific expression in mammals, *bmp15* was found to be expressed in various tissues including the ovary. In the ovary, BMP15 could be detected in both oocyte and follicle cells, at both RNA level and protein level. Expression of *bmp15* was found in follicles at all stages of development with no significant changes during folliculogenesis. By incubating the follicles with antiserum against zfBMP15, it was found that the rate of oocyte maturation increased. On the contrast, when the follicles were incubated with recombinant human BMP15, it was found that human chorionic gonadotropin (hCG)-induced oocyte maturation was suppressed (166). In a following study, zebrafish were injected with BMP15 antiserum and it was found that the number of early stages follicles was decreased while the number of mature follicles was increased. Furthermore, BMP15 antiserum caused precocious oocyte development and maturation. Knockdown of BMP15 in oocytes using antisense zfBMP15 morpholino was performed, which resulted in an increase in oocyte maturation. On the contrast, when over-expressing BMP15 in oocytes, MIH or hCG-induced oocyte maturation was significantly reduced, implying a role of BMP15 in suppressing oocyte maturation. While over-expression of BMP15 had no effect on spontaneous oocyte maturation, it prevented oocyte maturation by reducing the sensitivity of the follicles to MIH (167). GDF9, on the other hand, is another BMP family member that has been cloned and characterized in the zebrafish ovary (168). In contrast to *bmp15* which has ubiquitous tissue distribution, *gdf9* was shown to be expressed exclusively in the gonads, and the expression was restricted to the oocyte in the ovary, similar to its counterpart in mammals as an oocyte-derived factor in the ovary. Furthermore, the expression of *gdf9* exhibited dynamic changes during folliculogenesis with the

level being the highest in the primary growth (PG) stage followed by a gradual decrease during follicle development toward the full-grown (FG) stage.

1.7 Objectives of the study

In the current study, we use zebrafish as the model for investigating the role of BMPs in female reproduction. Zebrafish is becoming a more popular model in development in the past decades. It serves as an excellent model for female reproduction for several reasons. First, zebrafish has small size and it is easy to maintain. Second, it has asynchronous ovary, with follicles of different stages available throughout the year. Also, more information on the genome of zebrafish is available nowadays, facilitating our study.

As of today, only BMP15 and GDF9 have been characterized in the zebrafish ovary. Little is known about other members in the BMP family. In this study, we choose to study *bmp2a*, *bmp2b*, *bmp4*, *bmp6* and *bmp7a* because some of them have been characterized or partly characterized in the zebrafish. We will also study the two type II receptors, *bmpr2a* and *bmpr2b*. We will first investigate whether they are expressed in the ovary, followed by revealing their spatiotemporal expression in the ovary. We will find out how their expression level changes during folliculogenesis, which will provide important information on their potential roles in the ovary. Also, we will find out the localization of the BMP ligands and receptors in the follicle, in order to find out how BMPs signals in the ovary.

After investigating the spatiotemporal expression of the BMP ligands and receptors, we would like to find out the functional role of the BMPs in zebrafish ovary. To carry out functional studies, it is essential that we have the recombinant BMP proteins. Therefore, our next step is to produce recombinant BMP proteins, followed by assaying their activities. An assay will be designed to serve this purpose. With the help of the recombinant BMPs, we will investigate the functional role of the BMPs in the zebrafish ovary, as well as finding out how they will regulate other important genes that are well-known to be regulators in zebrafish ovarian development. Throughout this comprehensive study, we hope to gain a more thorough understanding on the role of BMPs in the zebrafish ovary, as well as revealing the importance of BMPs in reproduction on top of their well known functions in other developmental aspects.

Table 1.1 Alternative names for BMP family members

Ligand	Alternative names
BMP2	BMP2A
BMP3	Osteogenin
BMP3b	GDF10
BMP4	BMP2b
BMP6	Vgr1
BMP7	OP1
BMP8a	OP2
BMP8b	OP3
BMP9	GDF2
BMP12	GDF7, CDMP3
BMP13	GDF6, CDMP2
BMP14	GDF12
BMP15	GDF9B
BMP16	Nodal
GDF3	Vgr2
GDF5	CDMP1
GDF15	PLAB, MIC1, PDF, PTGF-β

Vgr, Vg-related protein; OP, osteogenic protein; CDMP, cartilage-derived morphogenetic protein; PLAB, placental bone morphogenetic protein; MIC, macrophage inhibiting cytokine; PDF, prostate differentiation factor; PTGF-β, placental TGF-β (71)

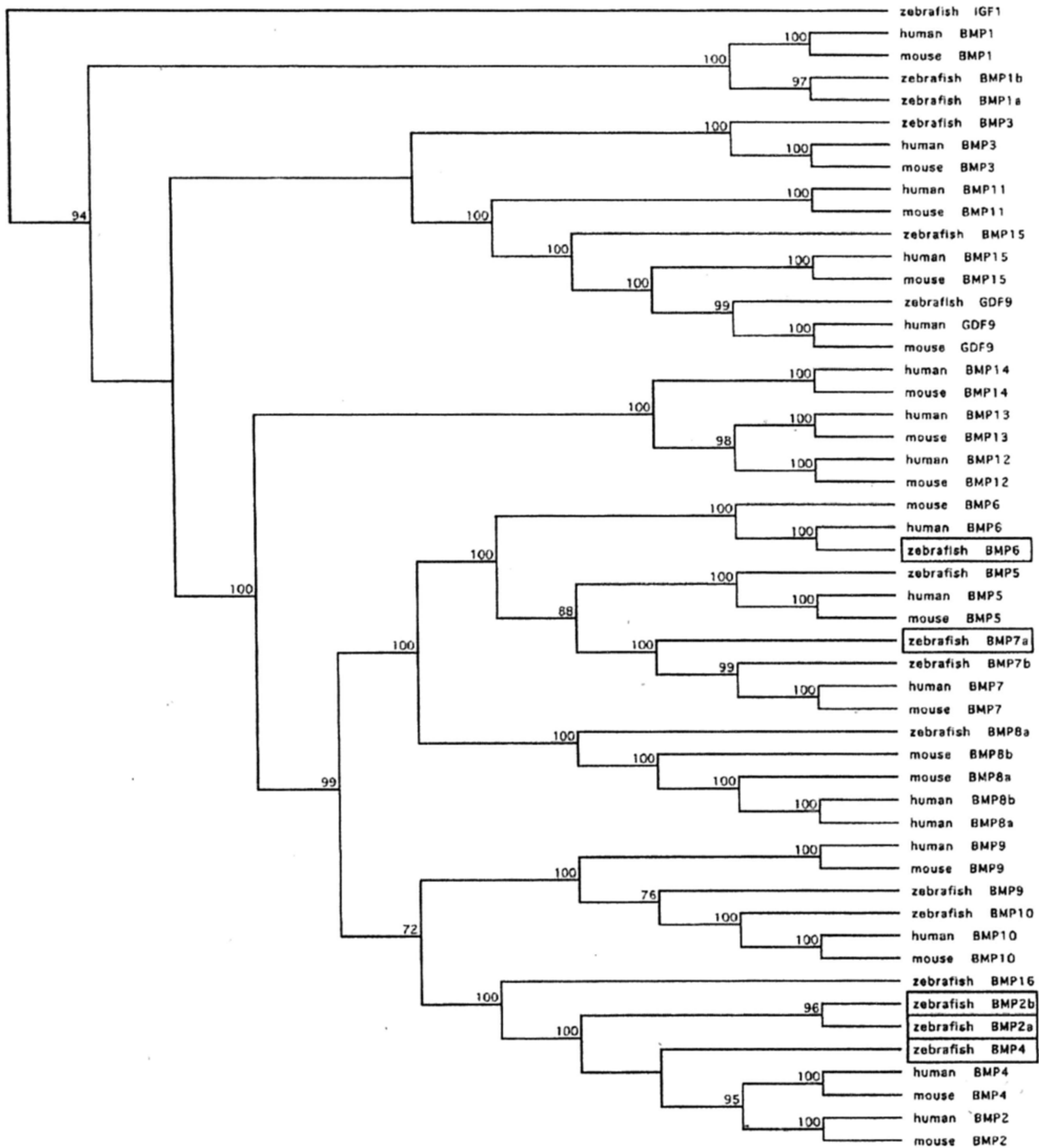


Fig. 1.1 Phylogenetic relationship of zebrafish BMP ligands with their counterparts from the human and mouse as demonstrated by bootstrapping analysis using neighbor joining method. The numbers at the forks are the bootstrap proportions. The BMP ligands investigated in this study are boxed.

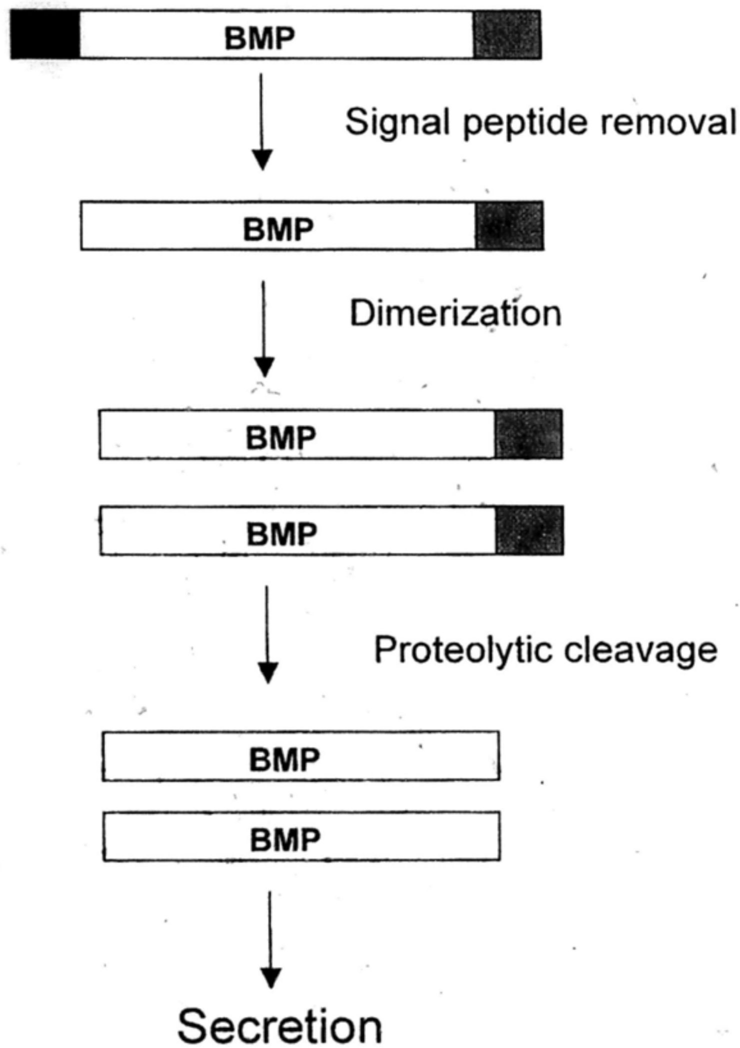


Fig. 1.2 Processing and secretion of BMP. After the signal peptide is removed, BMP dimerize. The proprotein undergo proteolytic cleavage to generate the mature BMP protein. The mature BMP protein is then secreted.

Chapter 2

Spatiotemporal Expression of the BMP Family in Zebrafish Ovary

2.1 Introduction

Numerous studies have reported that BMP family plays an important role in zebrafish development, particularly during embryogenesis. For example, *bmp2a* and *bmp2b* have been detected in the developing median fins of zebrafish, suggesting their potential roles in fin development (81). *bmp6* has also been demonstrated to be involved in fin regeneration (82). *bmp2b* also plays a critical role in morphogenesis of semicircular canals in zebrafish inner ear (83), while *bmp4* is important for establishing left-right asymmetry (84). The two BMP type II receptors, *bmpr2a* and *bmpr2b,* are also believed to establish left-right asymmetry, supporting the presence of BMP signaling in the zebrafish (169). In the *bmp7* mutant *snailhouse*, strongly dorsalized phenotype was observed (85, 86). In addition to establishing dorsal-ventral axis and organ development, BMPs are also important for post-embryonic development. A newly found homolog, *bmp7b,* was found to be expressed in the eyes, ears, pronephic kidney and the gastrointestinal system, suggesting the potential roles of *bmp7b* in post-embryonic development (87).

Though some BMP ligands have been characterized in the zebrafish and have their roles identified in development, their roles in reproduction are known to a much lesser extent. Only GDF9 and BMP15 have been characterized in zebrafish reproduction (166, 168). *gdf9* has been cloned, with its spatiotemporal expression characterized (168). *gdf9* was gonad-specific, and within the ovarian follicle, it was expressed exclusively in the oocyte. Its temporal expression profile showed a gradual decrease in expression during folliculogenesis with the lowest level at the full-grown (FG) stage. When incubating FG follicles and ovarian fragments with hCG, it was observed that the expression of *gdf9* was down-regulated by hCG. Therefore, *gdf9* might be under the regulation by pituitary gonadotropins.

BMP15 is another member in the BMP family being characterized in the zebrafish ovary (166). zfBMP15 has been cloned and found to be expressed in various tissues, including the

ovary. In contrast to GDF9, BMP15 was detected in both the oocyte and the follicle layer, with no significant change in expression during folliculogenesis. With regard to its function, BMP15 was found to have an inhibitory role in oocyte maturation as demonstrated by knockdown studies (167).

There is a new perspective on ovarian follicle development in recent years. Instead of being passively regulated by endocrine hormones, there are actually differentiation factors released by the oocyte that regulate its own growth and development. In mammalian studies, GDF9 has been identified to be an oocyte-specific factor which modulates ovarian development in the mouse and human (21, 170). While in the zebrafish, GDF9 has also been identified as an oocyte-derived factor (168). In addition, members of the EGF family including *egf*, *tgfa*, *hbegf* and *btc* have also been identified to be oocyte factors in the zebrafish, potentially acting on their receptor, *egfr*, on the follicle layer through a paracrine manner (171). In the present study, we examined the spatiotemporal expression profiles of other BMP family members including the ligands *bmp2a*, *bmp2b*, *bmp4*, *bmp6* and *bmp7* (*bmp7a* rather than the newly found homolog *bmp7b* was studied (87)), as well as the two BMP type II receptors, in the zebrafish ovary. We added on top of *gdf9* and the EGF family another group of differentiation factors, the BMP family, as oocyte-specific factors which act on follicle cells to modulate follicle development. Our results strengthen the belief that instead of being passively nurtured by the endocrine hormones, the oocyte actually plays an active role by releasing various differentiation factors that act on the follicle cells in a paracrine manner.

2.2 Materials and methods

2.2.1 Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), Invitrogen (Carlsbad, CA) and USB (Cleveland, OH) unless otherwise stated.

2.2.2 Animals

Zebrafish (*Danio rerio*) were purchased from local pet stores and maintained in flow-through aquaria at 28±1°C on a 14-h light/10-h dark photoperiod. The fish were fed thrice a day with commercial tropical fish food. The animals were anaesthetized on ice and sacrificed by decapitation before dissection. All experiments were performed under license from the

Government of the Hong Kong Special Administrative Region and endorsed by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong.

2.2.3 Isolation of ovarian follicles

Zebrafish ovary was dissected in Cortland's medium and dispersed gently using plastic pipette. Follicles were manually separated using fine forceps and grouped according to size and morphology: full-grown (FG; ~0.65 mm), mid-vitellogenic (MV; ~0.5 mm), early vitellogenic (EV; ~0.40 mm), pre-vitellogenic (PV; ~0.3 mm) and primary growth follicles (PG; ~0.1 mm). Follicles were stored in Tri-Reagent (Molecular Research Center, Cincinnati, OH) for RNA extraction, followed by reverse transcription and real-time qPCR for detection of transcript levels.

2.2.4 Separation of oocytes and follicle layers

Zebrafish ovary was dissected and the full-grown immature follicles were selected and placed in Cortland's medium devoid of calcium and magnesium to weaken the attachment of the follicle layer to the oocyte as described in our previous report (171). The follicle layer was carefully peeled off from the oocyte using fine forceps under microscope. Five follicle layers or five denuded oocytes were pooled and stored in Tri-Reagent until RNA extraction. To ensure the cleanliness of the separation, molecular markers specifically expressed in the two compartments were used. *gdf9* was the marker expressed only in the oocytes while *lhcgr* was the marker exclusively expressed in the follicle layers (168, 172).

2.2.5 Ovarian fragment incubation

Ovaries were dissected from zebrafish and put in Cortland's medium supplied with 0.1% glucose. The follicles were dispersed gently using plastic pipette and dissection blades, resulting in 7-8 follicles in one clump (called ovarian fragments below). The ovarian fragments were incubated in a 24-well plate (BD, Franklin Lakes, NJ) with 500 µl medium per well in the presence or absence of actinomycin D (1 µg/ml) for 24 h at 28°C. After the incubation period, the ovarian fragments were collected in Tri-Reagent for RNA extraction.

2.2.6 Embryo collection

Sexually mature male and female zebrafish were put in the same tank in a sex ratio of 1:1 the night before embryo collection. Embryos were collected the next morning when the lights of the aquarium were on. Embryos were observed under microscope and collected according to different developmental stages. Embryos at stages of 4-cell, 16-cell, 32-cell, 256-cell, high, sphere, shield, tailbud, 10 somites, 25 hpf and 2 dpf were collected at different time points (5 embryos per sample). As a reference, full-grown follicles were also collected from a freshly dissected zebrafish on the same day. Embryos and full-grown follicles were stored in Tri-Reagent until RNA extraction.

2.2.7 RNA isolation and reverse transcription

Total RNA was isolated from the tissues, ovarian follicles, ovarian fragments, follicle layers, denuded oocytes and embryos using Tri-Reagent according to manufacturer's instruction. Various organs or tissues were dissected for RNA extraction, including the brain, ovary, testis, gill, kidney, liver and muscle. Organs/tissues and embryos were homogenized before RNA isolation. Total RNAs from the tissues and ovarian follicles of different stages as well as ovarian fragments were quantitated and 3 μg from each was used for reverse transcription in a 10 μl volume containing 1 x M-MLV RT buffer, 0.5 mM of each dNTP, 0.5 μg oligo(dT), 0.1 mM DTT and 100 U M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA). The entire total RNA from five follicle layers and five denuded oocytes as well as the embryos were reverse transcribed using the same reagents. Reverse transcription was performed at 37°C for 2 h.

2.2.8 Real-time and semi-quantitative PCR

Real-time qPCR was used to monitor the expression changes of different target genes during folliculogenesis. Real-time qPCR was carried out on the iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) in a volume of 30 μl containing 10 μl of 1:20 diluted RT reaction mix, 1 x PCR buffer, 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, 0.2 μM of each primer, 0.75 U Taq Polymerase, 0.5 x EvaGreen (20 x concentrated; Biotium, Hayward, CA) and 20 nM fluorescein (Bio-Rad). The reaction profile consisted of 40 cycles of 95°C for 30 sec, 56°C-60°C for 30 sec (annealing temperature varies for different target genes), 72°C for 40 sec and 84°C for 7 sec for signal detection. A melt curve analysis consisting of 180 cycles of 7 sec with temperature increase of 0.2°C/cycle was performed at the end of the reaction to demonstrate the

specificity of the reaction. Results were normalized to the housekeeping gene *ef1a*. Semi-quantitative PCR was used to detect the expression in the tissues, follicle layers and denuded oocytes. For semi-quantitative PCR, the reaction was carried out in a volume of 15 µl, which consisted of 1 x PCR buffer, 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, 0.2 µM of each primer and 0.6 U Taq polymerase, using the Thermal Cycler (Bio-Rad). For the follicle layers and denuded oocyte samples, 33 cycles were performed under the following conditions: 94°C for 30 sec, 56°C-60°C for 30 sec (annealing temperature varies for different target genes) and 72°C for 40 sec. The cycle numbers used for different genes were listed in Table 2.1. The PCR products were visualized on a 1.5% agarose gel containing ethidium bromide. The primers for PCR were designed according to the sequences available in the GenBank and synthesized by Integrated DNA Technologies (IDT, Coralville, IA). Primers used for amplification of different target genes were listed in Table 2.1.

2.2.9 Statistical analysis

All real-time qPCR data were normalized to the housekeeping gene *ef1a*. All values were expressed as the mean ± SEM and the data were analyzed by one-way ANOVA followed by Newman-Keuls test for comparisons of all pairs of groups using the GraphPad Prism 5 for Mac OS X (GraphPad Software, San Diego, CA).

2.3 Results

2.3.1 Validation of semi-quantitative PCR quantification

Five members of the BMP ligands (*bmp2a, bmp2b, bmp4, bmp6* and *bmp7a*) and the two type II receptors (*bmpr2a* and *bmpr2b*) have been cloned and their nucleotide sequences were obtained from the GenBank. The primers were designed for each target gene and were synthesized by Integrated DNA Technologies (IDT, Coralville, IA) (Table 2.1). The optimal number of cycles for amplification was determined for each gene. The middle points in the curve were chosen as the optimal cycle numbers. The optimal cycle number for *bmp2a* and *bmp2b* was 34 while that for *bmp4, bmp6, bmp7a* and the two receptors (*bmpr2a* and *bmpr2b*) was 33 (Fig. 2.1).

2.3.2 Tissue distribution of BMP family in the zebrafish

All the BMP ligands and type II BMP receptors (*bmpr2a* and 2b) were found to be ubiquitously expressed in all the tissues examined, though the expression levels seemed to vary among different molecules and tissues. Inhibin α (*inha*) was used as a marker for gonadal tissues as it has been shown to be expressed in the gonads only (173). The exclusive expression of *inha* indicated that the other tissues were free from contamination by the gonadal tissues. The housekeeping gene, *ef1a*, was abundantly expressed in all tissues as expected. *bmp2a* and *bmp2b* seemed to have more abundant expression in most tissues as compared to other ligands and the receptors. The expression of *bmp7a* was relatively low, especially in kidney, liver and muscle (Fig. 2.2).

2.3.3 Temporal expression patterns during folliculogenesis

The temporal expression profiles of BMP ligands and receptors during folliculogenesis were analyzed using real-time qPCR. The expression of *bmp2a*, *bmp2b*, *bmp4* and *bmp6* increased significantly from PG to PV, though the increment varied to different degrees with *bmp2b* showing the most dramatic change during this transition. After reaching the peak at PV, the expression levels declined steadily toward the end of folliculogenesis. The expression of *bmp7a* did not show much change during folliculogenesis. *bmp2b* and *bmp4* were relatively more abundant as compared to other BMP ligands, while *bmp7a* appeared to be the least abundant. In contrary to the BMP ligands, the expression levels of *bmpr2a* and *bmpr2b* increased their expression steadily from PG to MV and the level surged at the FG stage (Fig. 2.3).

2.3.4 Spatial distribution of the BMP ligands and receptors within the follicle

The zebrafish follicle consists of a developing oocyte and a surrounding follicle layer containing granulosa and theca cells. To demonstrate the localization of BMP ligands and receptors in the two follicle compartments, we separated the two compartments manually followed by RNA extraction from each and RT-PCR detection. The oocyte marker gene *gdf9* and follicle cell marker gene *lhcgr* were used to ensure the cleanliness of the oocyte and follicle layer samples (168, 172). As a specific marker of the oocyte, the expression of *gdf9* was demonstrated in both the intact follicles and the denuded oocytes, but not the follicle layers. In contrast to *gdf9*, *lhcgr* was expressed exclusively in the follicle layers. *bmp2a*, *bmp2b*, *bmp6* and

bmp7a were found to be expressed only in the denuded oocytes under the current experimental conditions, while the expression of *bmp4* was found in both compartments. On the contrary, the receptors *bmpr2a* and *bmpr2b* were found to be located exclusively in the follicle layers under the current experimental conditions (Fig. 2.4).

2.3.5 Transcription dependence of the expression of BMP ligands and receptors in the ovary

Actinomycin D, as a pharmacological drug which blocks transcription, was applied on the ovarian fragments. It was observed that the BMP ligands (*bmp2a*, *bmp2b*, *bmp4*, *bmp6* and *bmp7a*) which were oocyte-derived factors do not have significant changes in expression upon the blockade of transcription, suggesting they have low turnover rate in the oocyte. On the contrast, the two BMP receptors, which were located exclusively in the follicle layer under the current experimental conditions, showed a drastic drop in expression upon incubation with actinomycin D. As the receptors in the follicle layer have high turnover rate, it is hypothesized that the genes that are located in the follicle layer are more likely to be subjected to regulation (Fig. 2.5).

2.3.6 Temporal expression patterns during embryogenesis

The BMP ligands showed a distinct expression pattern during embryogenesis. Expression of *bmp2a* increased gradually, while that of *bmp2b* peaked at sphere stage and *bmp4* peaked at shield stage. *Bmp6* exhibited an irregular expression pattern, while *bmp7a* peaked at sphere and shield stage. For the BMP receptors, both *bmpr2a* and *bmpr2b* dropped their expression to zero at 4-celled stage and gradually increased as the embryo developed (Fig. 2.6).

2.4 Discussion

In the mammals, the locations of the BMP ligands and receptors are diverse in different species, and sometimes controversial. A comprehensive study was performed on the spatiotemporal expression of the BMP family in adult rats (174). Through *in situ* hybridization analysis, it was found that BMP2 was located in the granulosa cells, while BMP3, BMP3b, BMP4 and BMP7 were in the theca cells. BMP6 was found in both the oocyte and granulosa cells with increasing expression during folliculogenesis. BMP15, however, was oocyte-specific.

While the BMP ligands were localized in various compartments, BMPR-IA and BMPR-IB were found in oocytes, granulosa cells and theca cells, whereas BMPR-II was restricted to the granulosa cells and weak expression in oocytes only. In another study using rat as model, it was demonstrated that BMP5 was located in the granulosa cells and it exerted stimulatory actions on granulosa cell proliferation and inhibitory actions on progesterone production (151). BMP3 was studied in the human ovary (175). Also localizing in the granulosa cells, human BMP3 was subjected to regulation by hCG. On the contrary, human GDF9 and its novel homolog GDF9B (BMP15) were restricted to the oocyte in primary follicles (170). In sheep, BMPR-IB was found in oocyte and granulosa cells, while BMPR-II was found in theca cells, in addition to the oocyte and granulosa cells (176). In another study using ewe as the model identified BMPR-IA, BMPR-IB and BMPR-II in all three compartments from primary to preovulatory stage (152). Therefore, in mammals, the localization of the BMP ligands and receptors was highly diverse in different species. Yet in the zebrafish, only GDF9 and BMP15 are known for their spatial distribution in the ovarian follicle (166, 168), but much less is known about other members in the BMP family.

The present study revealed the presence of *bmp2a*, *bmp2b*, *bmp4*, *bmp6* and *bmp7a* in the oocyte compartment, identifying the BMPs as oocyte-derived factors. While the receptors *bmpr2a* and *bmpr2b* were located exclusively in the follicle layer under the current experimental conditions, it is suggested that there is a potential paracrine BMP signaling going from the oocyte towards the follicle layer, as opposed to the traditional belief that the oocyte is passively nurtured by the follicle layer for its growth and development. Instead, the oocyte may play an active role by releasing various growth factors to regulate ovarian development. In contrast to BMP15 which was found in both the oocyte and follicle layer compartment (166), the BMP ligands that we studied were exclusively in the oocyte under the current experimental conditions, except for *bmp4* which was found in the follicle layer as well. There was further evidence supporting our results in the localization study. In the embryogenesis experiment, the BMP ligands exhibited a distinct expression pattern, with the highest expression at different stages. This not only implies their importance in embryogenesis, but also provides us some insights on the spatial distribution of the BMP ligands and receptors. We observed that from full-grown follicle to 4-celled stage embryo, the expression of the BMP ligands did not change significantly. This is most likely because the BMPs are maternal factors that were carried over from the oocyte

through fertilization into embryonic stage. On the contrary, the expression of BMP receptors dropped to zero at 4-celled stage followed by a gradual increase. The disappearance of these receptors can be explained by the localization of the receptors on the follicle layer. During ovulation, the follicle layer was shredded off. Therefore, we observed the expression of the receptors at an undetectable level. The embryogenesis experiment provided indirect evidence to support the results of our localization studies.

We also performed another experiment to further validate our localization studies. While hormones such as the gonadotropins act on the follicle layer to regulate the oocyte, the oocyte also releases growth factors and exerts its actions on the follicle layer in a paracrine manner. Thus the follicle layer appears as a converging point where the endocrine and paracrine signals act and interact. In other words, the follicle layer is more likely to be subjected to regulation and genes expressed in the follicle layer are expected to have a higher turnover rate as compared to those in the oocyte. From the experiment using actinomycin D which blocks transcription in the follicles, the expression of the two BMP receptors dropped drastically, indicating a high turnover rate. This also suggests that these genes are more likely to be subjected to regulation. On the contrast, the BMP ligands did not show significant change in expression. Therefore, they were more likely to be in the oocyte, which is subjected to regulation to a lower extent than the follicle layer. Our study added the BMP family as another group of oocyte-derived growth factors in addition to GDF9 and the EGF family in the zebrafish (168, 171), in contrast to BMP15 which was found in both oocyte and follicle layer compartment (166).

The distribution of the BMP ligands and receptors in this study suggests a paracrine signaling from the oocyte to the follicle layer, and we will provide further evidence in the next chapter that the follicle layer is the target for BMP signaling. Under the experimental conditions in this study, we showed that all the BMP ligands studied were located exclusively in the oocyte except for *bmp4* which was found in both compartments. This situation is similar to that of EGF family, of which *btc* was found in both the oocyte and the follicle layer (171). Studies in mammals suggested BTC as one of the downstream mediators of LH in the ovarian follicle, strongly arguing for its presence in the follicle layer (177). Whether *btc* and *bmp4* have a similar role in mediating the action of gonadotropins will be an interesting issue for us to investigate in the future. Also, we have to be cautious that the spatial expression was based on FG follicles only in this study, and might not apply to other stages. It will be interesting to investigate the

spatial distribution of follicles at earlier stages to see if the spatial distribution is consistent throughout different stages in order to get a more comprehensive picture.

The temporal expression pattern of most members in BMP family has not been studied in the zebrafish except for BMP15, which did not show a significant change over the course of folliculogenesis. Yet, *bmp4*, *bmp7* and *gdf9* had been characterized in the rainbow trout (164). The expression of *bmp4* and *bmp7* peaked at pre-vitellogenic stage, which was similar to our findings in the zebrafish with expression of most BMP ligands peaked at pre-vitellogenic stage. The pre-vitellogenic stage represents the time that the follicle is activated and starts incorporating vitellogenin for its growth. It represents a very important transition during folliculogenesis. This is crucial to the whole developmental process of the follicle, because arrest in early stage of folliculogenesis will result in failure of the follicle to continue development. Participation in this important transition may require co-operation of various factors. The peak at pre-vitellogenic stage might suggest a potential role of BMPs in promoting primordial follicle development. In fact, in mammalian studies, BMP4 and BMP7 had been shown to promote early folliculogenesis in the rat and mouse model respectively (57, 150). Whether BMPs in zebrafish exhibit a similar functional role in the ovary needs our further investigation in the future.

Interestingly, the two BMP receptors surged at the full-grown stage during folliculogenesis, which is similar to that of *egfr* (171). EGF and TGF-α, as oocyte-derived factors, have been shown to promote oocyte maturation (178). With their common receptor, *egfr*, reaching peak level at FG stage so as the BMP receptors, it will be interesting to investigate whether BMP signaling exerts an important role in regulating oocyte maturation. Also, the rise in expression of BMP receptors during folliculogenesis paralleled that of the gonadotropin receptors (172). In the hamster, it was reported that FSH up-regulated BMP receptors in the perinatal ovary (179). Whether the gonadotropins exert a similar effect on the BMP receptors remains a question to be investigated in the future.

In conclusion, several members in the BMP family have been characterized, including *bmp2a*, *bmp2b*, *bmp4*, *bmp6* and *bmp7a*, as well as the two type II receptors, *bmpr2a* and *bmpr2b*. They were all expressed in the zebrafish ovary, and within the ovarian follicle, spatial distribution studies under our experimental conditions suggested the BMP ligands located exclusively in the oocyte and follicle layer, except for *bmp4* which was found in both

compartments. We are unsure why *bmp4* has a different localization pattern from other BMP ligands, and this is worth further investigation in our study. In contrast to the BMP ligands, the BMP receptors were located exclusively in the follicle layer, suggesting a potential paracrine signaling from the oocyte towards the follicle layer. In addition, the temporal expression pattern revealed BMPs increased from PG to PV stage, while the receptors surged at the full-grown stage, arguing for a potential role of BMPs as oocyte-derived factors actively participating in modulating ovarian function by promoting early follicle development as well as regulating oocyte maturation.

Table 2.1 Primers used in semi-quantitative RT-PCR and real-time qPCR

Gene	Accession no.	Sequence	Expected Size (bp)	Cycle No. used
bmp2a	NM 131359	GCAGAGCCAACACTATCAGGAG	335	34
		CCACTTTAATACAGCAGGAGTTACG		
bmp2b	NM 131360	GCACAAGTATGAACAAGAAGAGGC	339	34
		CCGAACATATTGAGCAAGCGTAG		
bmp4	NM 131342	CCAACACCGTGAGAGGATTCC	379	33
		TCCACAGCAAGGCCATGATTAG		
bmp6	NM 001013339	AACCGCAACCGCTCCAATAG	376	33
		AACGCACCACCATGTTCCTG		
bmp7a	NM 131321	ACCTTCCATGTCAGCGTGTTC	335	33
		TGACCTTTCTGTTTGCCTCCTG		
bmpr2a	NM 001039817	ACCGCCAGCAGTTCACTAATG	322	33
		TCCGTCTTAACCAGCACATTCC		
bmpr2b	NM 001039807	GGCTCTGCTCACTGCTTCTG	298	33
		TGCGATGGCGTTGTGGTAAC		
ef1a	NM 131263	GGCTGACTGTGCTGTGCTGATTG	409	24
		CTTGTCGGTGGGACGGCTAGG		
gdf9	NM 001012383	TCTCCATCACTCCTTCTCTATCTT	102	30
		ATCCCAATGGTTTGATGTCAGAT		
lhcgr	NM 205625	GACGGCCTGAAAGGAGTAAG	279	32
		GCGCAGATTCAGGTTATCAC		
inha	XM 693951	AGCCTCCTCTGCCAGTGTTG	301	26
		ATGTTGATGGAAGCGATGGTCTC		

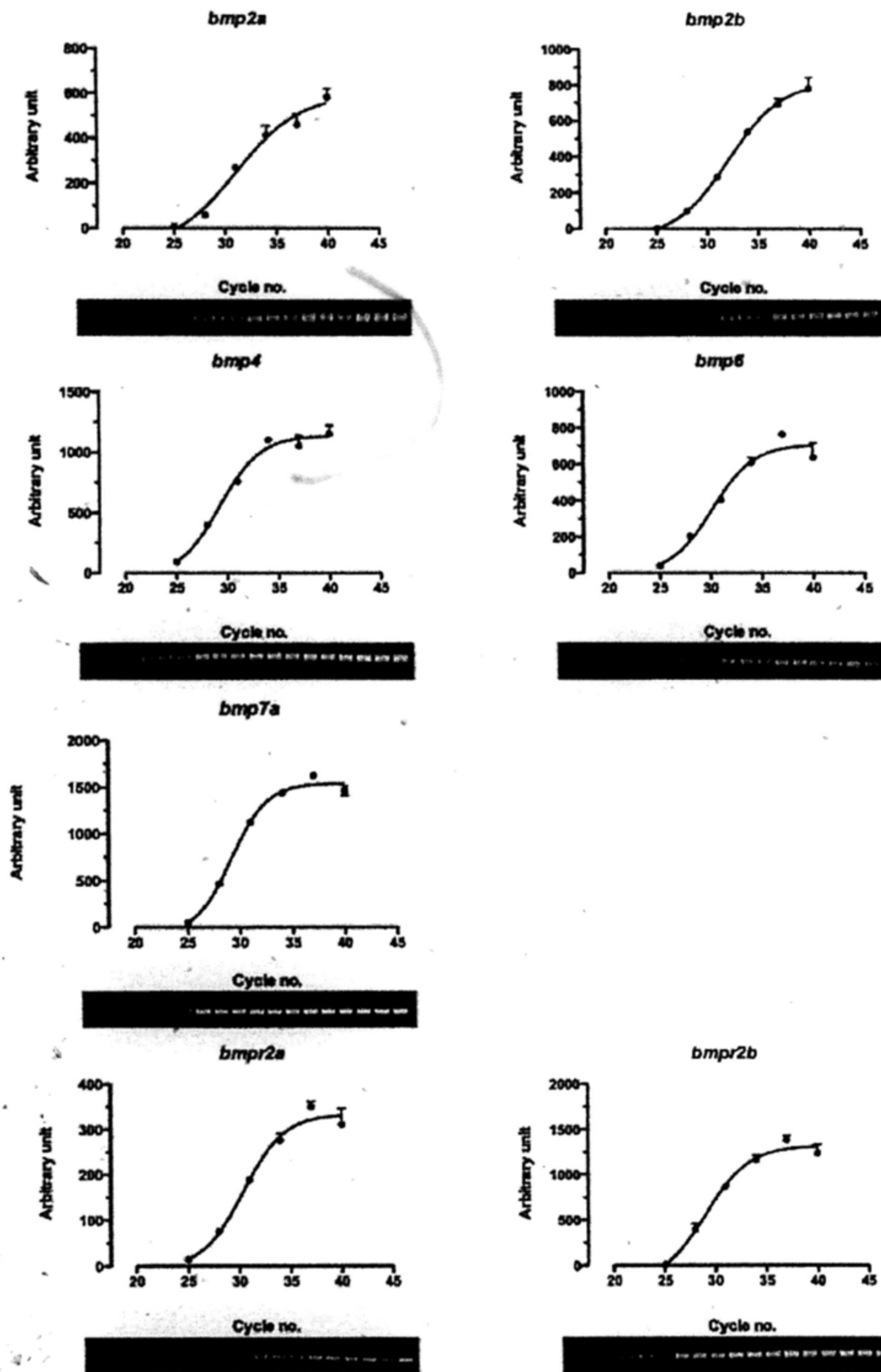
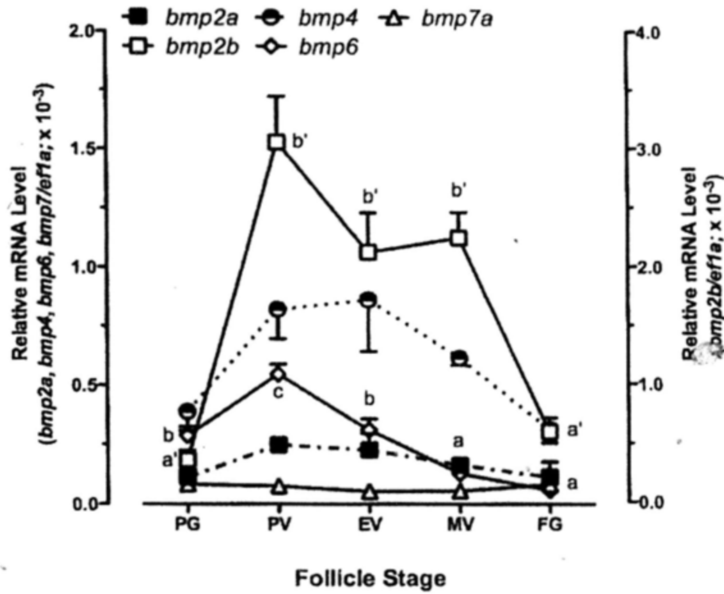


Fig. 2.1 Validation of semi-quantitative RT-PCR assay for zebrafish BMP ligands (*bmp2a*, *bmp2b*, *bmp4*, *bmp6* and *bmp7a*) and the two BMP type II receptors (*bmpr2a, bmpr2b*). RT-PCR was performed on the RNA isolated from whole ovary for different cycle numbers.

Fig. 2.2 Tissue distribution of the BMP family ligands and receptors in the zebrafish. B, brain; O, ovary; T, testis; G, gill; K, kidney; L, liver; M, muscle; +, RT with reverse transcriptase; -, RT without reverse transcriptase. *ef1a,* housekeeping gene; *inha,* marker specific for the gonads; *bmp2a, bmp2b, bmp4, bmp6, bmp7a,* BMP ligands; *bmpr2a, bmpr2b,* BMP receptors.

(A)



(B)

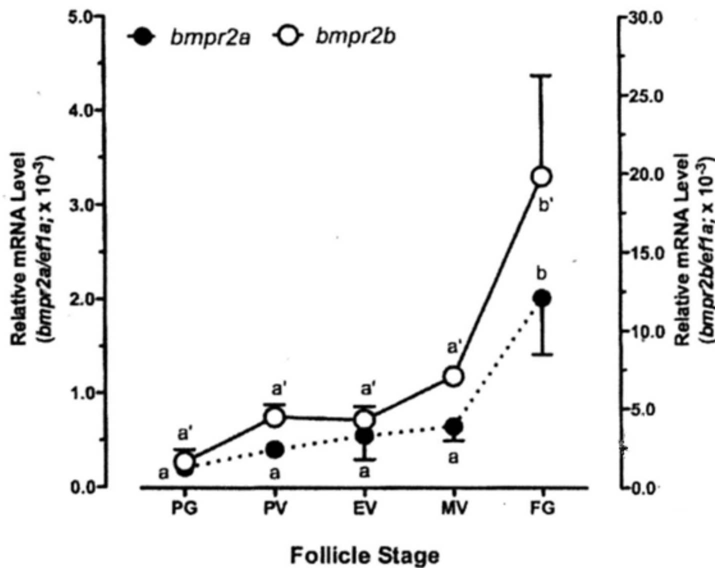


Fig. 2.3 Temporal expression profiles of BMP ligands *bmp2a*, *bmp2b*, *bmp4*, *bmp6* and *bmp7a* (**A**) and type II BMP receptors *bmpr2a* and *bmpr2b* (**B**) during folliculogenesis. The relative mRNA levels were determined by real-time qPCR and normalized to the housekeeping gene *ef1a*. The values are the mean ± SEM (n=3) from a representative experiment. Letters indicate statistical significance (P<0.05). PG, primary growth; PV, pre-vitellogenic; EV, early vitellogenic; MV, mid-vitellogenic; FG, full-grown.

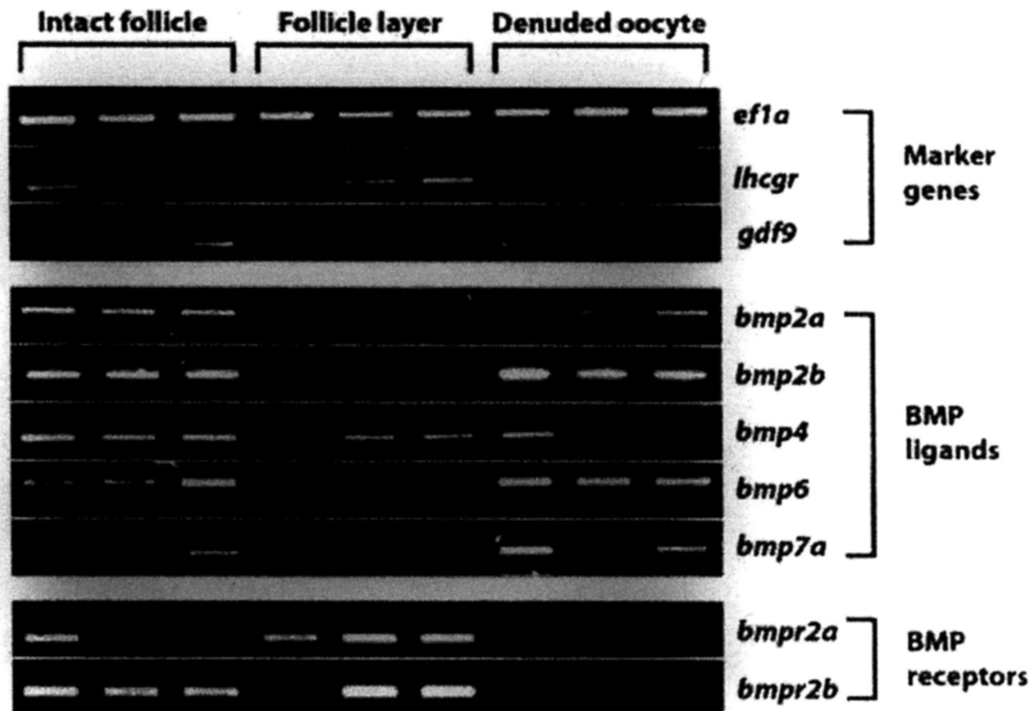
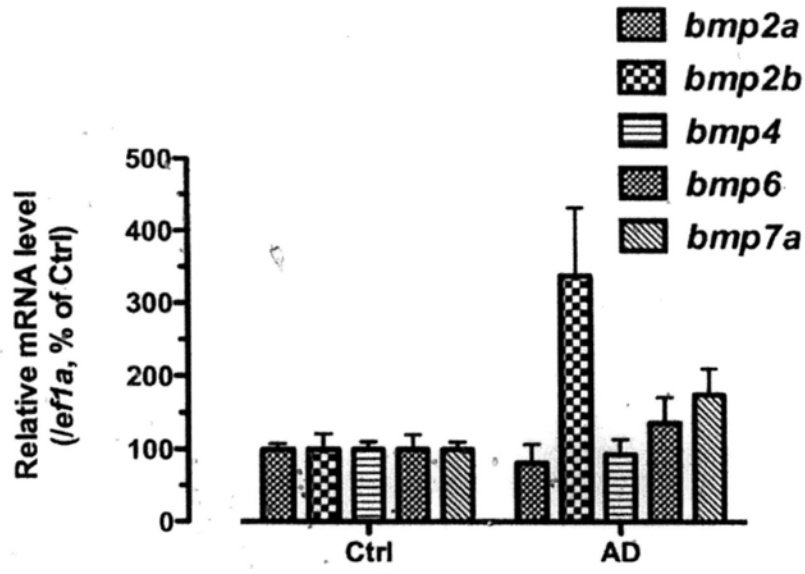


Fig. 2.4 Intrafollicle distribution of the BMP ligands and receptors in the full-grown follicle. The housekeeping gene, *ef1a*, was expressed in both compartments whereas the follicle cell-specific marker (*lhcgr*) and oocyte-specific marker (*gdf9*) were only detected in the follicle layers and denuded oocytes, respectively. Under the current experimental conditions, the BMP ligands (*bmp2a*, *bmp2b*, *bmp6* and *bmp7a*) were exclusively detected in the denuded oocytes whereas *bmp4* was detected in both compartments. On the contrast, the two type II receptors, *bmpr2a* and *bmpr2b*, were detected exclusively in the follicle layers.



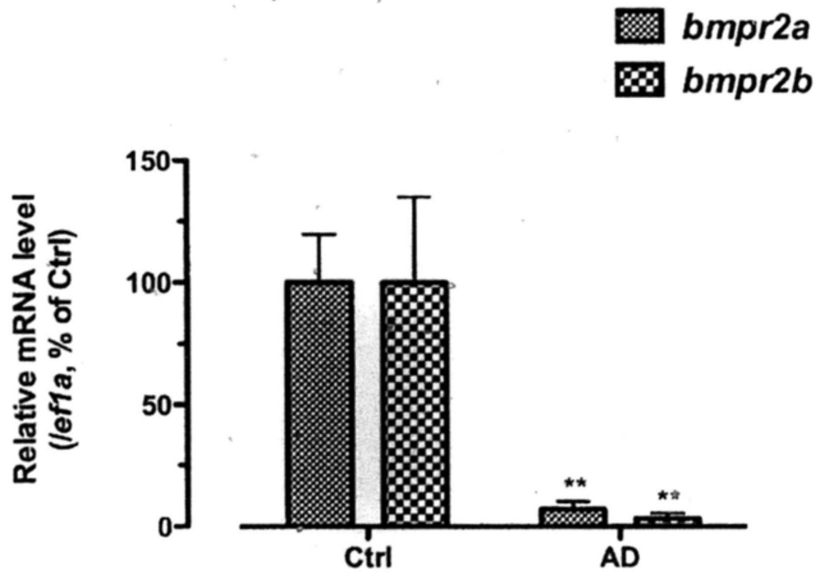
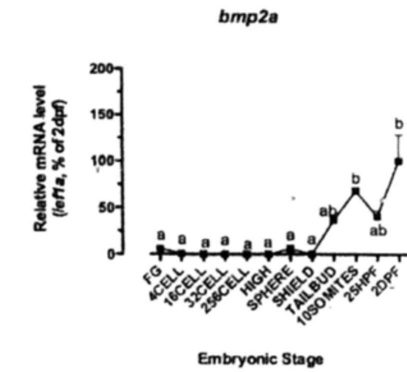
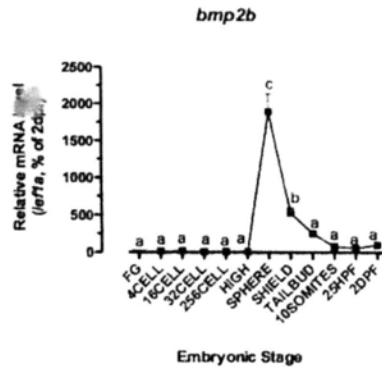
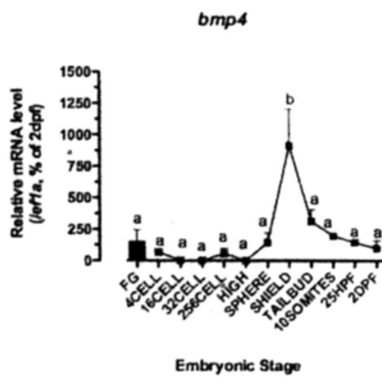


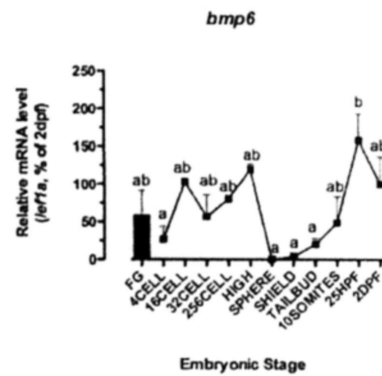
Fig. 2.5 Expression of BMP ligands (*bmp2a*, *bmp2b*, *bmp4*, *bmp6*, *bmp7a*) and the BMP type II receptors (*bmpr2a and bmpr2b*) of ovarian fragments upon incubation of Actinomycin D (1000 ng/ml) for 24 h. The values are the mean ± SEM (n=3) from a representative experiment. Asterisk (*) indicates statistical significance (**, $P < 0.01$ vs. control).

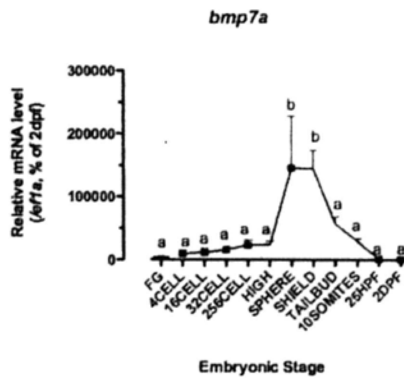
(A)

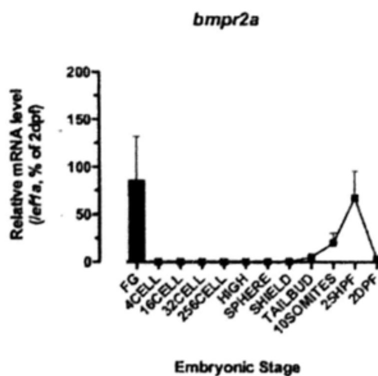


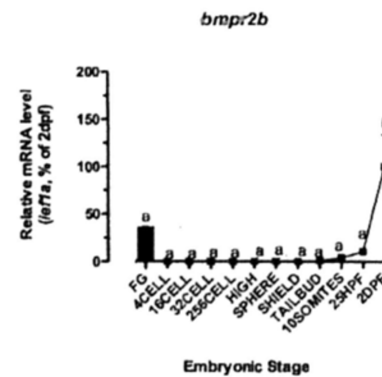












(B)

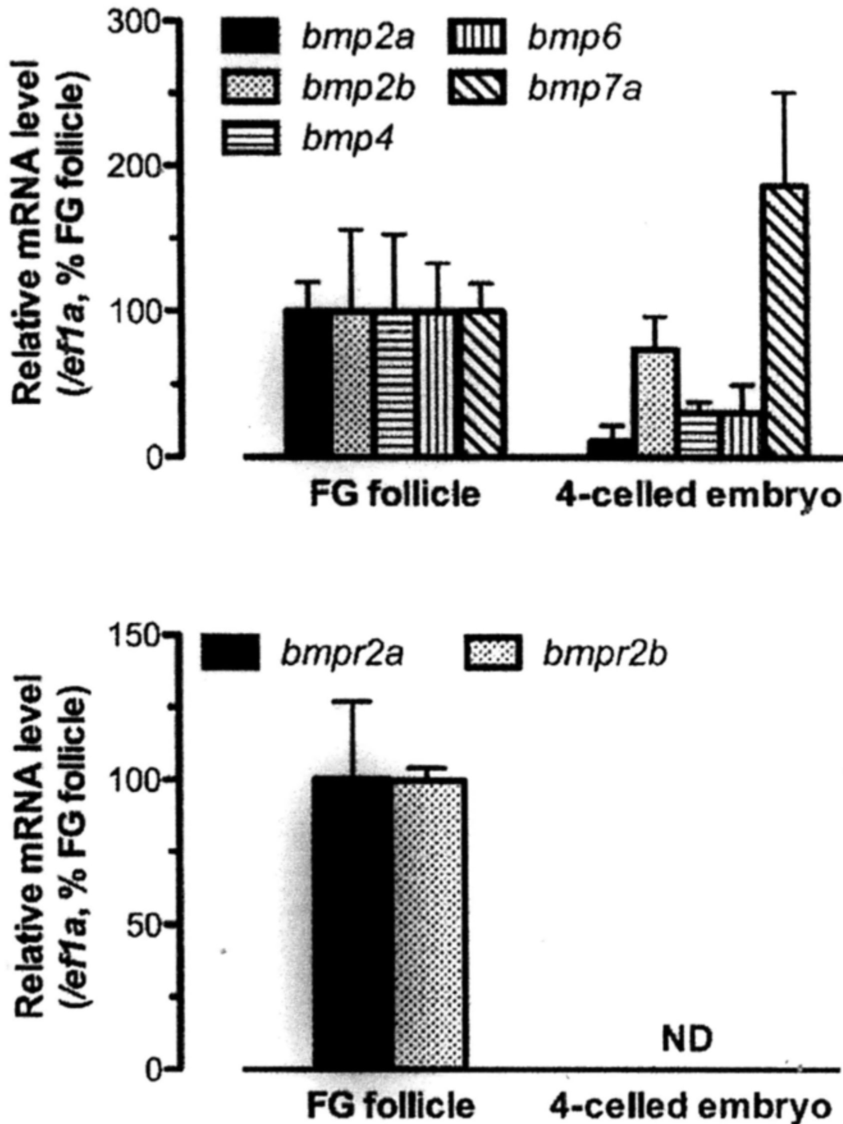


Fig. 2.6 Expression of BMP ligands (*bmp2a*, *bmp2b*, *bmp4*, *bmp6*, *bmp7a*) and the BMP type II receptors (*bmpr2a* and *bmpr2b*) during embryogenesis (A). Follicles at FG stage were also taken as a reference **(B).** The relative mRNA levels were determined by real-time qPCR, normalized to the housekeeping gene *ef1a*, and expressed as percentage change compared with the levels at 2 dpf. The relative mRNA level of FG stage and 4-celled stage were also compared separately. The values are the mean ± SEM (n=3) from a representative experiment. Letters indicate statistical significance (P<0.05).

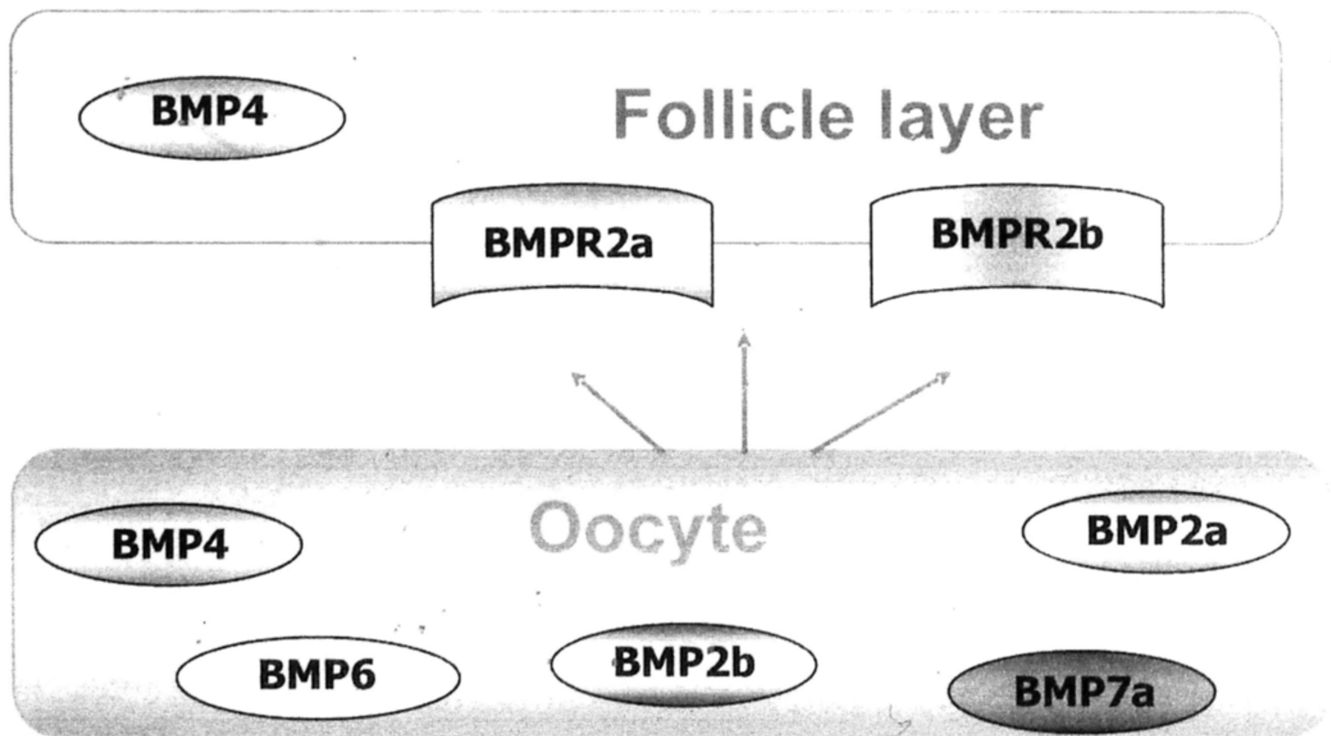


Fig. 2.7 Schematic diagram of spatial localization of BMP ligands (*bmp2a*, *bmp2b*, *bmp4*, *bmp6*, *bmp7a*) and receptors (*bmpr2a*, *bmpr2b*) in the zebrafish follicle. Under our experimental conditions, BMP ligands were found to be located exclusively in the oocyte (except for *bmp4* which was found in both compartments) while BMP receptors were located exclusively in the follicle layer, suggesting a potential paracrine signaling from the oocyte towards the follicle layer.

Chapter 3

Recombinant Production and Functional Characterization of Zebrafish BMP2b and BMP4

3.1 Introduction

Having investigated the spatiotemporal expression of BMP family, our next step would be to unravel the functional roles of BMPs in the ovary. Yet before we can study their function, it is essential that we have the recombinant BMPs available. BMPs can be purified from bone, yet the yield is very low and the purification procedure is complex (180). Several reports have tried to produce recombinant BMPs using prokaryotes or eukaryotes. For example, BMP2 was produced from *E. coli*, though refolding or solubilization was required (181-184). Expression of the ovine type II BMP receptor ectodomain was also done in *E. coli* followed by purification process (185). *Xenopus* BMPs were also expressed using a baculovirus expression system by infecting the insect cells (186, 187). Mammalian Chinese Hamster Ovary (CHO) cells have also been reported to produce *Xenopus* BMP4 with demonstrated bioactivity (188). Each system has their advantages and disadvantages.

Currently, some forms of BMPs are commercially available. Yet, they are from species other than zebrafish. We cannot assume that these commercially available BMPs are biologically active and exhibit the same function in the zebrafish model. In addition, some of them are made in bacterial system, which lacks post-translational modification or proper folding. Because of this, the ED_{50} of these recombinant products is often unexpectedly high. Also, the high cost of these commercially available recombinant proteins is another hindrance in our study. Therefore, we decided to make our own recombinant proteins in this study.

In our present study, we have chosen CHO cells as the bioreactor for producing zebrafish BMPs. Early in 1992, the production of human BMP2 in CHO cells was reported (64). As BMPs always form homodimers or heterodimers, a mammalian system was chosen for this study since in the mammalian system, refolding of protein and post-translational modification can be done. Also, CHO cells are easy to maintain and the cost is relatively low.

Flp-In CHO cells were used to produce zebrafish recombinant BMP proteins. Homologous recombination occurred between the FRT sites in the host cells and pcDNA5/FRT (the construct with cDNA sequence of zebrafish BMPs cloned in) through the help of the Flp recombinase expressed by pOG44, the plasmid that was co-transfected. In this study, BMP2b and BMP4 were chosen for recombinant protein production because BMP2b displayed the most dynamic change in expression during folliculogenesis and BMP4 was the only ligand examined that showed expression in both oocyte and follicle layer compartments. To ensure the recombinant proteins are active, a bioassay is needed. The present study has developed an assay to demonstrate the bioactivity of the recombinant BMPs by making use of Smad1/5/8 phosphorylation in cultured follicle cells. It is well established that Smad1/5/8 are downstream mediators of BMPs, and Smad1/5/8 phosphorylation is classical in BMP signaling (128, 129). Therefore, to test the bioactivity of the recombinant BMPs, we investigated whether these recombinant BMPs could induce Smad1/5/8 phosphorylation in cultured follicle cells. Further, we tested whether zfBMP4 had a functional role in oocyte maturation.

Recently, the two BMP type II receptors, *bmpr2a* and *bmpr2b*, have been reported to be involved in establishing the left-right asymmetry in the zebrafish, indicating that these two receptors were functional in the zebrafish (169). In our study, we demonstrated the presence of these BMP receptors in the ovary. Yet, whether they are involved in BMP signaling in the ovary remains to be questioned. We use bioinformatics tools to predict the extracellular domains of the two receptors, and produced the truncated forms of them using CHO cells, similar to what we did for recombinant zfBMP2b and 4. Ovine type II BMP receptor ectodomain has previously been expressed by bacterial system as mentioned earlier (185). Furthermore, a truncated BMP receptor has been reported to affect dorsal-ventral patterning in *Xenopus* embryos (114). To prove that the BMP receptor ectodomains were bioactive, we tested them by evaluating their ability to attenuate BMP signaling in cultured follicle cells through their binding with BMP ligands.

3.2 Materials and methods

3.2.1 Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), Invitrogen (Carlsbad, CA) and USB (Cleveland,OH) unless otherwise stated.

3.2.2 Animals

Zebrafish (*Danio rerio*) were purchased from local pet stores and maintained in flow-through aquaria at 28±1°C on a 14-h light/10-h dark photoperiod. The fish were fed thrice a day with commercial tropical fish food. The animals were anaesthetized on ice and sacrificed by decapitation before dissection. All experiments were performed under license from the Government of the Hong Kong Special Administrative Region and endorsed by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong.

3.2.3 Total RNA isolation and reverse transcription

Total RNA was isolated from CHO cells using Tri-Reagent according to manufacturer's instruction. Total RNAs was used for reverse transcription in a 10 μl volume containing 1 x M-MLV RT buffer, 0.5 mM of each dNTP, 0.5 μg oligo(dT), 0.1 mM DTT and 100 U M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA). Reverse transcription was performed at 37°C for 2 h.

3.2.4 Construction of expression constructs

To produce recombinant zebrafish BMP2b and BMP4, the open reading frames of *bmp2b* and *bmp4* were cloned into pcDNA5/FRT (Fig. 3.1) respectively, provided in the Flp-In Expression system (Invitrogen). The restriction sites of BamHI and XhoI as well as Kozak sequence were added in the primers used. To produce the truncated forms of zebrafish type II BMP receptors (*bmpr2a* and *bmpr2b*) containing extracellular domain only, the region corresponding to the ectodomain of each receptor was predicted by ExPASy (Expert Protein Analysis System, Pròteomics Server of the Swiss Institute of Bioinformatics) and cloned into pcDNA5/FRT as described above for BMP2b and BMP4. Similarly, the restriction sites of BamHI and XhoI as well as Kozak sequence were added in the primers. The restriction enzymes BamHI and XhoI were purchased from Promega. Zebrafish ovaries were dissected and RNA extracted, followed by reverse transcription and amplification. KAPA HiFi DNA polymerase (Kapa Biosystems, Woburn, MA) was used for amplification because of its high fidelity. After successful cloning into the vector, the expression constructs were sequenced by Tech Dragon Limited DNA Sequencing Service to confirm the identity of the constructs.

3.2.5 Cell culture and transfection of Flp-In CHO cells

The Flp-In Chinese Hamster Ovary (CHO) cells (Invitrogen) were used for recombinant protein production. F-12 medium (Gibco) supplied with 6 g/L HEPES (USB, Cleveland, OH) and 10% FBS (Hyclone) containing antibiotics (streptomycin, 100 μg/ml; penicillin, 100 U/ml) was used to culture the CHO cells. Transfection was performed in 6-well plates (BD, Franklin Lakes, NJ), when the growing CHO cells reached 80% confluency. The expression constructs of zfBMP2b, zfBMP4, or the ectodomains of zfBMPR-IIA and zfBMPR-IIB (1 μg each) were transfected into the Flp-In CHO cells using Transfectin Lipid Reagent (Bio-Rad). The plasmid pOG44 (Invitrogen) that expresses the Flp recombinase for homologous recombination between the FRT sites in the host cells and pcDNA5/FRT was also co-transfected to enhance the integration of the expression constructs into the host genome. The transfected CHO cells were re-plated into 10-cm culture dishes (BD) the next day and underwent hygromycin B (Invitrogen) selection at 500 μg/ml (optimized dose) for 1 month. The transfected CHO cells were plated into 96-well plate (BD) after 1-month selection at the density of 1 to 2 cells per well. One week after, the wells that contained single clones were selected and the cells from each well were re-plated into a new 10-cm culture dish. One clone for each protein (BMP2b: #B12; BMP4: #E7; BMPR-IIA: #A1 and BMPR-IIB: #E6) was selected for establishing stable cell lines for recombinant protein production. RNA extraction and reverse transcription were performed to confirm the expression of *bmp2b*, *bmp4*, *bmpr2a* and *bmpr2b* respectively. The cells were also transfected with empty pcDNA5/FRT in a similar way to generate a control cell line.

3.2.6 Real-time qPCR and semi-quantitative PCR

Real-time qPCR was used to detect the copy number of *bmp2b* and *bmp4* per 5,000,000 recombinant CHO cells. Real-time qPCR was carried out on the iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) in a volume of 30 μl containing 10 μl of 1:20 diluted RT reaction mix, 1 x PCR buffer, 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, 0.2 μM of each primer, 0.75 U Taq Polymerase, 0.5 x EvaGreen (20 x concentrated; Biotium, Hayward, CA) and 20 nM fluorescein (Bio-Rad). The reaction profile consisted of 40 cycles of 95°C for 30 sec, 58°C for 30 sec, 72°C for 40 sec and 84°C for 7 sec for signal detection. A melt curve analysis consisting of 180 cycles of 7 sec with temperature increase of 0.2°C/cycle was performed at the end of the reaction to demonstrate the specificity of the reaction. Semi-quantitative PCR was also

performed. For semi-quantitative PCR, the reaction was carried out in a volume of 15 μl, which consisted of 1 x PCR buffer, 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, 0.2 μM of each primer and 0.6 U Taq polymerase, using the Thermal Cycler (Bio-Rad). For the CHO cells, 33 cycles were performed under the following conditions: 94°C for 30 sec, 58°C for 30 sec and 72°C for 40 sec. The PCR products were visualized on a 1.5% agarose gel containing ethidium bromide. The primers for checking the expression of *bmp2b* and *bmp4* were designed according to the sequences available in the GenBank and synthesized by Integrated DNA Technologies (IDT, Coralville, IA) and they are listed in Table 3.1.

3.2.7 Recombinant protein production

The established clones of CHO cells stably transfected with *bmp2b*, *bmp4*, and truncated forms of *bmpr2a* and *bmpr2b* were used for recombinant protein production. The established cell lines were sub-cultured into 175 cm^2 flasks (Nunc, Rochester, NY) and allowed to grow for 3 days at 37°C with 5% CO_2 in 50 ml F-12 medium with 10% FBS (Hyclone). After 3 days, the cells were changed to serum-free condition and the culture continued for 5 days at a lower temperature of 28°C. The medium was then harvested and concentrated by 200 folds using Amplicon Ultra 10000 MWCO (Millipore, Billerica, MA). The same was done for the control cell line.

3.2.8 Primary follicle cell culture

Around 40 zebrafish ovaries were dissected and the follicles were dispersed slightly using plastic pipette. The follicles were washed with 60% L-15 (Gibco) several times in a 15 ml Falcon tube (BD) and filtered through a sieve to remove large size FG follicles. The filtered follicles were then washed several times with medium M199 (Gibco) and plated into 10-cm culture dishes. The follicles were grown for 3 days at 28°C in M199 with 10% FBS (Hyclone). Fresh medium of the same components was replaced after 3 days. After continuous incubation for 3 more days, the follicle cells were sub-cultured into 12-well plates (BD), with 400,000 cells per well. After 24-h incubation with the supplement of FBS, the medium was replaced with serum-free M199 for starvation purpose. The follicle cells were treated with the CHO cell-produced recombinant proteins or human BMP2 (Biovision, Mountain View, CA). The treated

follicle cells in each well were collected with 80 μl SDS sample buffer (62.5 mM Tris HCl [pH 6.8], 1% SDS, 10% glycerol, 5% 2-mercaptoethanol) for Western blotting.

3.2.9 Western blotting

The samples collected above were boiled at 95°C for 10 min before loading onto the acrylamide gel (12%) for SDS-PAGE. A biotinylated ladder (Cell Signaling, Danvers, MA) was used as size markers. After the proteins were separated by SDS-PAGE, they were transferred onto a nitrocellulose membrane at 90 V for 90 min. The membrane was blocked with 5% skim milk for 1 h after the blotting procedure, followed by washing twice with 1 x TBS with 1% Tween-20 (USB, Cleveland, OH) (called TTBS below) for 10 min. The primary antibody against phospho-Smad1/Smad5/Smad8 (Cell Signaling) was diluted at 1:1000 in 5% skim milk and incubated with the membrane overnight at 4°C. On the next day, the membrane was washed twice with TTBS for 10 min, followed by incubation with the secondary antibody (bovine anti-rabbit-HRP, Santa Cruz Biotechnology, Santa Cruz, CA) at 1:2000 dilution for 1 h at room temperature. The membrane was washed twice with TTBS for 10 min before detection using the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Rockford, IL). The chemiluminescent signal was visualized and analyzed with the Lumi-imager and the software LumiAnalyst 3.1 (Roche Applied Science, Indianapolis, IN).

3.2.10 Oocyte maturation assay

Full-grown follicles of size around 0.65 mm were isolated and placed in Cortland's medium. After the healthy follicles were selected, they were transferred into a 24-well plate, with 40 follicles per well, in a total of 400 μl medium. The follicles were subjected to different treatments, with 3 replicates for each treatment. The treatments included 17α, 20β-dihydroxy-4-pregnen-3-one (DHP, 5 ng/ml, Sigma-Aldrich), the control medium from a CHO cell line transfected with the vector only (25 μl/ml), the conditioned medium from the CHO cell line producing recombinant zebrafish BMP4 (25 μl/ml), or combination of DHP (5 ng/ml) with either control medium or recombinant zebrafish BMP4 (25 μl/ml). A control for spontaneous maturation was also included with no treatment. The follicles were incubated at 28°C for 12 h and scored for germinal vesicle breakdown (GVBD), which is a marker for oocyte maturation.

The follicles that have undergone GVBD exhibited translucent appearance as a result of ooplasmic clearing due to cleavage of yolk proteins.

3.3 Results

3.3.1 Establishment of stable CHO cell lines that express recombinant zfBMP2b and zfBMP4

The result on the distribution of BMPs and their receptors in the follicle strongly suggests that the somatic follicle cells are likely the target of BMP ligands which mostly come from the oocyte. To provide evidence for direct actions of BMP ligands on the follicle cells, we established two stable CHO cell lines that expressed recombinant zebrafish BMP2b and BMP4. These two BMP ligands were chosen because *bmp2b* was most abundantly expressed in the follicle and its expression level exhibited most significant change during folliculogenesis, whereas *bmp4* was the only ligand expressed in both oocytes and follicle cells. To confirm the expression of *bmp2b* and *bmp4* in the established CHO cell lines, RNA was extracted from these CHO cells for quantitative analysis by qPCR. A negative control was included in each assay by eliminating the reverse transcriptase, M-MLV, in the RT reaction. As shown in Fig. 3.2 and 3.3, both cell lines expressed the target genes; however, the expression level of *bmp4* was higher than that of *bmp2b* (Fig. 3.4).

3.3.2 Smad1/5/8 phosphorylation in cultured follicle cells in response to recombinant zebrafish BMP2b and BMP4

As BMPs signal through the Smad1/5/8 pathway, cultured zebrafish follicle cells were treated with 50 ng/ml human BMP2 (Biovision, Mountain View, CA) to test the response of Smad1/5/8 in the follicle cells. Using an antibody for phospho-Smad1/5/8 (Cell Signaling, Danvers, MA), we were able to detect an increase in Smad1/5/8 phosphorylation at 60 kDa in the cells treated with human BMP2. The effect was detectable at 30 min of treatment and increased significantly at 1 h (Fig. 3.5). Similarly, both recombinant zebrafish BMP2b and BMP4 produced from the CHO cells could also significantly induce phosphorylation of Smad1/5/8 dose-dependently at 2 h of treatment (Fig. 3.6 and 3.7).

3.3.3 Blockade of zfBMP4-induced Smad1/5/8 phosphorylation in cultured follicle cells by truncated BMP receptors

Two isoforms of type II BMP receptors were analyzed in the present study (BMPR-IIA, *bmpr2a;* BMPR-IIB, *bmpr2b*). To provide evidence for their being cognate receptors of BMPs in the zebrafish, we produced truncated forms of zebrafish BMPR-IIA and BMPR-IIB corresponding to the extracellular domains of zfBMPR-IIA and zfBMPR-IIB (Fig. 3.8 and 3.9). As shown in Fig. 3.10, the zfBMP4-induced phosphorylation of Smad1/5/8 was reduced by inclusion of the conditioned media containing BMPR-IIA and BMPR-IIB ectodomains in the treatment.

3.3.4 Suppression of spontaneous oocyte maturation by zfBMP4

It was reported previously that BMP15 had an inhibitory effect on final oocyte maturation in the zebrafish (167). To test if other forms of BMP have similar effects, we examined the rate of oocyte maturation in the presence or absence of recombinant zebrafish BMP4 together with DHP. As a positive control, DHP significantly promoted oocyte maturation compared to the spontaneous maturation, while the control medium exerted little effect on spontaneous maturation. By comparison, zfBMP4 could slightly but significantly suppress spontaneous maturation. However, zfBMP4 had no significant effect on DHP-induced oocyte maturation (Fig. 3.11).

3.4 Discussion

While recombinant proteins are needed for functional studies, researchers make use of various prokaryotic and eukaryotic systems to produce recombinant proteins, although they face different challenges when utilizing different systems.

E. coli is a widely used bacterial system for recombinant protein production. One of the major challenges in using the bacterial systems for recombinant protein production is the production of inclusion bodies, which are inactive aggregates that might affect the biological activities of the proteins produced. In 2002, the production of human BMP2 (hBMP2) in *E. coli* was reported (181). Since the recombinant protein hBMP2 was produced as inclusion bodies, they isolated the inclusion bodies followed by solubilization. *In vitro* refolding and purification was followed. Later, another group also attempted to produce hBMP2 in *E. coli* (182). In view

of the complicated refolding procedures and the expensive reagents, they developed a new approach to refold the protein by a specialized refolding buffer after the isolation of inclusion bodies and purification. Investigators have been trying to develop simple and inexpensive methods to produce hBMP2 in soluble form without the need for refolding or solubilization steps. To increase solubility, Ihm et al. co-expressed thioredoxin as a separate protein using the same vector, avoided unnecessary tags, and expressed hBMP2 in *E. coli* Rogetta-gamiB (DE3), allowing the formation of dimer through the disulfide bridges. No renaturation or solubilization steps were required, yet purification was needed (184).

To demonstrate that the recombinant protein is biologically active, an assay is needed. hBMP2 was assayed by their induction of alkaline phosphatase activity in C2C12 cells, which is a murine myoblast cell line (181-184). hBMP2 was also assayed by its ability to induce FGF-receptor 3 (FGFR3) (181). A study by Bessa et al., who also produced hBMP2 in *E. coli*, demonstrated for the first time that recombinant hBMP2 produced in *E. coli* is bioactive in human cell cultures by ability of hBMP to increase the activity of the early osteogenic marker in human fat-derived stem cells (183).

There are advantages and disadvantages using *E. coli* for recombinant BMP production. Since the proteins are produced in the form of inclusion bodies, protein production is enriched. Also, the recombinant protein can be protected from degradation and allows easy purification. On the other hand, it is time-consuming to go through the solubilization, refolding and purification steps. These processes are complicated and often result in low yield. Also, producing the recombinant BMPs in *E. coli* may encounter post-translation problems. In addition, the protein may be improperly folded and thus resulting in inactive form. Therefore, other eukaryotic expression systems may be considered for production of recombinant BMPs.

Early in 1995, researchers used the baculovirus expression system to produce heterodimeric BMPs. It involves transfecting insect cells to generate recombinant virus. The production of homo- and heterodimer BMP using baculovirus as a vector was reported (186). *Xenopus* BMP2, 4 and 7 (xBMPs) were generated, as well as the heterodimer xBMP4/7. The baculovirus system allows simultaneous expression of multiple genes, and they demonstrated the heterodimer xBMP4/7 to be most active as assayed by the mouse osteoblastic cell line (MC3T3-E1) for differentiation markers. The same group also demonstrated the bone-inducing activity of

the *Xenopus* BMPs that they produced (187). The advantages of using the baculovirus system are the higher yield and the production of either homodimeric or heterodimeric BMPs.

Yet, using the mammalian expression systems for recombinant protein production is still the choice for many researchers because it allows proper processing of the proteins. Israel et al. expressed human BMP2 in Chinese Hamster Ovary cells and had it characterized (64). hBMP2 was expressed with proper modifications such as signal and propeptide cleavage, N-linked glycosylation and dimerization. Yet, the authors also stated some limitations in producing mature BMPs from CHO cells. For example, since only one-third of the open reading frame encodes the mature region of BMP2, large amount of proteins expressed were actually byproducts. *Xenopus* BMP4 was also expressed in CHO cells as demonstrated by Northern and Western blotting (188). Dimerization and processing of xBMP4 also occurred correctly using CHO cells as the bioreactors and was shown to be bioactive.

Although not as many literatures have reported using mammalian expression system, for example CHO cells, to produce BMPs than in prokaryotic system, for example *E. coli*, we have finally decided to use CHO cells as the bioreactors for producing recombinant proteins for several reasons. First, in the mammalian system, appropriate post-translational modifications are expected. The protein can be properly folded and assembled. This is especially important as BMPs occur as dimers. This saves us the time for solubilization, renaturation and purification processes, as these steps are time-consuming and involve expensive reagents. On the other hand, CHO cells are easy and cheap to maintain. Although the production yield of recombinant protein might not be high, our project does not require huge amount of recombinant proteins for the study. A lab scale production would be sufficient for our purpose. Therefore, the low yield does not pose a problem for us.

CHO cells have been used as a bioreactor for the production of various recombinant proteins. While environmental effects such as pH, pO_2 and osmolarity would affect the yield of recombinant protein, culture temperature is also an essential factor. CHO cells are usually cultured at 37°C, the optimal temperature for mammalian cells. Yet, researchers have used lower culture temperature in an attempt to increase the production of recombinant proteins (57, 189, 190). Although low culture temperature decreases the specific growth rate, it can maintain high viability of cells for longer period (191). Also, by reducing glucose and glutamine consumption rate, less metabolic waste is produced (192, 193). Low culture temperature can

also reduce O_2 uptake and protease activity (194), as well as increasing tolerance against stress (195) . The effect of low culture temperature on the production of erythropoietin (EPO) in CHO cells was investigated and proven to be effective (190).

Although growing the recombinant CHO cells at lower temperature has beneficial effects on productivity, the cell growth is slower, which increases the batch time, reducing the overall productivity. Therefore, researchers developed a new method to maximize production. A biphasic process was employed to maximize the production of interferon-γ (196). The recombinant CHO cells were first grown at 37°C to reach a high density, which afterwards were shifted to 32°C to reach high specific productivity. They developed and validated a model for predicting the optimal time to shift and found that if they did the temperature shift after 3 days at 37°C, the volumetric productivity was greater than growing at either 37°C or 32°C. Our lab has adopted this biphasic process to produce zebrafish gonadotropins in earlier years, and was found to be efficient (197). In our study, we employed a similar strategy to produce the recombinant zfBMPs in CHO cells. We first allowed the recombinant CHO cells to grow at 37°C for 3 days, followed by shifting the culture temperature to 28°C, hoping to maximize the productivity and improving viability. 37°C is the proliferation phase of the CHO cells, in which cell growth occurs. 28°C is the production phase of the CHO cells, in which recombinant proteins are secreted. Therefore, by shifting the recombinant CHO cells from 37°C to 28°C, we can maximize the production of recombinant proteins as well as allowing enough time for the CHO cells to proliferate.

In our study, we chose to produce zebrafish BMP2b and BMP4. Among all the BMP ligands that we studied, BMP2b is most abundant in the follicles, implicating its significance in the zebrafish ovary. BMP4 is the second most abundant in the follicles among the BMP ligands that we studied. Different from other BMP ligands in this study, it was found in both oocyte and follicle cell compartments. Its special localization pattern arouses our interest in finding out its functional role. Other BMP lignds including BMP2a, BMP6 and BMP7a showed relatively low abundance when compared to BMP2b and BMP4. Although we are interested in investigating their functions as well, due to the time limit, we decided to produce zfBMP2b as the representative of an oocyte-derived BMP, and zfBMP4 due to its special localization pattern compared to other ligands.

After making the zebrafish recombinant BMPs, we needed to demonstrate that they were biologically active. In this study, we have developed an assay to demonstrate that zfBMPs were active by their ability to induce Smad1/5/8 phosphorylation in cultured zebrafish follicle cells. Human BMP2 which is commercially available was first used to establish this assay. After demonstrating its ability to induce Smad1/5/8 in cultured follicle cells, the biological activity of the recombinant zebrafish BMPs made in CHO cells were evaluated in a similar manner, demonstrating their bioactivity in the zebrafish ovary. For the recombinant BMPs that were made in other systems, various assays have been used to demonstrate their biological activity. For example, hBMP2 made in *E. coli* was assayed by their induction of alkaline phosphatase activity in C2C12 cells, which is a murine myoblast cell line (181-184). In addition, hBMP2 could also induce FGF-receptor 3 (FGFR3), which can be used as an assay as well (181). Compared to other assays, the assay that we developed was simple and convincing. It is an assay developed from cultured zebrafish follicle cells, which is an excellent proof that the recombinant zebrafish BMPs worked in zebrafish ovary. On top of that, we had the phospho-Smad1/5/8 antibody available and Western blotting was easy and cheap to perform, making this assay favorable for our study. An improvement to this assay would be that a loading control, for example total Smad1/5/8 or a housekeeping gene, should be included in the assay to make our results more convincing.

In our study, we also made the truncated forms of the two type II receptors, BMPR-IIA and BMPR-IIB and demonstrated their ability to bind BMP4 as well as their involvement in BMP signaling. Both receptors were able to block the Smad1/5/8 phosphorylation by BMP4 in the cultured follicle cells, indicating their involvement in BMP signaling. Previous studies have reported ability of BMP ligands to bind their receptors. A novel type II receptor from human skin fibroblasts, tBRK3 (truncated BMP Receptor Kinase-3) was cloned, which could bind BMP4 (102). When expressed in COS cells, BRK3 specifically bound BMP4. Binding was enhanced by co-expression with type I receptor. In the same year, the binding of BMP2 and BMP7 to hBMPR-II was also reported. Similarly, BMP binding increased in concert with the type I receptors (ActR-I, BMPR-IB and BMPR-IA) (101).

We have made truncated forms of the BMP receptors and demonstrated that they were functionally active. Early in 1994, it was reported that a truncated BMP receptor that could affect the dorsal-ventral patterning in the developing *Xenopus* embryo (114). The truncated form

of BMPR-II was also used to demonstrate its involvement in GDF9 signaling (105). Regarding our study, we again used a simple assay to demonstrate the potential binding of BMPR-II to BMP4 and its involvement in BMP signaling. We simply incubated the ectodomains of BMPR-II with BMP4, and observed their ability to reduce phospho-Smad1/5/8 level that was induced by BMP4. This not only demonstrated potential binding of BMPR-II to reduce the ability of BMP4 to induce Smad phosphorylation, but also suggested involvement of BMPR-II in BMP signaling in the zebrafish ovary.

After testing the biological activity of the zfBMPs, we proceed to test the functional role of the zfBMPs in oocyte maturation. By incubating the full-grown follicles with zfBMP4 or DHP, or combination of zfBMP4 and DHP, we investigated the role of zfBMP4 on oocyte maturation *in vitro*. Although we are eager to test the biological activity of zfBMP2b as well, zfBMP2b was run out by the time we conducted this assay. In the future, the biological activity of zfBMP2b should be tested as well after more production of zfBMP2b from the CHO cells. Germinal vesicle breakdown (GVBD) is used as an indicator for oocyte maturation, in which the germinal vesicle migrates to the periphery and the follicle becomes translucent due to ooplasmic clearing and cleavage of yolk proteins. The oocyte maturation assay is an excellent assay in monitoring the biological activities of the CHO cell-produced recombinant zfBMPs, since the assay is relatively easy to perform and it does not require expensive reagents. Also, the result can be easily interpreted as GVBD is a clear indication that oocyte maturation occurs. In addition, a positive control is available for the assay, in which DHP can significantly promote oocyte maturation. Our results indicated that zfBMP4 slightly, yet significantly suppressed spontaneous oocyte maturation, though it did not have effect on DHP-induced oocyte maturation. Previously it was reported that BMP15 had a suppressive role in oocyte maturation, by decreasing hCG- or DHP-induced oocyte maturation (166, 167). Our results showed that zfBMP4 did not have significant effect on DHP-induced maturation. Therefore, BMP4 and BMP15 might have differential roles in suppressing oocyte maturation, which is worth investigating in the future.

In conclusion, we have produced recombinant zebrafish BMP2b and BMP4 for our study using CHO cells as the bioreactor. Both BMPs were demonstrated to be biologically active based on their ability to induce Smad phosphorylation in cultured follicle cells. In addition, this strengthened our belief that BMPs as oocyte-derived factors may signal from the oocyte and

exert their actions on follicle cells. BMPR-II ectodomains were also produced in a similar manner, and it was suggested that they bound to BMP4 to reduce its ability to induce Smad phosphorylation in cultured follicle cells, providing hints of their participation in BMP signaling. Finally, we have tested the functional activity of zfBMP4 and demonstrated its ability to suppress spontaneous oocyte maturation.

Table 3.1 Primers used in semi-quantitative PCR and real-time qPCR

Gene	Accession no.	Sequence	Expected Size (bp)
bmp2b	NM 131360	CGGGGATCCGCCACCATGGTCGCCGTGGTCC GC	197
		CCGAACATATTGAGCAAGCGTAG	
bmp4	NM 131342	CCAACACCGTGAGAGGATTCC	379
		TCCACAGCAAGGCCATGATTAG	
bmpr2a	NM 001039817	CGGGGATCCGCCGCCACCATGCAGA	414
		GCGCTCGAGTCATTCCTCTCTGTGCA	
bmpr2b	NM 001039807	CGGGGATCCGCCGCCACCATGCAGA	420
		GCGCTCGAGTCACTCATCTCTGTGTA	

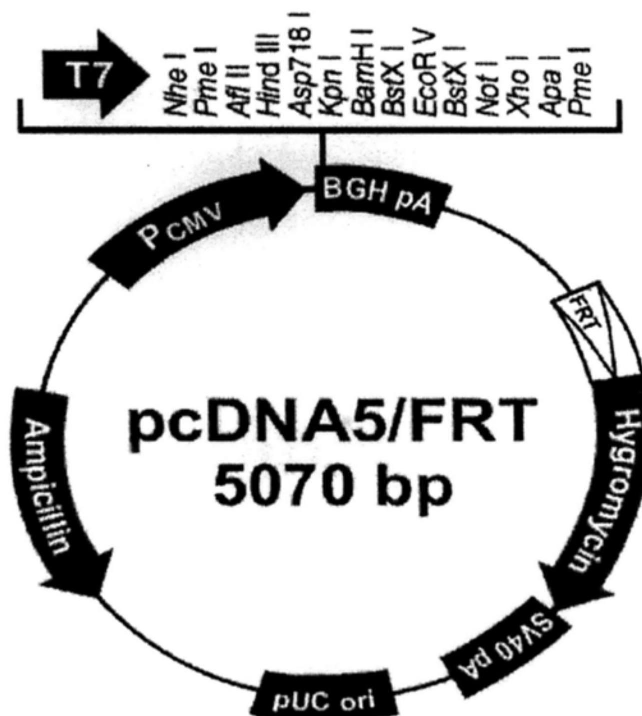
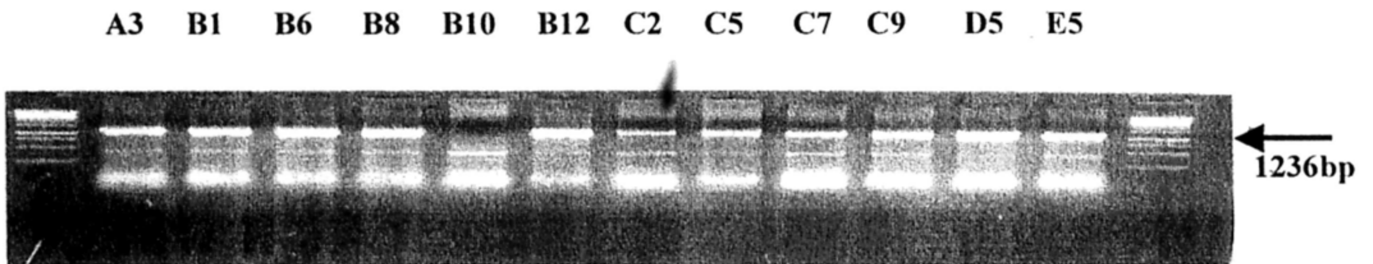


Fig. 3.1 pcDNA5/FRT expression vector used for cloning opening reading frames of *bmp2b* and *bmp4.* The inserts were cloned into the restriction sites BamHI and XhoI. The FRT site allows homologous recombination event to occur between the host cell line and the expression vector.

(A)



(B)

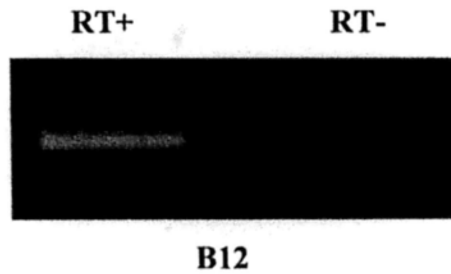
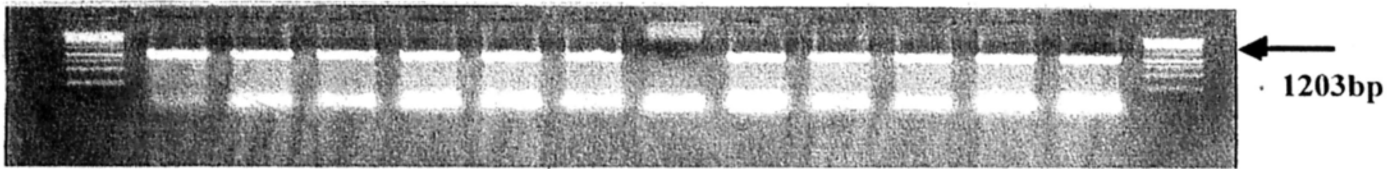


Fig. 3.2 (A) 12 clones were generated for *bmp2b*, of which 11 of them (except B10) were positive for *bmp2b* by RT-PCR. **(B)** Clone B12 was chosen to generate a cell line stably expressing *bmp2b*.

(A)



(B)

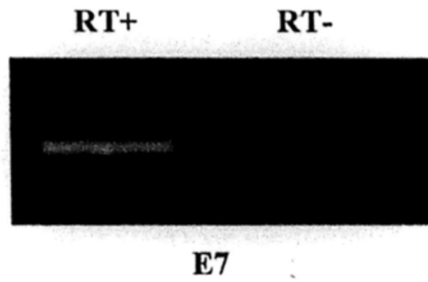
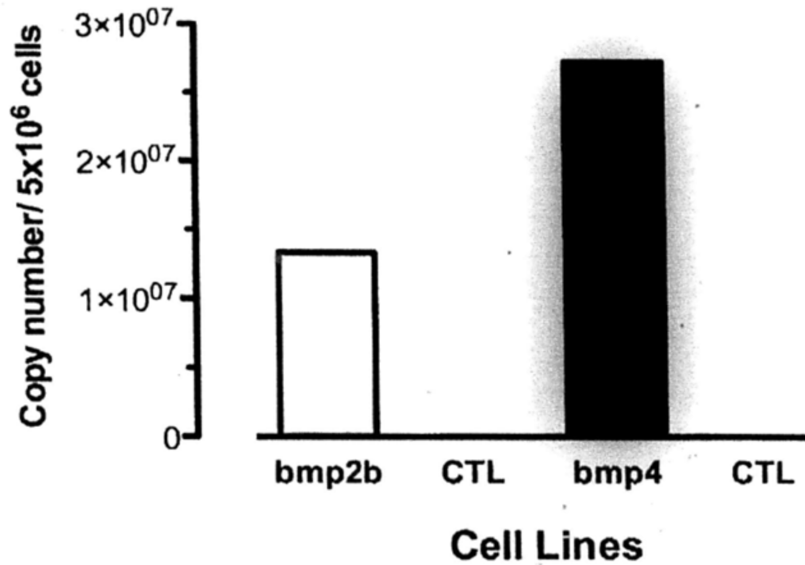
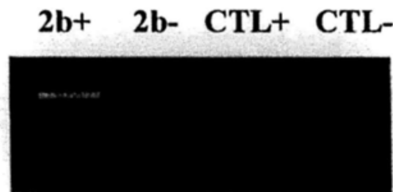


Fig. 3.3 **(A)** 12 clones were generated for *bmp4*, of which 11 of them (except E5) were positive for *bmp4* by RT-PCR. **(B)** Clone E7 was chosen to generate a cell line stably expressing *bmp4*.

(A)



(B)



(C)

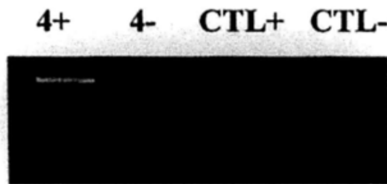


Fig. 3.4 (A) Copy number of BMP-producing cell lines per 5,000,000 cells vs. control. *bmp4* cell line has a higher expression than that of *bmp2b*. **(B)** RT-PCR result showing expression of *bmp2b*. From left: *bmp2b* RT+, *bmp2b* RT-, control RT+, control RT-. **(C)** RT-PCR result showing expression of *bmp4*. From left: *bmp4* RT+, *bmp4* RT-, control RT+, control RT-.

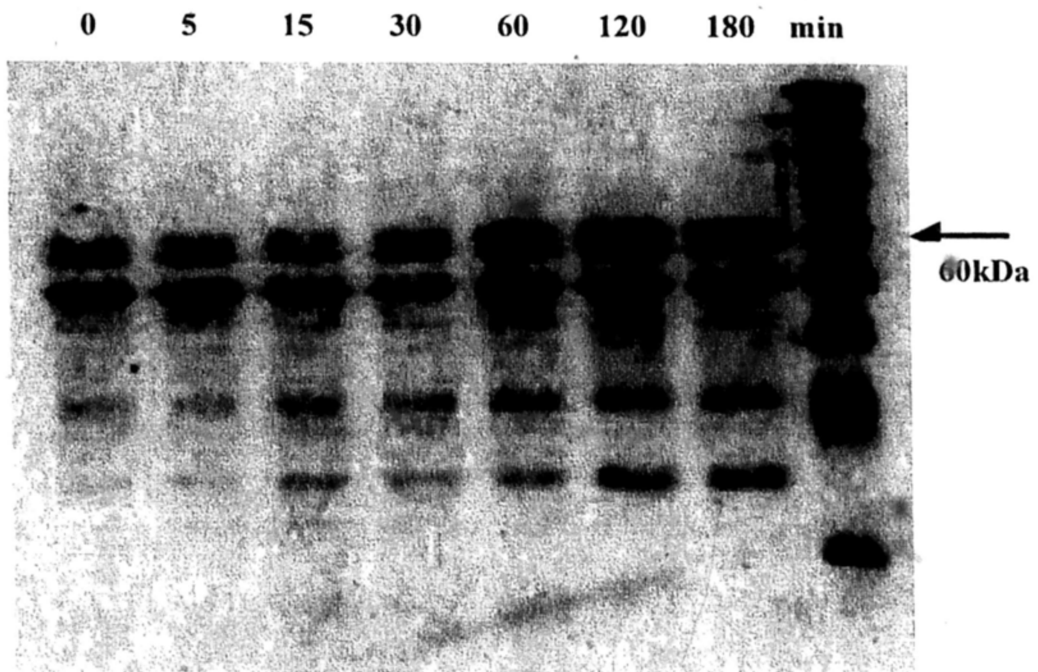


Fig. 3.5 Western blotting result showing recombinant hBMP2 inducing Smad1/5/8 phosphorylayion (60kDa) in cultured follicle cells at a concentration of 50 ng/ml. From left: 0 h, 5 min, 15 min, 30 min, 60 min, 120 min, 180 min.

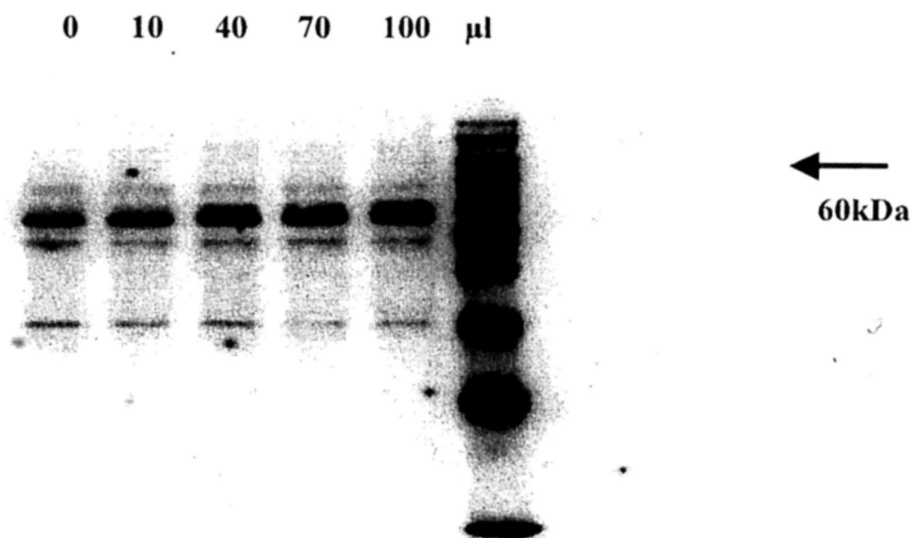


Fig. 3.6 Western blotting result showing zfBMP2b inducing Smad1/5/8 phosphorylation (60kDa) at 2 h. The zfBMP2b-containing medium (0 – 100 µl) was mixed proportionally with different amounts of control medium (from control CHO cells) in a total of 100 µl and added to the follicle cells resulting in a final volume of 1 ml per well. From left: 0 µl, 10 µl, 40 µl, 70 µl, 100 µl.

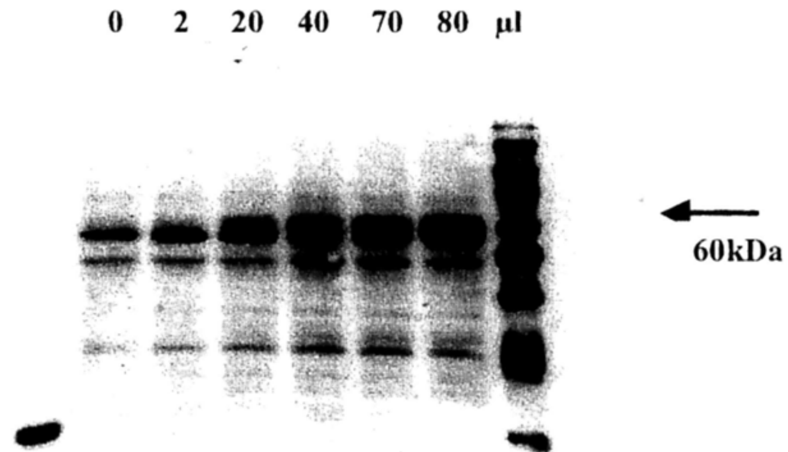
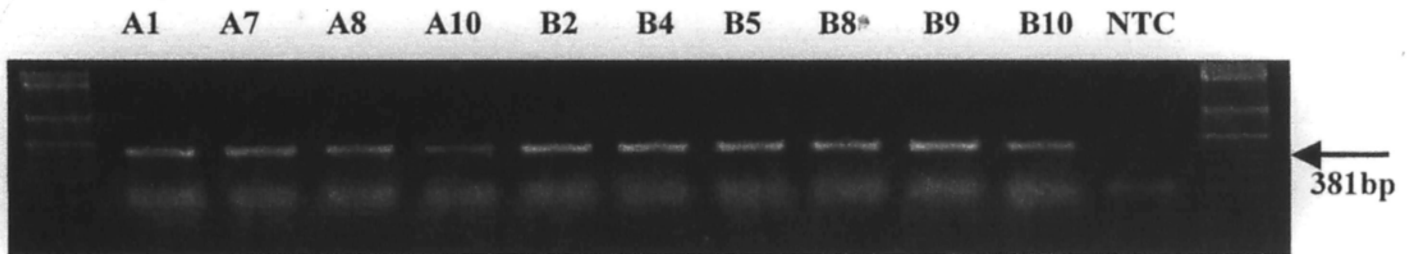


Fig. 3.7 Western blotting result showing zfBMP4 induced Smad1/5/8 phosphorylation (60kDa) at 2 h. The zfBMP4-containing medium (0 – 80 μl) was mixed proportionally with the control medium in 80 μl before adding to the follicle cells, resulting in a total of 1 ml. From left: 0 μl, 2 μl, 20 μl, 40 μl, 70 μl, 80 μl.

(A)



(B)

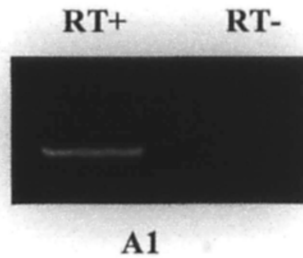
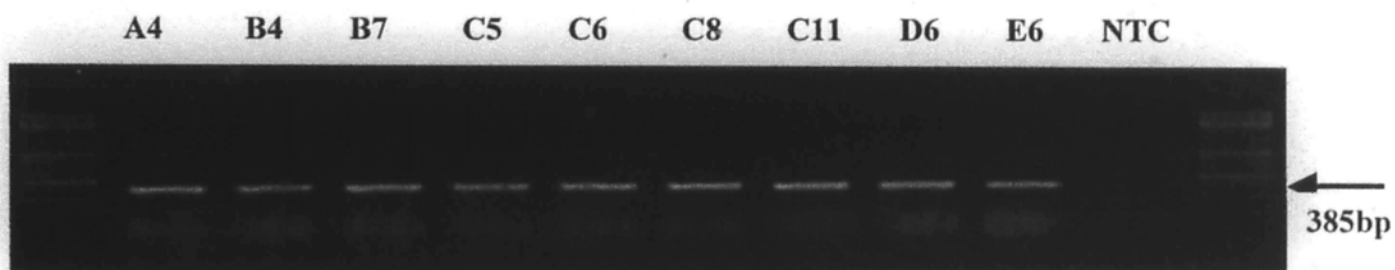


Fig. 3.8 (A) 10 clones were generated for *bmpr2a*, of which all of them were positive for *bmpr2a* by RT-PCR. **(B)** Clone A1 was chosen to generate a cell line stably expressing *bmpr2a.*

(A)



(B)

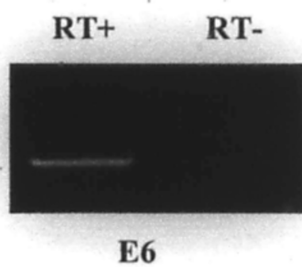
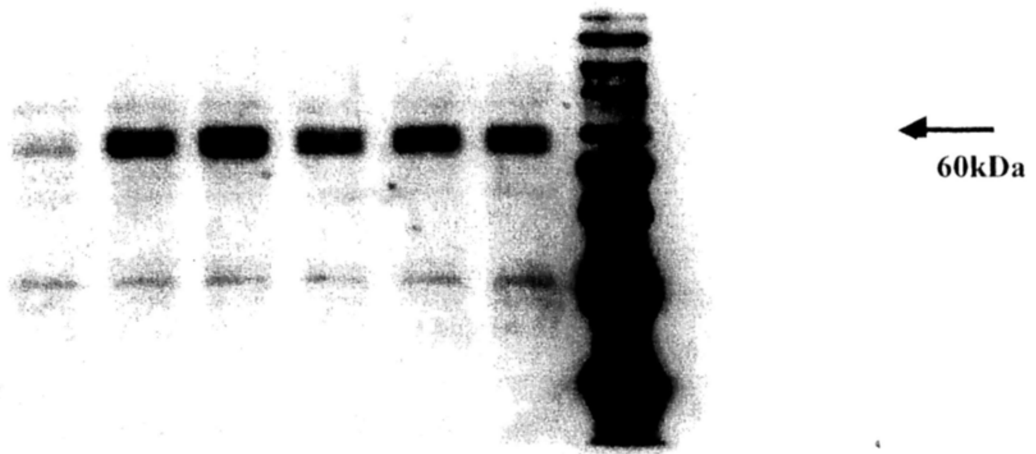


Fig. 3.9 **(A)** 9 clones were generated for *bmpr2b*, of which all of them were positive for *bmpr2b* by RT-PCR. **(B)** Clone E6 was chosen to generate a cell line stably expressing *bmpr2b.*



10	**5**	**-**	**-**	**-**	**-**	**Control (µl/ml)**
-	**5**	**10**	**5**	**5**	**5**	**zfBMP4 (µl/ml)**
-	**-**	**-**	**5**	**-**	**2.5**	**BMPR-IIA ED (µl/ml)**
-	**-**	**-**	**-**	**5**	**2.5**	**BMPR-IIB ED (µl/ml)**

Fig. 3.10 Suppression of zfBMP4-induced Smad1/5/8 phosphorylation in the follicle cells by recombinant ectodomains (ED) of type II BMP receptors BMPR-IIA and BMPR-IIB. The control medium, zfBMP4 and receptor ectodomains-containing media were mixed in different combinations in 10 µl and added to the follicle cells resulting in a total of 1 ml.

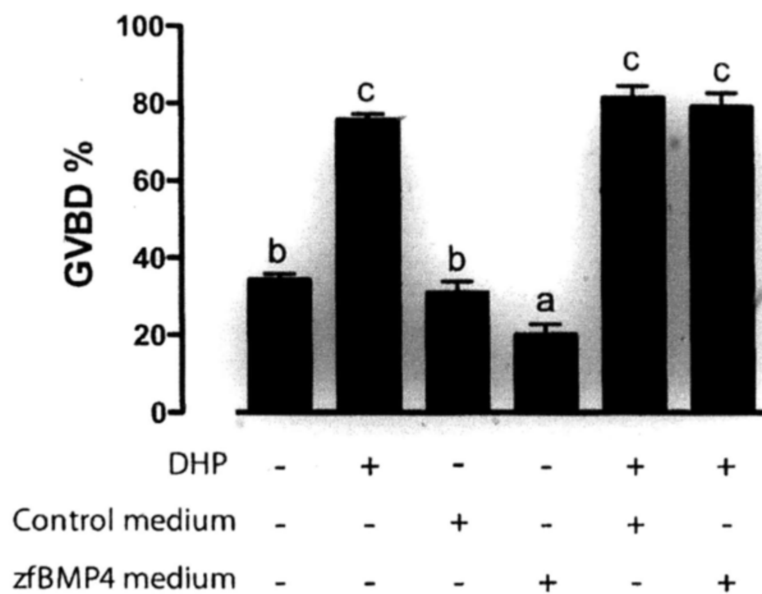


Fig. 3.11 Effect of recombinant zfBMP4 on oocyte maturation. Full-grown follicles were isolated and incubated for 12 h with different treatments. Germinal vesicle breakdown (GVBD) was used as an indicator for oocyte maturation. DHP (5 ng/ml) was used as a positive control. The control medium from the CHO cell line transfected with vector only (25 μl/ml) or conditioned medium from zfBMP4 cell line (25 μl/ml) was administered either alone or in combination with DHP (5 ng/ml). The values are the mean ± SEM (n=3) from a typical experiment representing 5 independent experiments, and different letters indicate statistical significance ($P < 0.05$).

Chapter 4

Regulation of the Ovarian Activin-Inhibin-Follistatin System by Zebrafish BMPs

4.1 Introduction

Although it is well established that gonadotropins play an indispensable role in ovarian development, the intraovarian local factors have been gaining increasing attention on their regulatory effects in the vertebrate ovary. In the zebrafish, the activin-inhibin-follistatin system has been well characterized as a group of local growth factors in the ovary, with their functional roles investigated or proposed, highlighting their significance in zebrafish ovarian development (34, 173, 198, 199).

During follicle development, the activin-inhibin-follistatin system exhibits dynamic changes in expression from primary growth (PG) to fully-grown (FG) stage. The expression of activin βA (*inhbaa*) is low at PG stage, but it increases dramatically during follicle growth, reaching the peak level at mid-vitellogenic (MV) stage and followed by a slight drop at FG stage. On the other hand, activin βB (*inhbb*) exhibits a slight but significant increase in expression starting at early vitellogenic (EV) stage, although the fold increase is much less than that of activin βA. The expression of follistatin (*fst*), the activin-binding protein, is low at PG stage, similar to that of activin βA. Yet, its expression increases drastically as it enters vitellogenesis, reaching the peak at MV stage. In contrast to activin βA, there is a sharp decline in expression of follistatin at FG stage (198). Inhibin α (*inha*), as the activin antagonist, has extremely low expression in PG stage. There is a steady increase in expression of inhibin α during vitellogenesis, followed by a drastic surge at FG stage prior to final oocyte maturation (173). The dynamic changes in expression of the activin-inhibin-follistatin system strongly suggest that it must be under regulation to increase or decrease expression at specific stages. This leads us to search for the factors that participate in regulation of the activin-inhibin-follistatin system.

In earlier years, our group has identified the expression of activin βA and activin βB in the follicle layer compartment, while follistatin was found to localize in the oocyte (198), although low expression in the follicle layer was observed in primary follicle cell culture (199). In recent years, our group has developed a more sensitive method to mechanically separate the follicle layer away from the oocyte compartment and verify the cleanliness of each compartment using specific markers, which provides more convincing results for the localization studies (168).

Using the new method, the expression of the activin subunits was confirmed in the follicle layer, while follistatin was confirmed to localize in the oocyte compartment (171), which might modulate the activities of activin by binding and neutralization. The localization of inhibin α was also studied using the new method, and it was found to localize exclusively in the follicle layer (173).

In the previous chapters, we have identified BMPs as oocyte-derived factors, while the BMP receptors were located exclusively in the follicle layer. Therefore, we hypothesize that BMPs signal from the oocyte towards the follicle layer in a paracrine manner. However, the targets of BMPs in the follicle layer remain unknown. Unlike the EGF family members which are mostly located in the oocyte (171), or the IGF family which is located in both the follicle layer and oocyte (Yu and Ge, unpublished), activin subunits and inhibin α are exclusively expressed in the follicle layer (171, 198). Together with their dynamic changes during folliculogenesis, these two pieces of evidence suggest them to be potential targets of BMP signaling from the oocyte. Although follistatin was found to localize in the oocyte, there was low expression detected in primary follicle cell culture (199), suggesting that it can be potential targets of BMP signaling as well. In fact, it has been reported that EGF family members (EGF, BTC, TGFα, HB-EGF) derived from the oocyte up-regulate activin βA and βB in the follicle layer (171), demonstrating a paracrine regulation from the oocyte to the follicle layer. As a potential BMP-mediated oocyte-to-follicle cell communication is observed, we hypothesize the activin-inhibin-follistatin system to be a potential target of BMP signaling.

There were a few reports documenting the regulation of activin-inhibin-follistatin system in the ovary by BMPs. Such studies were found in different species, including chicken, human, rat, bovine and sheep (152, 153, 200-202). In the chicken ovary, BMP6 was expressed in the theca cells. Pre-ovulatory granulosa cells were cultured with theca-derived BMP6, and it was found that BMP6 could enhance basal and gonadotropin-induced inhibin A production, as well as increasing basal inhibin α and βA subunits in F1, F2, F3/4 follicles and increased basal βB subunit in F3/4 cells. BMP6 also enhanced FSH/LH-induced α subunit in all follicles as well as FSH-induced βA subunit in F2, F3/4 cells. When the cells were co-treated with gonadotropins and BMP6, the expression of βB subunit also increased. Collectively, these studies on the regulation of activin-inhibin subunits and production of inhibin by BMP6 revealed a functional BMP system in the avian ovary (200).

In human granulosa cell culture, BMP6 expression was detected. Addition of BMP6 stimulated the expression of activin βA and βB subunits, but not the α subunit (201). On the contrast, in rat granulosa cell culture, BMP6 decreased α and β subunits mRNA (153). In bovine granulosa cell culture, BMP4, 6 and 7 enhanced inhibin A and activin A production (202), while in sheep granulosa cells, BMP2 could also enhance inhibin A production (152). Therefore, in mammals, BMPs were shown to modulate the activin-inhibin-follistatin system in ovary development. However, the significance of the regulation was not discussed in detail in these studies.

In this study, we have developed a novel approach to study the regulation of the activin-inhibin-follistatin system by BMPs. By co-incubating the zfBMP-producing CHO cells (Chapter 3) with zebrafish primary follicle cells, the BMPs secreted from the CHO cells would act on the follicle cells in a paracrine manner, mimicking the BMPs secreted from the oocyte and acting on the neighboring somatic cells. The siRNA targeting zfBMP4 was introduced into the CHO cells cells by transfection to knock down the expression of zfBMP4, demonstrating the specificity of the effect of zfBMP4 on inhibin α and follistatin. In order to provide further evidence for a paracrine signaling mechanism by the BMP family, microinjection experiment was also performed using morpholino to target the splicing site of *bmp2b*. We attempted to knock down expression of BMP2b in the oocyte and by using inhibin α as the marker gene on the follicle layer, we expected a drop in expression of inhibin α along with the knockdown of BMP2b in the oocyte. By investigating the regulatory roles and the signaling mechanism of BMPs on the activin-inhibin-follistatin system, our understanding of the physiological importance of BMPs in the zebrafish ovary can be enhanced.

4.2 Materials and Methods

4.2.1 Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), Invitrogen (Carlsbad, CA) and USB (Cleveland, OH) unless otherwise stated.

4.2.2 Animals

Zebrafish (*Danio rerio*) were purchased from local pet stores and maintained in flow-through aquaria at 28±1°C on a 14-h light/10-h dark photoperiod. The fish were fed thrice a day

with commercial tropical fish food. The animals were anaesthetized on ice and sacrificed by decapitation before dissection. All experiments were performed under license from the Government of the Hong Kong Special Administrative Region and endorsed by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong.

4.2.3 Primary follicle cell culture

Around 40 zebrafish ovaries were dissected and the follicles were dispersed slightly using plastic pipette. The follicles were washed with 60% L-15 (Gibco) several times in a 15 ml Falcon tube (BD) and filtered through a sieve to remove large FG follicles. The filtered follicles were then washed several times with medium M199 (Gibco) and plated into 10-cm culture dishes. The follicles were grown for 3 days at 28°C in M199 with 10% FBS (Hyclone). Fresh medium of the same components was replaced after 3 days. After continuous incubation for 3 more days, the follicle cells were sub-cultured into 24-well plates (BD), with 200,000 cells per well. After 24-h incubation with the supplement of FBS, the medium was replaced with serum-free M199 for starvation purpose. The follicle cells were treated with recombinant zebrafish BMP4 (R&D, Cat no. 1128-BM) or BMP2a (R&D, Cat no. 111-BM)). The treated follicle cells in each well were collected with 200 μl Tri-reagent for RNA extraction and reverse transcription.

4.2.4 Culture of CHO cells

The CHO cell lines expressing recombinant zebrafish BMP2b and BMP4 were established as described in the previous chapter. A control cell line expressing the empty vector (pcDNA5) was also established. The CHO cell lines were maintained in F-12 medium (Gibco) supplied with 6 g/L HEPES (USB, Cleveland, OH) and 10% FBS (Hyclone) supplied with antibiotics (Streptomycin, 100 μg/ml; penicillin, 100 U/ml). The CHO cells were maintained in 10-cm culture dishes and passed twice a week when the cells reached 100% confluency.

4.2.5 Co-incubation of primary follicle cells and CHO cells

Recombinant zfBMP2b and zfBMP4-producing CHO cells, as well as the control CHO cells, were seeded in 12-well plates, with 200,000 cells in 0.5 ml F-12 medium with 10% FBS. The CHO cells were allowed to attach within a 24-h incubation period at 37°C. On the next day, 200,000 follicle cells from the step 4.2.3 were added to each well in 0.5 ml F-12 medium with

10% FBS (incubation medium was changed from M199 to F-12 during subculture at the step 4.2.3 when resuspending the pellet). The addition of the zebrafish follicle cells to the CHO cells already plated resulted in a total of 1 ml F-12 medium containing 400,000 cells in total (200,000 CHO cells and 200,000 follicle cells). The incubation temperature was reduced to 28°C, which is the optimal temperature for the growth of zebrafish follicle cells and production of recombinant zfBMP by the CHO cells. After 24 h of co-incubation, the follicle cells became attached to the well surface and the medium was changed to 0.5 ml of serum-free F-12 for another 24 h after washing once with 1 ml of F-12. The 24 h incubation period was followed by RNA extraction with Tri-reagent and reverse transcription. For the control setting, 200,000 control CHO cells (transfected with empty vector) were seeded, followed by adding 200,000 follicle cells. The lower dose included 100,000 BMP-producing CHO cells plus 100,000 control CHO cells, followed by adding 200,000 follicle cells, resulting in a total of 400,000 cells. The higher dose included 200,000 BMP-producing CHO cells added with 200,000 follicle cells, reaching a fixed total of 400,000 cells.

4.2.6 siRNA transfection

siRNA targeting zebrafish *bmp4* (Duplex of 5' AUGGGAAAGUUUCGACGUUUU 3' and 5' AACGUCGAAACUUUCCCAUUU 3') was designed by ABI siRNA Target finder and synthesized by Integrated DNA technologies (IDT, Coralville, IA). The negative control targeting zebrafish *gdf9* (Duplex of 5' GCAGUUCUCGGACUGUCAA 3' and 5' UUGACAGUCCGAGAACUGC 3') was designed by Invitrogen's online BLOCK-iT RNAi Designer and synthesized by IDT. For the siRNA transfection, the reverse transfection protocol rather than the forward transfection protocol (Invitrogen) was used, and was performed in 12-well plates. Six pmol RNAi duplex was diluted with 100 μl F-12 free of serum and antibiotics. One μl of Lipofectamine RNAiMAX (Invitrogen) was added to the diluted RNAi duplex, followed by mixing and incubating at room temperature for 20 min. The recombinant or control CHO cells at 200,000 cells/500 μl were added to the complex (~100 μl), resulting in a total of 600 μl and final RNAi concentration of 10 nM. The transfected CHO cells were allowed to incubate for 24 h at 37 °C. Follicle cells were added on the transfected CHO cells the next day and incubated for 24 h at 28 °C. Starvation was performed as previous described. The co-cultured cells were collected in Tri-reagent for RNA extraction followed by reverse transcription.

4.2.7 Microinjection

Zebrafish ovaries were dissected and around 200 FG follicles of size around 600 μm were isolated in Cortland's medium. Healthy follicles were selected for microinjection. Needles for microinjection were prepared from glass capillaries (World Precision Instruments, Inc, Sarastoa, FL). Glass capillaries were pulled into needles by a micropipette puller (Model # PC-10) (Narishige, International USA, Inc., East Meadow, NY) and had the tips of needles polished using a microgrinder (EG-400) (Narishige). Microinjection was performed using the Nanoliter Injector (Drummond Scientific Co., Broomall, PA, Cat no. 3-000-203-XV). Control morpholino (5' CCTCTTACCTCAGTTACAATTTATA 3') and morpholino targeting the splicing site of intron 2-3 and exon 3 of *bmp2b* (5' ATATCAAATCAGGCCTCACCTTCGT 3') (Gene Tools, LCC, Philomath, OR) were designed by the online program of the manufacturer. Figure 4.1 illustrates the targeting site of morpholino. The follicles were injected with 4.6 nl of around 200 μM of morpholinos, with the injection volume approximately equaled to 1% of the follicle volume and reached the final concentration of 10 μM inside the follicles. Water was also injected as a control. The injection experiment was repeated for 3 days with the order of injection (water, control morpholino, *bmp2b*-targeting morpholino) rotated for each day. The follicles were incubated for either 2 h or 15 h after injection. Ten healthy follicles were collected for each group on each day in Tri-reagent for RNA extraction and reverse transcription.

4.2.8 RNA isolation and reverse transcription

Total RNA was isolated from the cultured follicle cells, co-cultured cells or injected FG follicles using Tri-Reagent according to manufacturer's instruction. Total RNAs from the follicle cells, co-cultured cells or FG follicles were used for reverse transcription in a 10 μl volume containing 1 x M-MLV RT buffer, 0.5 mM of each dNTP, 0.5 μg oligo(dT), 0.1 mM DTT and 100 U M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA). Reverse transcription was performed at 37°C for 2 h.

4.2.9 Real-time qPCR

Real-time qPCR was used to monitor the changes in expression of the activin-inhibin-follistatin system under regulation by the zfBMPs. Real-time qPCR was carried out on the iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) in a volume of 30 μl

containing 10 μl of 1:20 diluted RT reaction mix, 1 x PCR buffer, 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, 0.2 μM of each primer, 0.75 U Taq Polymerase, 0.5 x EvaGreen (20 x concentrated; Biotium, Hayward, CA) and 20 nM fluorescein (Bio-Rad). The reaction profile consisted of 40 cycles of 95°C for 30 sec, 56°C-60°C for 30 sec (annealing temperature varies for different target genes), 72°C for 40 sec and 84°C for 7 sec for signal detection. A melt curve analysis consisting of 180 cycles of 7 sec with temperature increase of 0.2°C/cycle was performed at the end of the reaction to demonstrate the specificity of the reaction. Results were normalized to the housekeeping gene *ef1a*. The primers for PCR were designed according to the sequences available in the GenBank and synthesized by Integrated DNA Technologies (IDT, Coralville, IA). Primers used for amplification of different target genes were listed in Table 4.1.

4.2.10 Statistical analysis

All real-time qPCR data were normalized to the housekeeping gene *ef1a*. All values were expressed as the mean ± SEM and the data were analyzed by one-way ANOVA followed by Dunnett's test or by Newman-Keuls test for comparisons of all pairs of groups using the GraphPad Prism 5 for Mac OS X (GraphPad Software, San Diego, CA).

4.3 Results

4.3.1 Regulation of *inhbaa, inhbab, inhbb, fst* and *inha* expression by zfBMP2b and zfBMP4

When recombinant zfBMP2b or zfBMP4-producing CHO cells (200,000 cells) were incubated with zebrafish follicle cells for 24 hours, all activin β subunits (*inhbaa, inhbab, inhbb*) were down-regulated, while the activin-binding protein, follistatin (*fst*), and the activin antagonist, inhibin α (*inha*), were up-regulated. zfBMP2b could reduce the expression of *inhbaa* and *inhbb* to around 50%, and increase the expression of *inha* to around 400%. However, the effect of zfBMP2b on *inhbab* and *fst* was not significant. The effect of zfBMP4 was more potent when compared to that of zfBMP2b, which might be due to the higher expression level of *bmp4* in the CHO cell line as shown in previous chapter (Chapter 3). zfBMP4 could reduce the expression of all activin β subunits to less than 50%, while increasing the expression of *fst* to around 300% and that of *inha* to 1500%, respectively (Fig. 4.2).

4.3.2 zfBMP2b and zfBMP4 dose-dependently down-regulated activin β subunits and up-regulated *fst* and *inha* at 24 h

When zfBMP-producing CHO cells were incubated with zebrafish follicle cells for 24 hours, both zfBMP2b and zfBMP4 down-regulated all activin β subunits (*inhbaa*, *inhbab*, *inhbb*) in a dose-dependent manner (0, 100,000 and 200,000 zfBMP2b or zfBMP4-producing CHO cells together with 200,000 follicle cells), although down-regulation of *inhbab* was not statistically significant by zfBMP2b. zfBMP2b at low concentration (100,000 CHO cells/well) reduced the expression of the activin β subunits to around 75%, while at the same concentration, zfBMP4 could reduce those to around 50%. With 200,000 zfBMP2b-producing CHO cells, the expression of the activin β subunits could be reduced to around 50%, while 200,000 zfBMP4-producing CHO cells could reduce *inhbab* and *inhbb* to around 50% but *inhba* by less than 50%. While both zfBMP2b and zfBMP4 could down-regulate all activin β subunits, they up-regulated *fst* and *inha* in a dose-dependent manner (0, 100,000 and 200,000 zfBMP2b or zfBMP4-producing CHO cells together with 200,000 follicle cells). zfBMP2b at low concentration (100,000 CHO cells/well) did not significantly up-regulated *fst*, while 200,000 zfBMP2b-producing CHO cells could increase expression of *fst* to around 150%. With 100,000 zfBMP2b-producing CHO cells, expression of *inha* was increased to 200%, while at higher concentration (200,000 CHO cells/well), *inha* expression was increased to 300%. Similar dose-dependent effect was also observed for zfBMP4, but it was much more potent than that of zfBMP2b. zfBMP4 at low concentration (100,000 CHO cells/well) could increase expression of *fst* to around 250%, while with 200,000 zfBMP4-producing CHO cells, expression of *fst* was increased to close to 300%. While low concentration of zfBMP4 (100,000 CHO cells/well) could increase expression of *inha* to slightly less than 1500%, a higher concentration of zfBMP4 (200,000 CHO cells/well) could increase *inha* to slightly more than 1500% (Fig. 4.3).

4.3.3 zfBMP2b and zfBMP4 down-regulated activin β subunits and up-regulated *fst* and *inha* at 48 h

When zfBMP2b-producing CHO cells were incubated with zebrafish follicle cells for 48 hours, all activin β subunits were down-regulated. However, only *inhbab* was down-regulated in a dose-dependent manner (0, 100,000 and 200,000 zfBMP2b-producing CHO cells together with 200,000 follicle cells). When zfBMP4-producing CHO cells were incubated with zebrafish

follicle cells for 48 hours, all activin β subunits were down-regulated in a dose-dependent manner (0, 100,000 and 200,000 zfBMP4-producing CHO cells together with 200,000 follicle cells). Both concentrations of zfBMP4 (100,000 or 200,000 CHO cell/well) could reduce *inhbaa* and *inhbab* to around 50%, while reducing that of *inhbb* to around 70%, although significance was not shown at the concentration of 100,000 zfBMP4-producing cells. While the activin β subunits were down-regulated, *inha* was up-regulated in a dose-dependent manner by zfBMP2b (0, 100,000 and 200,000 zfBMP2b-producing CHO cells together with 200,000 follicle cells). Low concentration of zfBMP2b (100,000 CHO cells/well) could increase *inha* expression to 200%, while higher concentration of zfBMP2b (200,000 CHO cells/well) cells could increase *inha* to 300%. zfBMP2b produced no significant change for *fst* at 48 h, indicating that the incubation time is critical for the regulation to occur. The effect of zfBMP4 was more potent than that of zfBMP2b. When zfBMP4-producing CHO cells were incubated with zebrafish follicle cells for 48 hours, *fst* and *inha* were up-regulated in a dose-dependent manner (0, 100,000 and 200,000 zfBMP4-producing CHO cells, together with 200,000 follicle cells). At both concentrations (100,000 and 200,000 CHO cells/well), zfBMP4 could increase expression of *fst* to around 150% while increasing that of *inha* to around 1000%, slightly less than the percentage increase at 24 h (Fig. 4.4).

4.3.4 Effects of zfBMP4 and zfBMP2a (R&D) on *inha*

To confirm the effects of the recombinant zfBMP2 and zfBMP4 from co-cultured CHO cells, we also tested a commercially available recombinant zebrafish BMP4 and BMP2a (R&D). Treatment of the cultured zebrafish follicle cells with the commercial zfBMP4 also up-regulated *inha* at 24 h, consistent with our results from the co-culture experiment. The percentage increase, however, was much less than that obtained from co-culture experiment (expression of *inha* increased to 200%, compared to 1500% when using the co-culture approach). This is likely due to the low potency of the commercial molecule as it is produced in *E. coli* with ED_{50} 0.2-1 μg/ml (R&D). Also because of the low potency, we could not observe significant changes in the expression of other genes of the activin-inhibin-follistatin system. Despite this, the consistent response of *inha* not only confirmed our finding with the co-culture system but also indicated the advantages of animal cells as the bioreactor for recombinant protein production. Although we are interested to see whether commercial recombinant zebrafish BMP2b would give the same

result as the co-culture system, zfBMP2b is not commercially available. However, zfBMP2a was available instead. zfBMP2a with ED_{50} 100-400 ng/ml (R&D) could also up-regulate *inha* at 24 h, exhibiting a similar effect as that of zfBMP2b and zfBMP4 that we produced from CHO cells. This indicates that the BMPs might have a redundant role in regulation of *inha* in cultured zebrafish follicle cells (Fig. 4.5).

4.3.5 siRNA transfection on zfBMP4-producing CHO cells

To further confirm the specificity of the observed effects of zebrafish BMPs on activin-inhibin-follistatin expression in the follicle cells, we used siRNA to knock down the expression of zfBMP4 in the CHO cells before co-culturing them with the follicle cells. A blank control (with lipofectamine only) and a negative control (*gdf9* siRNA) were also included. A control with no transfection was also included. *bmp4* siRNA specifically targeted zfBMP4 production from the CHO cells, and thus diminished the up-regulatory effect on the expression of *inha* and *fst,* the two genes of the activin-inhibin-follistatin that responded to BMPs the most. Expression of *inha* was reduced by half when compared to the untransfected zfBMP4 control. Expression of *fst* was reduced to a greater extent, close to the basal level of the control CHO cells which were untransfected. Lipofectamine and *gdf9* siRNA did not have significant effect on the expression of *inha*, though slightly decreasing that of *fst*. However, the effect of *bmp4* siRNA on expression of *fst* was much more drastic when compared to that of lipofectamine and *gdf9* siRNA controls (Fig. 4.6).

4.3.6 Microinjection of morpholino to knock down expression of BMP2b in the oocyte

To provide evidence for the importance of the endogenous oocyte-derived BMPs in regulating follicle cells, we used morpholinos to knock down the expression of zfBMP2b in the oocytes of FG follicles. zfBMP2b was chosen because it has the highest expression level in the follicle as compared to others. Water, control morpholino and morpholino targeting *bmp2b* were injected into the oocytes. Expression of inhibin α, as a marker gene in the follicle layer, was approximately the same after injection of water and control morpholino. However, there was a slight yet insignificant drop of expression of inhibin α when morpholino targeting *bmp2b* was injected. The drop in expression was observed at both 2 h and 15 h post-injection (Fig. 4.7).

4.4 Discussion

The activin-inhibin-follistatin system, as a group of important regulators in ovarian development, has been characterized in the zebrafish. The dynamic changes in the expression pattern of each of them during folliculogenesis (34) strongly suggest they are under tight regulation. Further evidence has shown that activin and inhibin subunits (α and β) are exclusively expressed in the follicle layer whereas follistatin is predominantly expressed in the oocytes (173, 198). These two pieces of evidence regarding spatiotemporal distribution suggest the activin-inhibin-follistatin system to be potential target genes of BMPs deriving from the oocyte which signal to the follicle cells. Therefore, in this study, we intend to investigate the regulation the activin-inhibin-follistatin system by BMPs in the follicle.

We have developed a novel approach to study the regulatory actions of BMPs on the activin-inhibin-follistatin system by making use of the recombinant zfBMP-producing CHO cells and the cultured zebrafish follicle cells. We co-incubated the zfBMP-producing CHO cells and the zebrafish follicle cells (Fig. 4.8). In this co-culture system, zfBMPs secreted from the CHO cells can act directly on the follicle cells in a paracrine manner. This would mimic the BMPs from the oocyte to act on the neighboring somatic follicle cells and exert regulatory effects on the target genes in these cells. As the activin subunits (βA and βB) and inhibin α are all expressed exclusively in the follicle layer (173, 198), we can investigate how the BMPs from the recombinant CHO cells would mimick the BMPs from the oocyte to regulate the expression of activin subunits and inhibin α in the follicle cells in a paracrine manner.

Besides mimicking the paracrine action in the ovarian follicle, the advantage of using the co-culture system is that it eliminates the need to concentrate and purify the recombinant proteins produced by the CHO cells. Furthermore, our result is confirmed by the commercial recombinant zebrafish BMP proteins, zfBMP4 and zfBMP2a (R&D), further validating this newly developed system. Also, transfection of the CHO cells by siRNA to knock down the production of recombinant BMPs is possible in the co-culture system, allowing further manipulations of the co-culture system (Fig. 4.6).

In our study, we found that both zfBMP2b and zfBMP4 produced from the CHO cells can up-regulate *inha* and *fst*, while down-regulating the three activin β subunits (*inhbaa, inhbab, inhbb*), with the effect of zfBMP4 being more potent. However, the more potent effect of zfBMP4 could probably only due to the high expression in the CHO cells. It should be noted

that both zfBMP2b and zfBMP4 caused the same response of the target genes, further supporting the specificity of the actions. The difference in their potency correlated well with the expression levels of the two proteins in the CHO cell lines (Chapter 3). The similar effects of the two BMP proteins also imply the redundant roles of the BMPs in ovarian development. This also brings up a question, i.e., why are there so many BMPs in the follicle? The answer to this question remains entirely unknown. Our speculation is that mutation or deletion of BMPs may lead to severe consequences as these proteins are important pleiotropic factors involved in many aspects of development and function (71, 72). Therefore, if some BMPs have redundant functions, even if one or more BMPs are mutated or deleted, other BMPs with the same functional role can compensate for the loss and ensure proper development.

In the microinjection experiment, we attempted to knock down the expression of BMP2b in the oocyte by targeting the splicing site of *bmp2b*. We chose to inject BMP2b morpholino because it is most abundantly expressed in the follicle. Any changes in expression of inhibin α on the follicle cells due to the knockdown in the oocyte would be more easily detected. Injecting BMP4 morpholino might not be a good choice because *bmp4* was found in both the oocyte compartment and the follicle cell compartment. Even if we knock down *bmp4* in the oocyte, expression of *bmp4* is still found on the follicle cells, and it might have regulatory role on inhibin α as well and thus making us difficult to interpret the results. Therefore, it might not be an appropriate approach to use BMP4 morpholino to demonstrate the paracrine signaling from the oocyte to the follicle cells.

With BMP2b knocked down in the oocyte, we expected to see a decrease in expression of inhibin α in the follicle layer as the up-regulatory effect by BMP2b was diminished. We observed a tendency of decrease in the expression of inhibin α after injection of morpholino targeting *bmp2b*, at both 2 h and 15 h post-injection. Yet, the effect was not significant. One possibility is that BMP2b is not the sole oocyte-derived BMP that up-regulates inhibin α. Other BMPs present in the oocyte may compensate the loss of BMP2b in stimulating inhibin α expression. Therefore, the single knockdown of one of the BMP ligands may not be sufficient in abolishing the up-regulatory actions in a significant manner. We suggest using a cocktail of morpholinos to perform multiple knockdowns in future studies in order to diminish the signal from the oocyte more completely. We might also perform over-expression experiment by injecting *bmp2b* mRNA into the oocyte. An increase in expression of inhibin α will be expected,

though we are unsure whether the presence of abundant mRNA binding proteins in the oocyte might bind to the injected mRNA and make translation inefficient.

Although in mammalian studies, it was reported that BMPs can regulate the activin-inhibin-follistatin system (152, 153, 201, 202), the reason behind was not discussed thoroughly. Here in zebrafish, we observed that BMPs up-regulated *inha* and *fst*, while down-regulating the activin β subunits. We therefore proposed a model to explain the regulation of BMPs on the activin-inhibin-follistatin system. This proposed model is partly based on our findings that in both goldfish and zebrafish, activin stimulated *fshb* (FSHβ) while suppressing *lhb* (LHβ) expression in the pituitary (203-205). When FSH reaches the ovary, it up-regulates inhibin α (173). This situation is the same in mammals, in which expression of inhibin α is regulated by FSH from the pituitary (206, 207). When the follicle develops to the full-grown stage and becomes ready to mature, inhibin would act as a signal from the ovary to travel as a hormone through the bloodstream to the pituitary, signaling the pituitary that the oocyte is ready for maturation. This is evidenced by the temporal expression profile that expression of inhibin α surged at the FG stage (173). When inhibin travels to the pituitary, activin is likely suppressed due to the antagonistic action of inhibin. With activin action reduced, FSH release is likely reduced as well, but LH release is increased, which is the master regulator that triggers the oocyte to undergo maturation. From our observation in this study, BMPs from the oocyte drastically up-regulate inhibin α, thus amplifying the signal in the pituitary to switch from producing FSH to LH in order to trigger oocyte maturation. This also explains why the expression of BMP receptors rose to the highest level towards maturation, since BMPs play a possible role in up-regulating the expression of inhibin α as the follicle approaches maturational stage. BMPs, by increasing the expression of inhibin α, also divert the synthesis from activins to inhibins in the follicle cells. BMPs also up-regulated follistatin, which is a binding protein of activins, to further modulate the activity of activins.

To support our proposed model, it is essential that we obtain evidence that inhibin secreted from the follicle does travel through the bloodstream to the pituitary and acts as an antagonist of activin to block FSH action and trigger LH action. We can try to inject an antibody raised against inhibin to the zebrafish ovary, in order to block inhibin from travelling to the pituitary. Due to the blockade of inhibin signaling, we expect to see oocyte maturation rate

being affected as LH action decreased. This is an on-going project in our lab and the results will surely provide more insights to our hypothesized model.

It was reported that activin promoted oocyte maturation (208). In chapter 3, we demonstrated that zfBMP4 suppressed spontaneous oocyte maturation, which is similar to the reported effect of BMP15 in the zebrafish. BMP15 was found to suppress DHP or hCG-induced oocyte maturation, yet it has no effect on spontaneous maturation (166, 167). However, our results demonstrated the opposite. zfBMP4 exerted a slight, but significant suppressive effect on spontaneous maturation; yet, it had no effect on DHP-induced oocyte maturation. This indicates that BMP15 and BMP4 may act through different mechanisms in suppressing oocyte maturation. One possibility is that BMPs may exert their inhibitory effects on oocyte maturation by modulating activin activity in the follicles as both zfBMP4 and zfBMP2b suppressed activin subunits but dramatically increased inhibin and follistatin.

This inhibitory tone may help to prevent precocious oocyte maturation while BMPs are activating the upper level of the HPG axis by increasing inhibin output. By preventing the follicle from precocious maturation, BMPs ensure synchronous maturation of the follicles. Once the follicle is ready for maturation, BMPs drastically induce the production of inhibin, which in turns signals to pituitary to switch the biosynthesis of FSH and LH, which is the master regulator controlling final oocyte maturation. This hypothetic role of oocyte-derived BMPs is further supported by the differential effects of BMPs on FSH and LH receptor expression in the follicle cells.

In conclusion, using a novel co-culture approach to co-incubate the BMP-producing CHO cells and zebrafish follicle cells, we have identified the regulatory effects of BMPs on the activin-inhibin-follistatin system. Our results indicated that BMPs down-regulated the activin subunits, while up-regulating follistatin and inhibin α. The significance of this finding is that BMPs are potentially important signals derived from the oocyte that up-regulate inhibin α on the follicle layer, which in turns signals the pituitary for the readiness of the oocyte to mature. By suppressing the actions of activins in promoting oocyte maturation, BMPs help to prevent the follicle from precocious maturation. Therefore, BMPs are important in ensuring synchronous maturation of the follicle, which is important in the reproductive process.

Table 4.1 Primers used in real-time qPCR

Gene	Accession no.	Sequence	Expected Size (bp)
inhbaa	NM 130916.1	AACAGGCAGAACAGACGGAGATC	180
		GCAGCCGAATGTTGACGTTAGC	
inhbb	NM 131068.2	TAGGGAGGACGGCAGGGTTG	150
		TCGTTGGAGATCAGAAAGTAGAGGC	
inhbab	NM 001018156.1	AGCCCTTCGAGATCATCACCTTC	103
		GCCTGCTCCACCACTGACAG	
inha	XM 693951	AGCCTCCTCTGCCAGTGTTG	301
		ATGTTGATGGAAGCGATGGTCTC	
fst	NM 131037.3	TAAAGAGACGTGTGATAATGTGGACTGTG	466
		ATCGCATGACTTGGCCTTGATG	
ef1a	NM 131263	GGCTGACTGTGCTGTGCTGATTG	409
		CTTGTCGGTGGGACGGCTAGG	

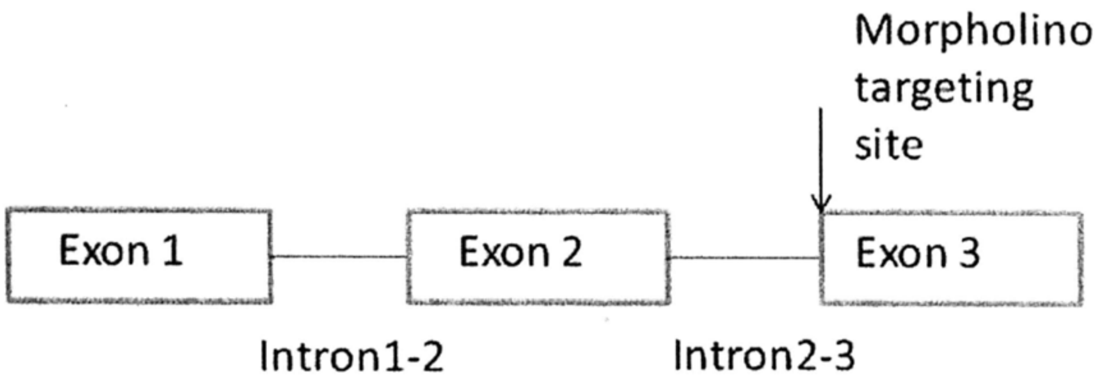


Fig. 4.1 Morpholino targeting splicing site of BMP2b. Morpholino was designed to target the splicing site between intron 2-3 and exon 3.

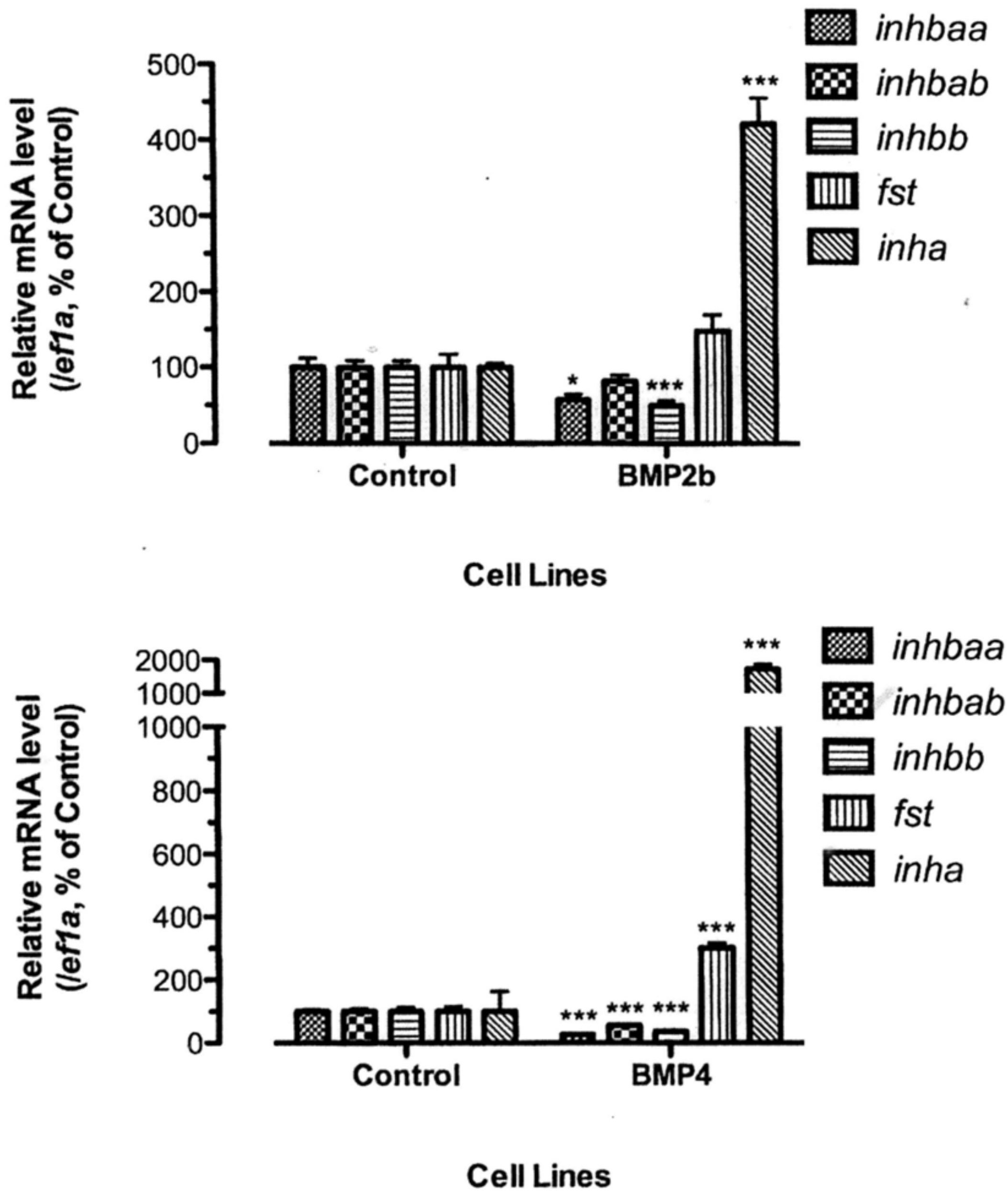
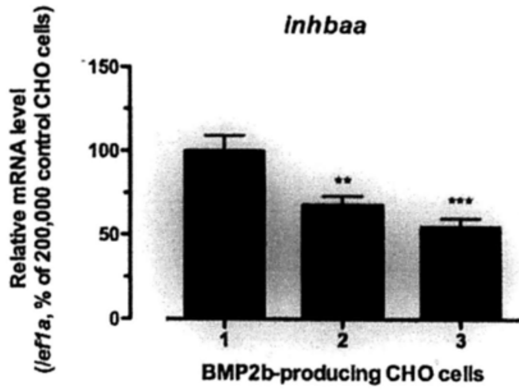
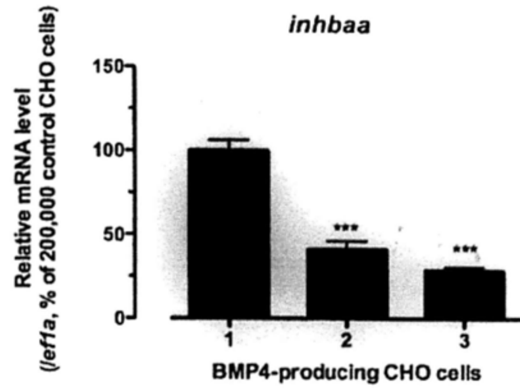
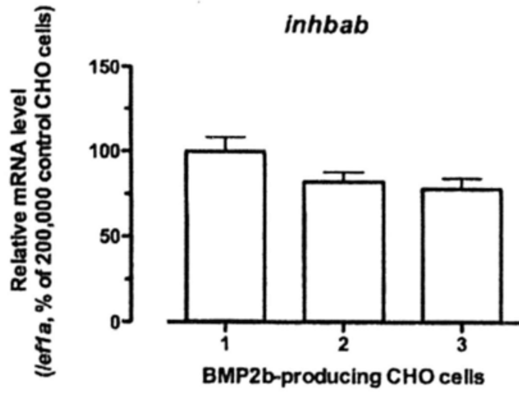
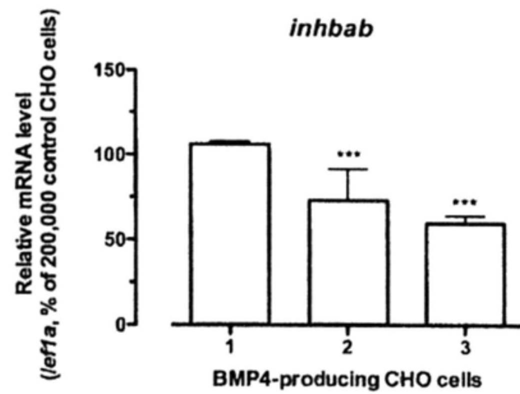


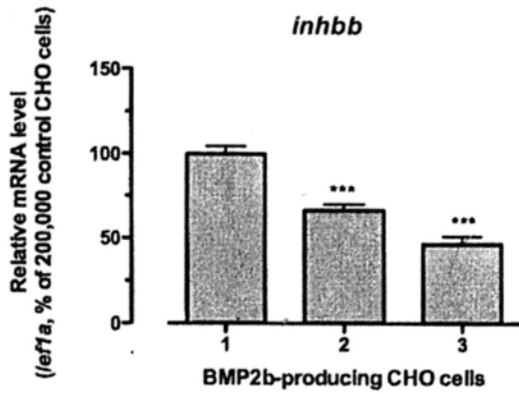
Fig. 4.2 Effects of recombinant zebrafish BMP2b and BMP4 on the expression of activin-inhibin-follistatin system in the follicle cells. CHO cell-produced zfBMP2b and zfBMP4 down-regulated activin β subunits (*inhbaa, inhbab and inhbb*), while up-regulating follistatin (*fst*) and inhibin α (*inha*) at 24 h. The effect of zfBMP4 is more potent than that of BMP2b. The values are the mean ± SEM (n=6) from a representative experiment. Asterisk (*) indicates statistical significance (*, P<0.05; ***, P<0.001 vs. control).

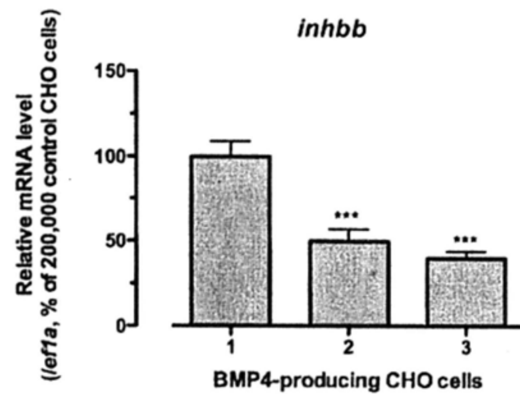




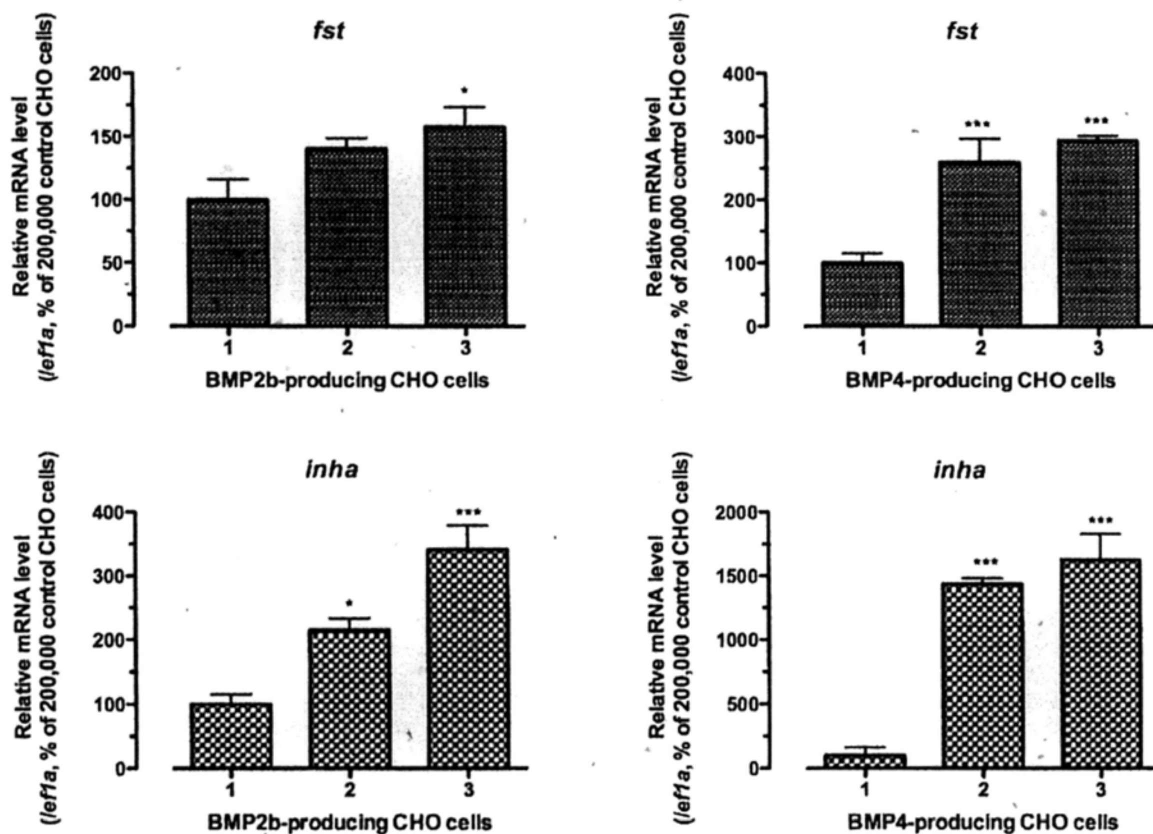






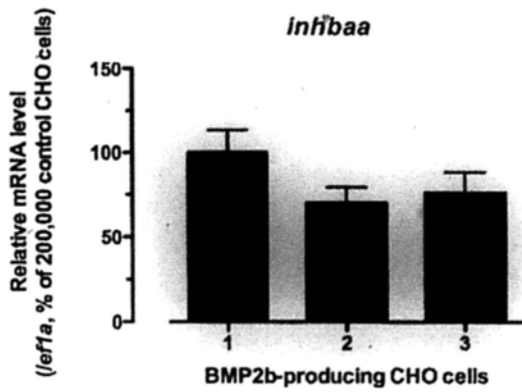


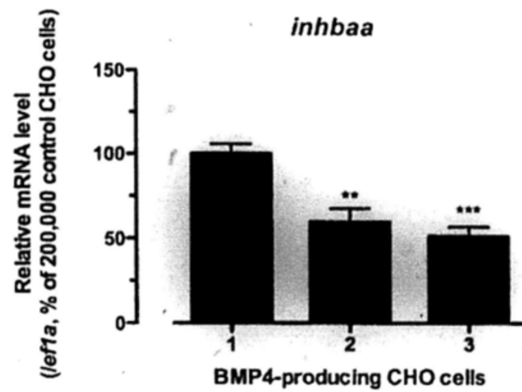
	1	2	3
Control CHO cells	**200,000**	**100,000**	**0**
zfBMP2b/zfBMP4-producing CHO cells	**0**	**100,000**	**200,000**

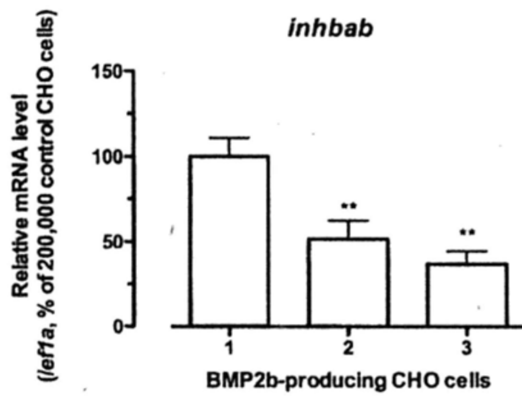


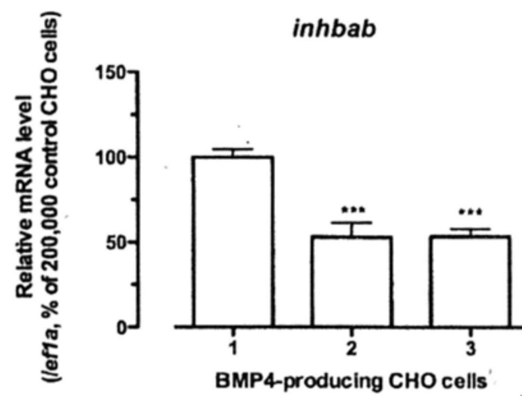
	1	2	3
Control CHO cells	**200,000**	**100,000**	**0**
zfBMP2b/zfBMP4-producing CHO cells	**0**	**100,000**	**200,000**

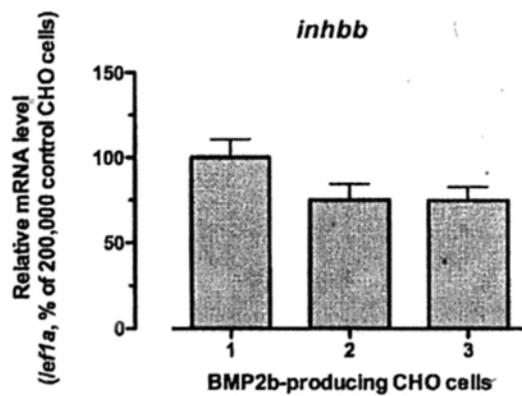
Fig. 4.3 Dose-dependent effects of zfBMP2b and zfBMP4 on the expression of activin-inhibin-follistatin system in the follicle cells at 24 h. CHO cell-produced zfBMP2b and zfBMP4 down-regulated activin β subunits (*inhbaa, inhbab, inhbb*) at 24 h in a dose-dependent manner, although statistical significance was not reached for the down-regulation of *inhbab* by BMP2b. CHO cells-produced zfBMP2b and zfBMP4 up-regulated *fst* and *inha* at 24 h in a dose-dependent manner The values are the mean ± SEM (n=6) from a representative experiment. Asterisk (*) indicates statistical significance (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ vs. control).

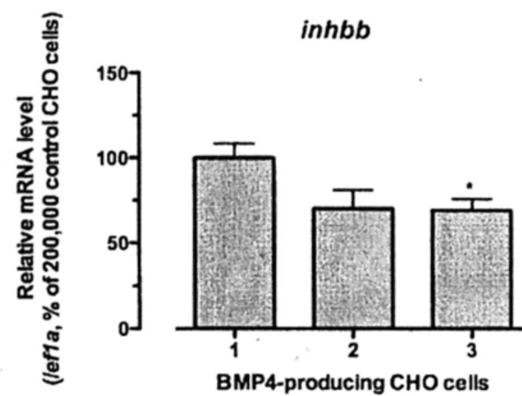




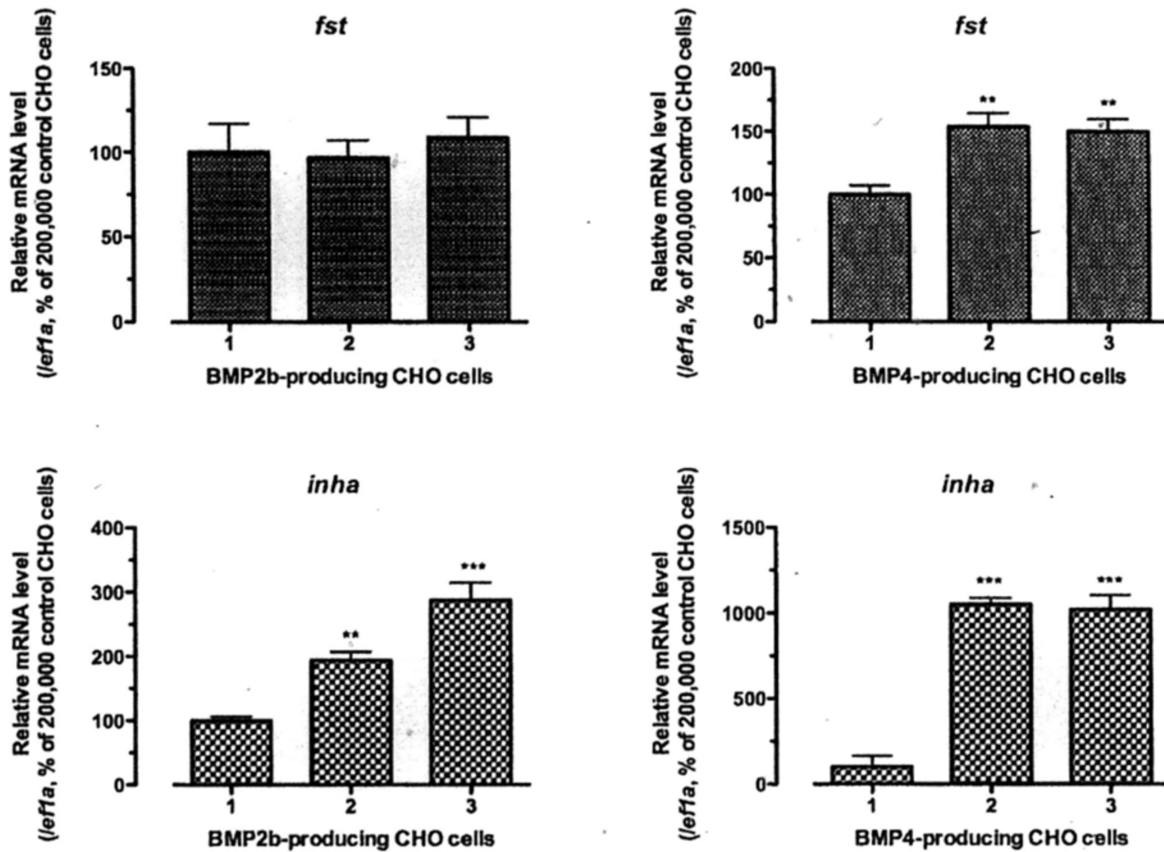








	1	2	3
Control CHO cells	**200,000**	**100,000**	**0**
zfBMP2b/zfBMP4-producing CHO cells	**0**	**100,000**	**200,000**



	1	2	3
Control CHO cells	**200,000**	**100,000**	**0**
zfBMP2b/zfBMP4-producing CHO cells	**0**	**100,000**	**200,000**

Fig. 4.4 Dose-dependent effects of zfBMP2b and zfBMP4 on the expression of activin-inhibin-follistatin system in the follicle cells at 48 h. CHO cell-produced zfBMP2b and zfBMP4 down-regulated activin β subunits (*inhbaa, inhbab, inhbb*) at 48 h in a dose-dependent manner, although not all of them showed significance. zfBMP2b up-regulated *inha* but had no significant effect on *fst*. zfBMP4 up-regualted both *fst* and *inha* at 48 h. The values are the mean ± SEM (n=6) from a representative experiment. Asterisk (*) indicates statistical significance (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ vs. control).

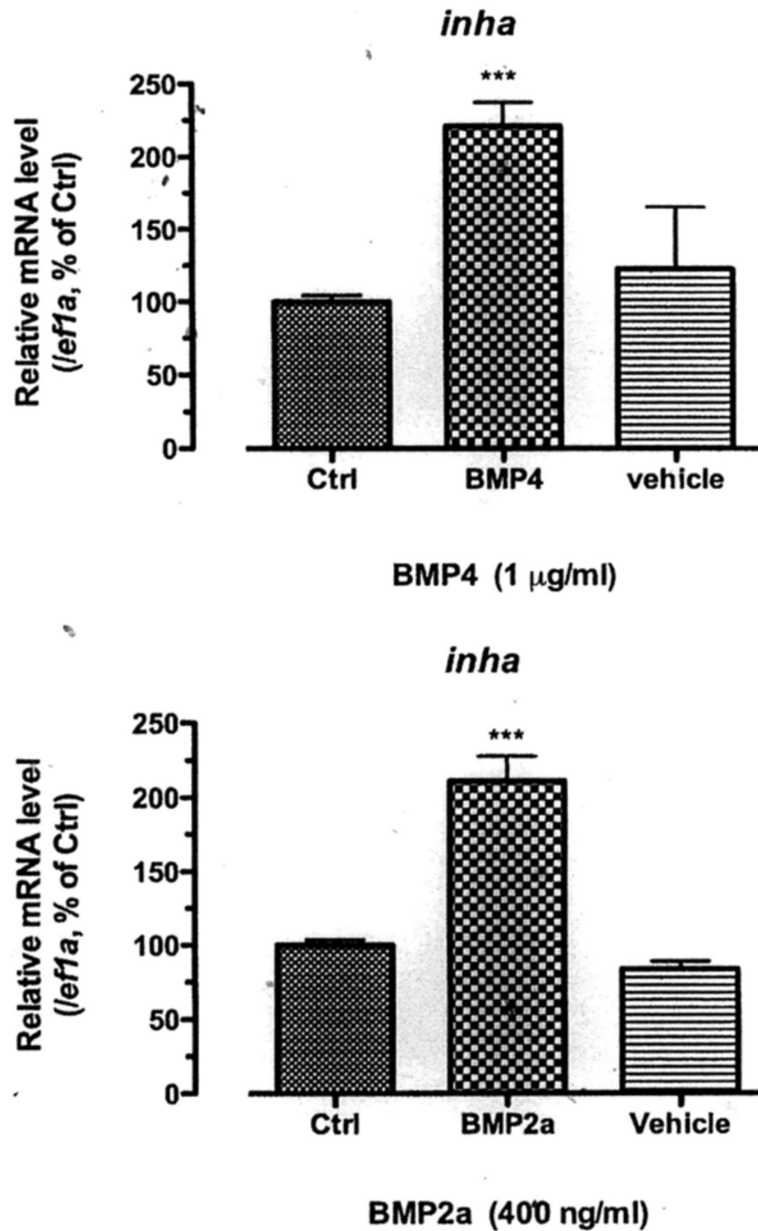
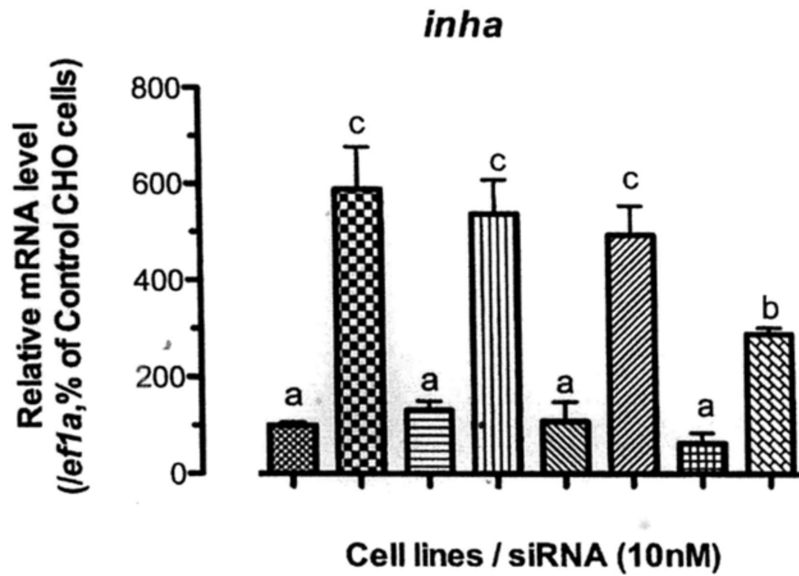
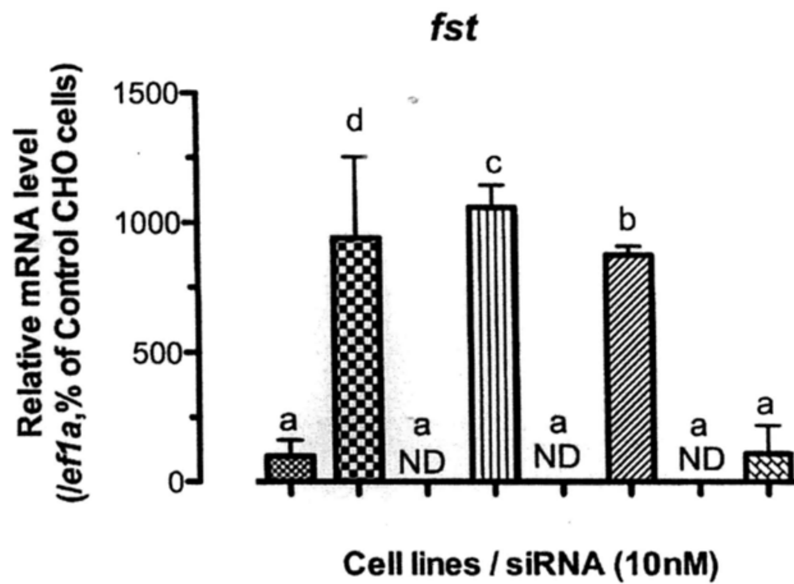


Fig. 4.5 Effects of recombinant zfBMP4 and zfBMP2a (R&D) on *inha*. 1 μg/ml of recombinant zfBMP4 or 400 ng/ml recombinant zfBMP2a (R&D) was administered to the cultured zebrafish follicle cells and incubated for 24 h. zfBMP4 and zfBMP2a increased the expression of inhibin α (*inha*), while the vehicle that was used to dissolve the recombinant protein was included as a control. The values are the mean ± SEM (n=6) from a representative experiment. Asterisk (*) indicates statistical significance (***, P<0.001 vs. control).





Control CHO cells	+	-	+	-	+	-	+	-
BMP4 CHO cells	-	+	-	+	-	+	-	+
Lipofectamine	-	-	+	+	+	+	+	+
gdf9 siRNA	-	-	-	-	+	+	-	-
bmp4 siRNA	-	-	-	-	-	-	+	+

Fig. 4.6 Blockade of zfBMP4 expression in the CHO cells by siRNA and its effect on *inha* and *fst* expression in the co-cultured follicle cells. zfBMP4 from CHO cells significantly up-regulated *inha* and *fst*. The blank control (with Lipofectamine only) and negative control (transfected with *gdf9* siRNA) were included, and they showed significant increase of *inha* and *fst*. However, when *bmp4* siRNA was transfected into the zfBMP4-producing CHO cells, expression of *inha* and *fst* were significantly knocked down. Expression of *inha* was knocked down by half when compared to the untransfected zfBMP4-producing cells. Expression of *fst* was knocked down to close to basal level of untransfected control CHO cells. The values are the mean ± SEM (n=6) from a representative experiment. Letters indicate statistical significance ($P<0.05$).

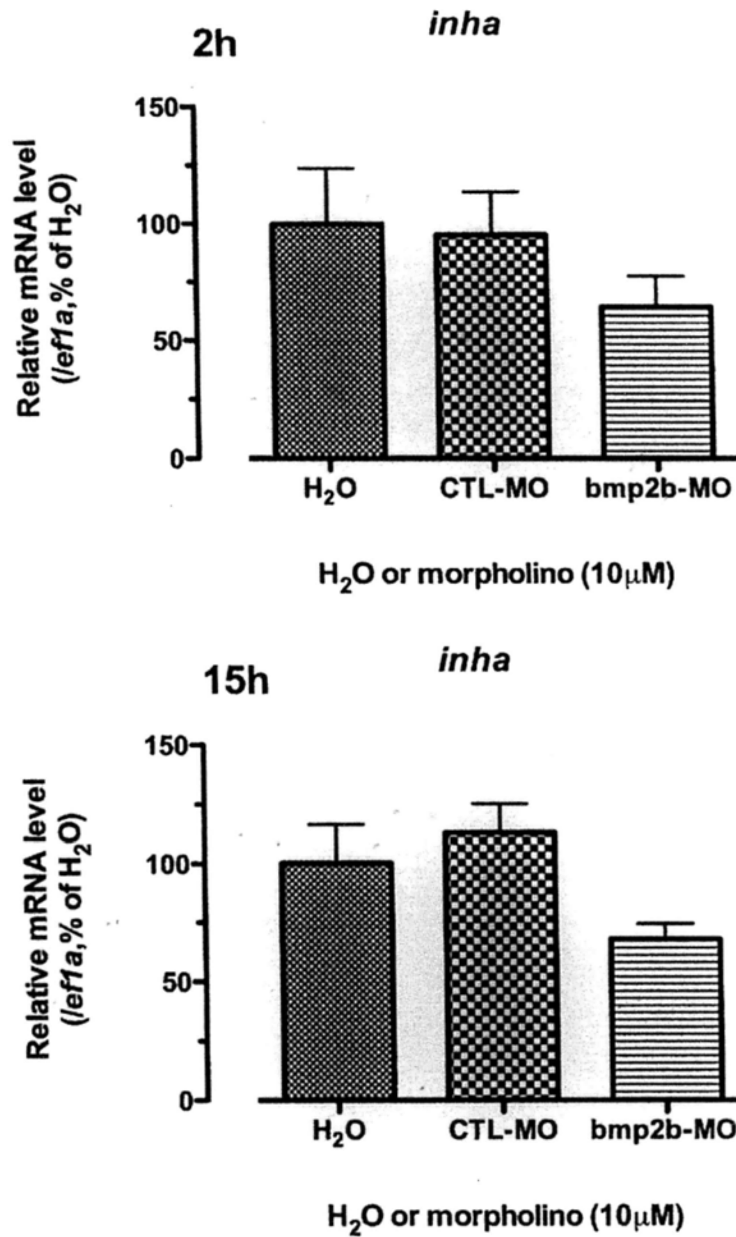


Fig. 4.7 Morpholino knockdown of *bmp2b* in oocytes. Control morpholino or morpholino targeting *bmp2b* were injected into oocytes. Water was also injected as a control. Inhibin α was used a marker to detect the drop of expression of genes located in the follicle layer. A slight, yet insignificant drop in expression of inhibin α was detected by injecting morpholino targeting *bmp2b*, compared to water and control morpholino. Similar results were obtained at 2 h or 15 h post-injection.

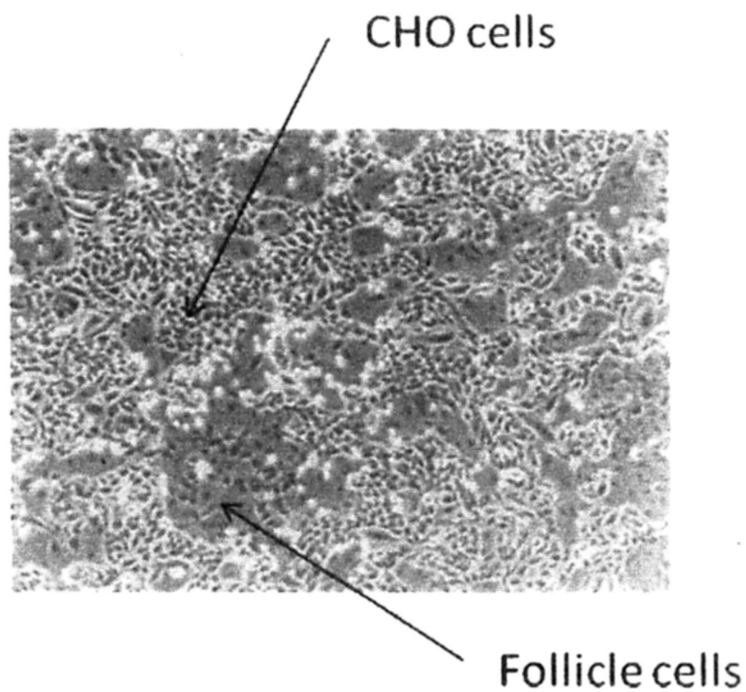


Fig. 4.8 Recombinant CHO cells and zebrafish follicle cells in the co-culture system. CHO cells were seeded one night before follicle cell subculture. The two types of cells were able to grow together in a healthy manner in F-12 medium at 28°C. Cell morphology and survival rate appeared to be normal for both kinds of cells.

Chapter 5

Regulation of Gonadotropin Receptors by Zebrafish BMP2b and BMP4

5.1 Introduction

Vertebrate folliculogenesis can be divided into two phases, namely growth and maturation, and it is subjected to regulation by gonadotropins. The pituitary gonadotropin, follicle-stimulating hormone, FSH, is responsible for follicle development, while luteinizing hormone, LH, is responsible for oocyte maturation and ovulation. In teleosts, as in other vertebrates, the folliculogenesis is also governed by pituitary hormones. Two types of gonadotropins have been identified in fish, namely FSH and LH (formerly termed GTH-I and GTH-II, respectively), which are homologous to FSH and LH in tetrapods (32).

With two gonadotropins isolated, two gonadotropins receptors were also found, which were designated as FSHR and LHCGR (formerly termed GTHR-I and GTHR-II respectively). They are both G-protein-coupled receptors, with large extracellular domain, seven transmembrane helices and a carboxy-terminal intracellular tail (209). A two-receptor model for salmon gonadotropins has been proposed. According to this model, FSHR is localized on the theca and granulosa cells, while LHCGR is on the granulosa cells only. Receptor specificity assay demonstrated that salmon FSHR bound both GTHs, with higher affinity for FSH, while LHCGR was specific for LH only. This is in contrast to mammals in which the two gonadotropin receptors have high specificity for their respective ligands (210).

In the zebrafish, FSHR (which is actually follicle-stimulating hormone receptor, Fshr) was first cloned by Laan et al. (211), which showed high similarity to tetrapod FSHR and FSHR from salmon and catfish (212, 213). When COS-7 cells were transfected with zfFSHR, there was an increase in cAMP in response to the treatments with carp pituitary extracts or human FSH, but not hCG (211).

Later, our group carried out the experiment to clone both zebrafish follicle-stimulating hormone receptor (Fshr) and luteinizing hormone receptor (Lhcgr). When CHO K-1 and COS-1 cells were transiently transfected with *fshr* and *lhcgr*, there was increase in SEAP expression in

both Fshr and Lhcgr-expressing cells when goldfish pituitary extract was administered. On the other hand, hCG could only increase the response of Lhcgr-expressing cells but not that of Fshr-expressing cells (172). Stable cell lines expressing the gonadotropin receptors were also generated and their specificity was also tested, with the conclusion that FSH is specific for Fshr only, while LH can act on both Fshr and Lhcgr (197).

The temporal expression patterns of the zebrafish gonadotropin receptors were investigated in order to get some hints on the functional roles of the gonadotropin receptors during follicle development (172). During follicle development, the expression of both *fshr* and *lhcgr* was initially low. The expression of *fshr* increases at PV stage, peaks at MV stage, but decreases at the FG stage prior to oocyte maturation and ovulation. The expression of *fshr* is not accompanied by *lhcgr*, whose expression lags behind that of *fshr*, with a significant increase at MV stage, followed by a surge at FG stage. Thus, there exists a differential regulation of the gonadotropin receptors during folliculogenesis; however, the mechanisms that control the expression of *fshr* and *lhcgr* in the zebrafish and in teleosts in general are largely unknown.

The gonadotropin receptors are subjected to control by various endocrine or paracrine factors including pituitary hormones, steroids and local growth factors (214-222). In the zebrafish ovary, the expression levels of *fshr* and *lhcgr* are clearly stage-dependent, suggesting that their expression must be controlled or modulated by certain internal factors from the follicle. Since our earlier studies have shown that the BMP family members are largely expressed in the oocyte and their receptors are exclusively expressed in the follicle cells, it is likely that the gonadotropin receptors that are exclusively expressed in the follicle cells may be subjected to the regulation by oocyte-derived factors such as BMPs. Using the co-culture system we developed and described in Chapter 4, we demonstrated that BMPs did participate in the regulation of gonadotropin receptors. It was found that both zfBMP2b and zfBMP4 produced from CHO cells could differentially regulate the two gonadotropin receptors, with *fshr* being down-regulated, while *lhcgr* being up-regulated in folliculogenesis by the BMPs. This study identified BMPs as one of the long-sought growth factors that differentially regulate the two gonadotropin receptors.

5.2 Materials and Methods

5.2.1 Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), Invitrogen (Carlsbad, CA) and USB (Cleveland, OH) unless otherwise stated.

5.2.2 Animals

Zebrafish (*Danio rerio*) were purchased from local pet stores and maintained in flow-through aquaria at 28±1°C on a 14-h light/10-h dark photoperiod. The fish were fed thrice a day with commercial tropical fish food. The animals were anaesthetized on ice and sacrificed by decapitation before dissection. All experiments were performed under license from the Government of the Hong Kong Special Administrative Region and endorsed by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong.

5.2.3 Primary follicle cell culture

Around 40 zebrafish ovaries were dissected and the follicles were dispersed slightly using plastic pipette. The follicles were washed with 60% L-15 (Gibco) several times in a 15 ml Falcon tube (BD) and filtered through a sieve to remove large size FG follicles. The filtered follicles were then washed several times with medium M199 (Gibco) and plated into 10-cm culture dishes. The follicles were grown for 3 days at 28°C in M199 with 10% FBS (Hyclone). Fresh medium of the same components was replaced after 3 days. After continuous incubation for 3 more days, the follicle cells were sub-cultured on top of the recombinant CHO cells (described below), with 200,000 cells per well.

5.2.4 Culture of CHO cells

The CHO cell lines expressing recombinant zebrafish BMP2b and BMP4 were established as described in the previous chapter. A control cell line carrying the empty vector (pcDNA5/FRT) was also established. The CHO cell lines were maintained in F-12 medium (Gibco) supplied with 6 g/L HEPES (USB, Cleveland, OH) and 10% FBS (Hyclone) supplied with antibiotics (Streptomycin, 100 μg/ml; penicillin, 100 U/ml). The CHO cells were maintained in 10-cm culture dishes and passed twice a week when the cells reach 100% confluency.

5.2.5 Co-incubation of primary follicle cells and CHO cells

Recombinant zfBMP2b and zfBMP4-producing CHO cells, as well as the control CHO cells, were seeded in 12-well plates, with 200,000 cells in 0.5 ml F-12 medium with 10% FBS. The CHO cells were allowed to attach within a 24-h incubation period at 37°C. On the next day, 200,000 follicle cells from the step 5.2.3 were added to each well in 0.5 ml F-12 medium with 10% FBS (incubation medium was changed from M199 to F-12 during subculture at the step 5.2.3 when resuspending the pellet). The addition of the zebrafish follicle cells to the CHO cells already plated resulted in a total of 1 ml F-12 medium containing 400,000 cells in total (200,000 CHO cells and 200,000 follicle cells). The incubation temperature was reduced to 28°C, which is the optimal temperature for the growth of zebrafish follicle cells and production of recombinant zfBMPs by the CHO cells. After 24 h of co-incubation, the follicle cells became attached to the well surface and the medium was changed to serum-free F-12 for starvation for another 24 h, after washing once with 1 ml of F-12 medium. The 24 h starvation period was followed by RNA extraction with Tri-reagent and reverse transcription. For the control setting, 200,000 control CHO cells (transfected with empty vector) were seeded, followed by adding 200,000 follicle cells. The lower dose included 100,000 zfBMP-producing CHO cells plus 100,000 control CHO cells, followed by adding 200,000 follicle cells, resulting in a total of 400,000 cells. The higher dose included 200,000 zfBMP-producing CHO cells added with 200,000 follicle cells, reaching a fixed total of 400,000 cells.

5.2.6 RNA isolation and reverse transcription

Total RNA was isolated from the co-cultured cells using Tri-Reagent according to manufacturer's instruction. Total RNAs from the co-cultured cells were used for reverse transcription in a 10 μl volume containing 1 x M-MLV RT buffer, 0.5 mM of each dNTP, 0.5 μg oligo(dT), 0.1 mM DTT and 100 U M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA). Reverse transcription was performed at 37°C for 2 h.

5.2.7 Real-time qPCR

Real-time qPCR was used to monitor the changes in expression of the gonadotropin receptors under regulation by the BMPs. Real-time qPCR was carried out on the iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) in a volume of 30 μl containing 10 μl of 1:20 diluted RT reaction mix, 1 x PCR buffer, 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, 0.2

μM of each primer, 0.75 U Taq Polymerase, 0.5 x EvaGreen (20 x concentrated; Biotium, Hayward, CA) and 20 nM fluorescein (Bio-Rad). The reaction profile consisted of 40 cycles of 95°C for 30 sec, 62°C for 30 sec, 72°C for 40 sec and 84°C for 7 sec for signal detection. A melt curve analysis consisting of 180 cycles of 7 sec with temperature increase of 0.2°C/cycle was performed at the end of the reaction to demonstrate the specificity of the reaction. The data were normalized to the housekeeping gene *ef1a*. The primers for PCR were designed according to the sequences available in the GenBank and synthesized by Integrated DNA Technologies (IDT, Coralville, IA). Primers used for amplification of the gonadotropin receptors were listed in Table 5.1.

5.2.8 Statistical analysis

All real-time qPCR data were normalized to the housekeeping gene *ef1a*. All values were expressed as the mean ± SEM and the data were analyzed by one-way ANOVA followed by Dunnett's test for comparisons of all pairs of groups using the GraphPad Prism 5 for Mac OS X (GraphPad Software, San Diego, CA).

5.3 Results

5.3.1 Regulation of *fshr* and *lhcgr* expression by zebrafish BMP2b and BMP4

When recombinant zfBMP2b or zfBMP4-producing CHO cells (200,000 cells) were incubated with zebrafish follicle cells for 24 hours, *fshr* expression was significantly down-regulated as compared to the follicle cells co-incubated with the control CHO cells, while *lhcgr* was up-regulated. The effect of zfBMP4 was more potent when compared to that of zfBMP2b, which might be due to the higher expression level of *bmp4* in the CHO cell line as shown in previous chapter (Chapter 3) (Figure 5.1).

5.3.2 zfBMP2b and zfBMP4 dose-dependently down-regulated *fshr* but up-regulated *lhcgr* expression at 24 h

When BMP-producing CHO cells were incubated with zebrafish follicle cells for 24 hours, both zfBMP2b and zfBMP4 down-regulated *fshr* expression in a dose-dependent manner (0, 100,000 and 200,000 zfBMP2b- or zfBMP4-producing CHO cells together with 200,000 follicle cells). In contrast, both forms of zebrafish BMPs could up-regulate the expression of

lhcgr. zfBMP2b from the recombinant CHO cells at the density of 100,000 cells/well could down-regulate *fshr* to around 75%, while 200,000 zfBMP2b-producing CHO cells could down-regulate *fshr* expression to around 60%. Similarly, the zfBMP4-producing CHO cells at 100,000 cells/well could down-regulate *fshr* mRNA level to around 65%, while 200,000 zfBMP4-producing CHO cells could down-regulate *fshr* expression to around 60%. On the contrast, zfBMP2b-producing CHO cells at lower dose (100,000 CHO cells/well) could up-regulate *lhcgr* expression to around 135%, although it was not significant, while 200,000 zfBMP2b-producing CHO cells could up-regulate *lhcgr* to around 150%. zfBMP4-producing CHO cells at 100,000 cells/well could up-regulate *lhcgr* to around 150%, while 200,000 zfBMP4-producing CHO cells per well could up-regulate *lhcgr* to around 160%. Collectively, the effect of zfBMP4 was more potent than that of zfBMP2b (Figure 5.2).

5.3.3 zfBMP2b and zfBMP4 dose-dependently down-regulated *fshr* and *lhcgr* at 48 h

When zfBMP2b or zfBMP4-producing CHO cells were incubated with zebrafish follicle cells for 48 hours, zfBMP2b still showed an inhibitory effect on *fshr* expression in a dose-dependent manner (0, 100,000 and 200,000 zfBMP2b-producing CHO cells). zfBMP2b-producing CHO cells at 100,000 cells/well could down-regulate *fshr* to around 50%, while 200,000 zfBMP2b-producing CHO cells per well could down-regulate *fshr* to slightly less than 50%. However, different from its effect on *lhcgr* expression at 24 h, the extended incubation with zfBMP2b for 48 hours also down-regulated *lhcgr* expression at both doses. Both 100,000 and 200,000 zfBMP2b-producing CHO cells could down-regulate *lhcgr* expression to around 75%. When zfBMP4-producing CHO cells were incubated with zebrafish follicle cells for 48 hours, *fshr* was down-regulated in a dose-dependent manner (0, 100,000 and 200,000 zfBMP4-producing CHO cells) as that observed at 24 h. zfBMP4-producing CHO cells at 100,000 cells/well could down-regulate *fshr* expression to slightly less than 50%, while 200,000 zfBMP4-producing CHO cells per well down-regulated *fshr* expression to around 35%. The effect of zfBMP4 on *fshr* expression level was more potent than that of zfBMP2b. However, no significant effect was observed on the expression of *lhcgr* (Figure 5.3).

5.4 Discussion

Similar to mammals, fish also possess two gonadotropins, although their physiological functions are not well-defined in most fish species. In salmonids, FSH and LH were well characterized. With their differential expression patterns during the reproductive cycle, FSH is most likely involved in recruitment and promoting follicle growth, while LH is most likely associated with oocyte maturation and ovulation (223, 224). However, this is not the case in some other fish, like in goldfish, which has a different mode of ovarian development (225). To find out the possible roles of the gonadotropins, we can investigate the expression patterns of their receptors during the reproductive cycle.

Our lab has cloned zebrafish follicle-stimulating hormone receptor (*fshr*) and luteinizing hormone receptor (*lhcgr*) and characterized their temporal expression profiles during folliculogenesis (172). The expression of *fshr* increased with vitellogenesis. It started to increase at PV stage and peaked at MV stage, followed by a drop at FG stage. The expression of *lhcgr*, on the other hand, lagged behind that of *fshr* with low expression during vitellogenesis but surged expression at FG stage, indicating its potential role in oocyte maturation and ovulation (172). The distinct expression patterns of the two gonadotropin receptors during folliculogenesis leads to the hypothesis that they may have differential roles in follicle development. What controls the expression of these gonadotropin receptors becomes an interesting question. What is the switch that turns off *fshr* at MV stage, while pushing up *lhcgr* after MV stage leading to the surge at FG stage, followed by oocyte maturation? The coordination of *fshr* and *lhcgr* expression and their precise control during folliculogenesis are believed to be important to assure proper ovarian development. In the zebrafish, we are still in search of the hormones and growth factors that differentially regulate these two gonadotropin receptors, though similar studies have been done in mammals. In rats, administration of estradiol to hypophysectomized rats led to an increase in FSHR and a decrease in LHR in granulosa cells (214). This represented one of the differential regulations by steroids. In another study, when LH was administered in immature hypophysectomized rats, LH caused a decrease in *fshr* and *lhr* in preovulatory follicles (215). Yet, another study showed that LH stimulated granulosa cell differentiation in immature hypophysectomized rats by inducing *lhr* (216). FSHR was also found to be increased by testosterone and 5alpha-dihydrotestosterone (DHT) in bovine granulosa cells (217). Thus, it appears that the gonadotropin receptors are mostly subjected to the regulation by endocrine hormones from the pituitary or steroids from the gonads.

However, there has been evidence that local factors such as insulin-like growth factor (IGF-I) (219), activin, follistatin (218, 220, 222) may also exert regulatory actions on the gonadotropin receptors. It has been recently shown that BMPs are also among the various ovarian growth factors that regulate the gonadotropin receptors.

BMP7 was shown to increase FSHR transcript level in neonatal mouse ovaries *in vitro* (57), while in human granulosa cells, BMP7 could up-regulate FSHR and suppress LHR gene expression (226). BMP6 was also reported to increase FSHR gene expression in human granulosa cells (201). In chicken granulosa cells, BMP6 could increase LHR gene expression in F1 cells, while increasing FSHR gene expression in F1, F2, F3/4 cells (200). With these studies reported in other vertebrates on the roles of BMPs on gonadotropin receptors, we wonder if BMPs could possibly regulate the *fshr* and *lhcgr* as well in the zebrafish ovary. We adopted the co-culture system as described in the previous chapters. Briefly, the zfBMP2b or zfBMP4-producing CHO cells were co-incubated with the zebrafish follicle cells followed by examining the expression of zebrafish *fshr* and *lhcgr*. Interestingly, after 24 hours of co-culture with zfBMP2b/4 from the CHO cells, *fshr* expression was found to be suppressed and *lhcgr* expression was found to be elevated in cultured zebrafish follicle cells.

As described in Chapter 2 the expression of BMP receptors increased steadily during folliculogenesis, reaching the peak at FG stage, indicating an increase in BMP signaling as the follicle develops. The peak of BMP signaling at FG stage coincides with the time when *fshr* expression drops and *lhcgr* expression rises prior to final oocyte maturation and ovulation. The timing of the expression of BMP receptors (*bmpr2a* and *bmpr2b*) and the differential effects of zfBMP2b and zfBMP4 on *fshr* and *lhcgr* expression suggests that the BMPs from the oocyte might participate in the differential expression of the two gonadotropin receptors at final oocyte maturation, and this regulation may represent an important signal from the oocyte for the readiness of the follicle to mature and ovulate.

In the previous chapter, we discussed that activin from the pituitary stimulates production of FSH, while suppressing that of LH (203). FSH acts on FSHR in the ovary to up-regulate the expression of inhibin α (173), which may act as a signal from the ovary to the pituitary for the readiness of the oocytes to mature. Inhibin acts at the pituitary to shut down activin signaling, leading to a decreased expression of FSH but an increase in LH. Thus LH is released, probably as a preovulatory surge, and acts on LHCGR in the ovary, triggering oocyte maturation and

ovulation. The fact that BMPs from the oocyte up-regulate inhibin α in the follicle cells further supports this hypothesis. While reducing *fshb* expression in the pituitary through inhibin, BMPs from the oocyte might as well down-regulate *fshr* on the follicle cells to help diminishing FSH actions. This happens when the follicle is close to maturation, i.e. MV stage, when BMP signaling is reaching towards the peak and *fshr* expression starts decreasing. Meanwhile, LH production increases in the pituitary again by inhibin, and the expression of its receptor *lhcgr* also increases in the FG follicle. BMPs from the oocyte might as well help to up-regulate *lhcgr* to facilitate the action of LH to help the oocyte to mature. This explains why BMP signaling reaches the highest at the FG stage, since it needs to perform the job of promoting oocyte maturation by boosting up LH production and action at both the pituitary and ovary levels. This hypothesis is supported by localization studies with BMPs located in the oocyte and the gonadotropin receptors located on the follicle layer, suggesting a paracrine regulation of BMPs from the oocyte to the gonadotropin receptors on the follicle layer. The temporal expression pattern further supports our hypothesis, with the expression of BMP receptors increased gradually and raised to the highest at FG stage, at the exact time when *fshr* expression decreases and *lhcgr* increases, to facilitate final oocyte maturation. Although we are not sure of the mechanism of how BMPs regulate the gonadotropin receptors, this will be an interesting question to investigate in the future. Moreover, from the co-culture experiment, we observed that the effect of zfBMP2b and zfBMP4 on *lhcgr* at 48 h was different from that at 24 h (zfBMP2b down-regulated *lhcgr* at 48 h, while zfBMP4 had no effect on *lhcgr* at 48 h). This indicated that the differential regulation occurred at the critical time of 24 h.

In conclusion, we have demonstrated that BMPs from the oocyte may serve as one of the long-sought growth factors that differentially regulate the two gonadotropin receptors during folliculogenesis in the zebrafish ovary. Both zfBMP2b and zfBMP4 suppressed the expression of *fshr* but increased the expression of *lhcgr* prior to oocyte maturation and ovulation. With BMPs localized in the oocyte and the gonadotropin receptors in the follicle cells, the BMPs from the oocyte may signal the follicle cells in a paracrine manner. The precise control of the expression of the gonadotropin receptors by the BMPs may help to ensure proper development of the ovary.

Table 5.1 Primers used in real-time qPCR

Gene	Accession no.	Sequence	Expected Size (bp)
fshr	NM 001001812.1	AACATGCACATAGAGAGGATTCCCAG	334
		GCTCAGTAAACAGCTCCAGGC	
lhcgr	NM 205625.1	TGAATACGCCACAATGAATCTCTT	470
		ATGACGATCCAATGACATCTGACTC	
ef1a	NM 131263	GGCTGACTGTGCTGTGCTGATTG	409
		CTTGTCGGTGGGACGGCTAGG	

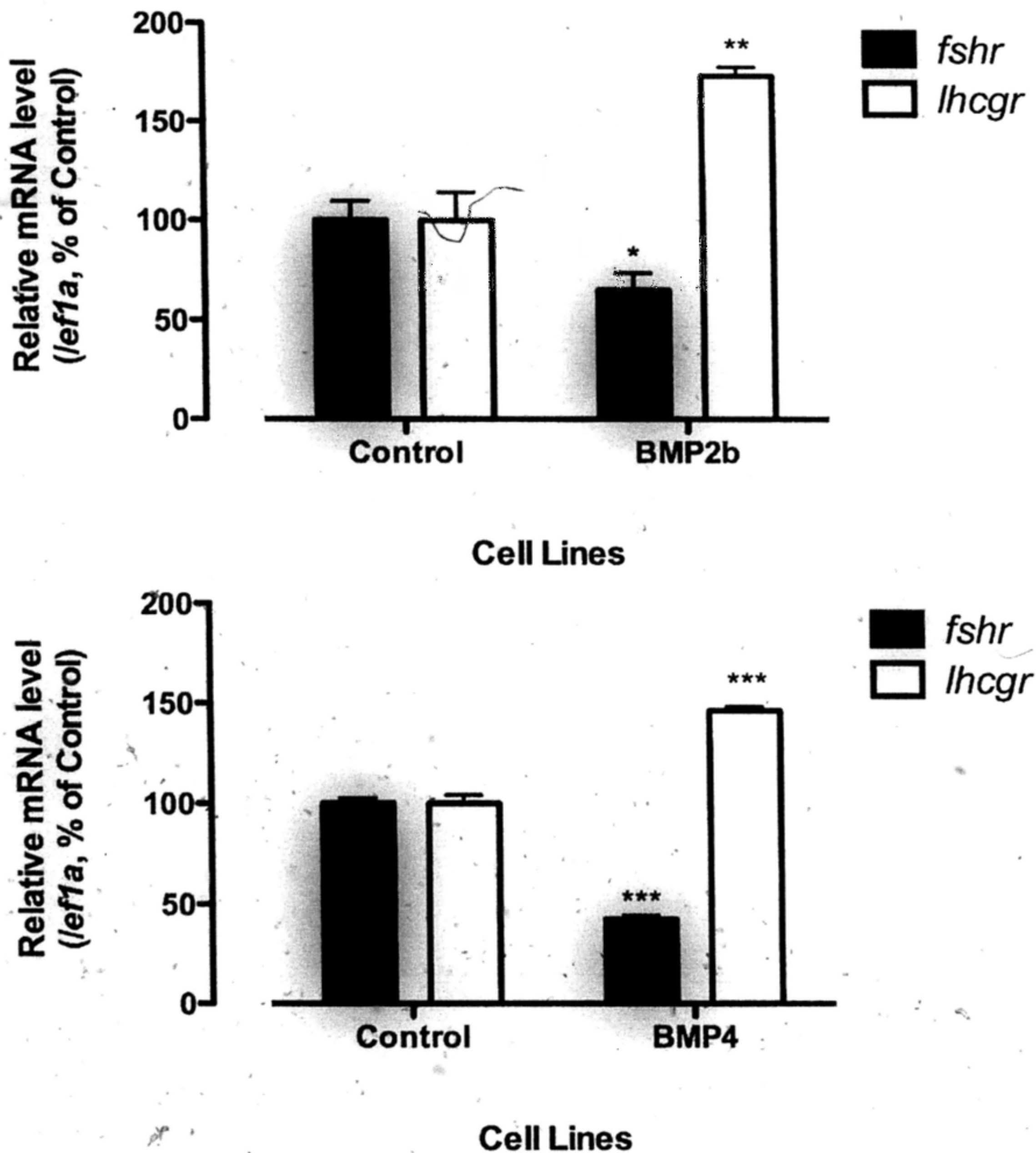
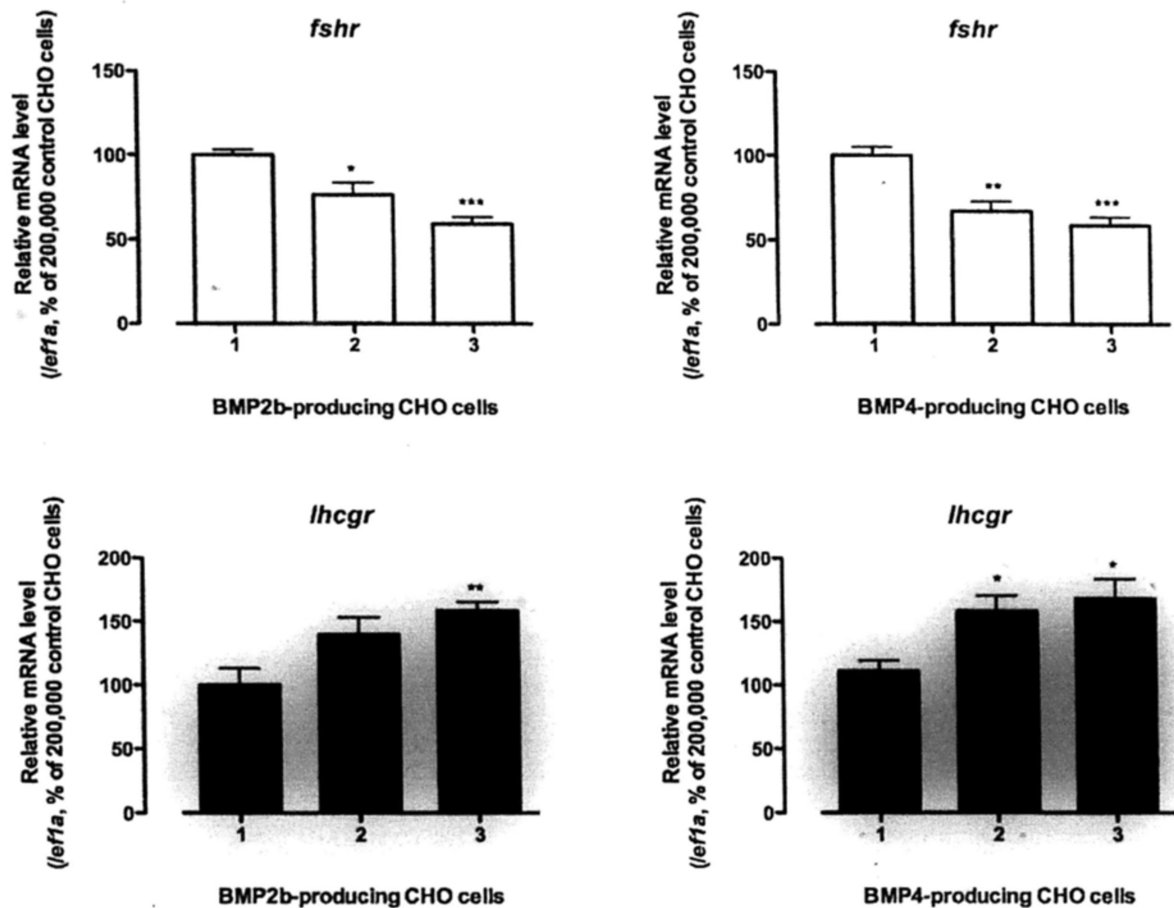
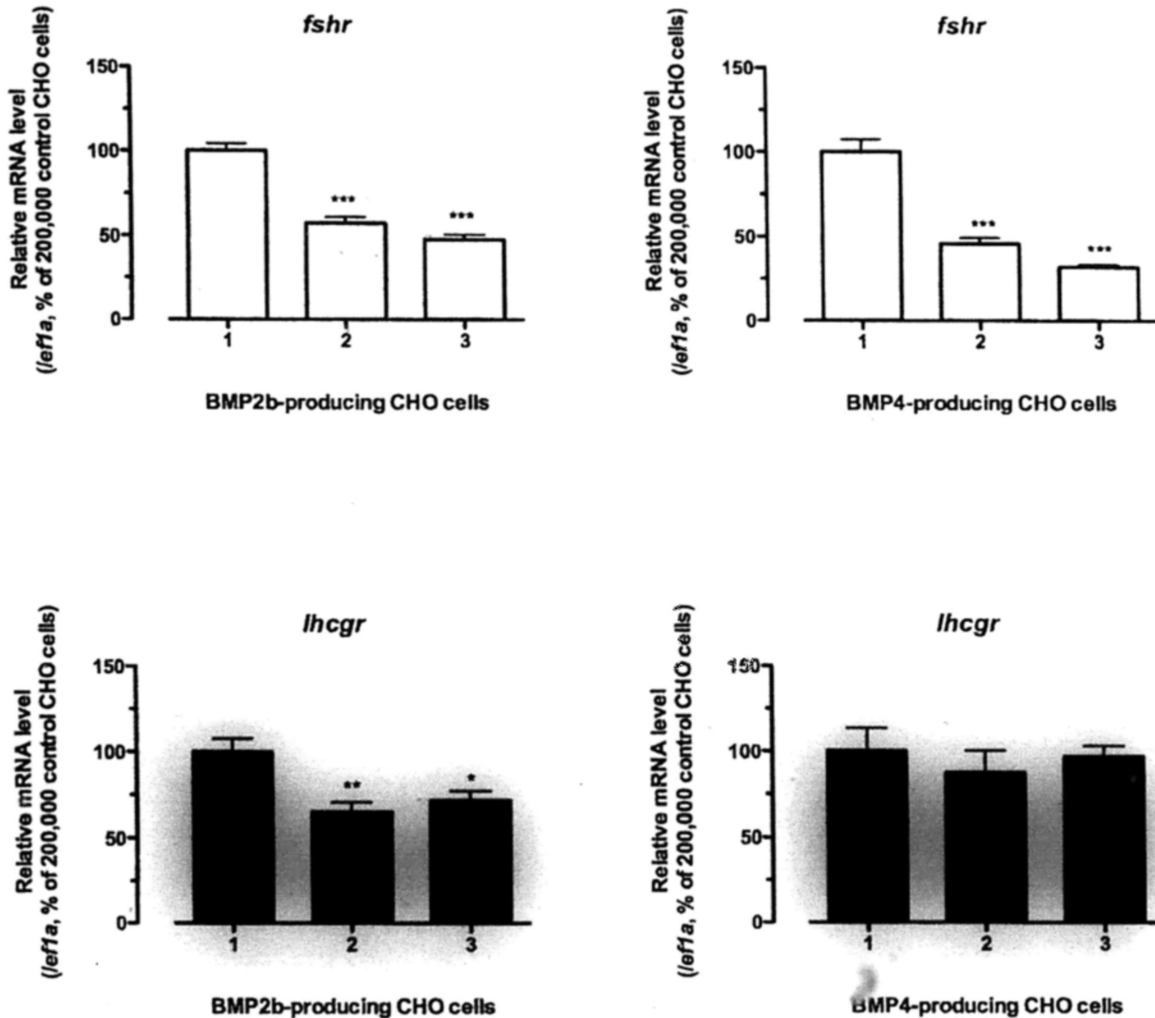


Fig. 5.1 Effects of zfBMP2b and zfBMP4 from the CHO cells on *fshr* and *lhcgr* expression. zfBMP2b and zfBMP4 differentially regulated the two gonadotropin receptors by down-regulating *fshr* and up-regulating *lhcgr* at 24 h. The values are the mean ± SEM (n=6) from a representative experiment. Asterisk (*) indicates statistical significance (*, P<0.05; **, P<0.01; ***, P<0.001 vs. control).



	1	2	3
Control CHO cells	**200,000**	**100,000**	**0**
zfBMP2b/ zfBMP4-producing CHO cells	**0**	**100,000**	**200,000**

Fig. 5.2 Dose response of zfBMP2b and zfBMP4 regulation of *fshr* and *lhcgr* expression in co-cultured follicle cells. Both CHO cell lines dose-dependently down-regulated *fshr* but up-regulated *lhcgr* at 24 h. The values are the mean ± SEM (n=6) from a representative experiment. Asterisk (*) indicates statistical significance (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ vs. control).



	1	2	3
Control CHO cells	**200,000**	**100,000**	**0**
zfBMP2b/ zfBMP4-producing CHO cells	**0**	**100,000**	**200,000**

Fig. 5.3 Effects of zfBMP2b and zfBMP4 from the CHO cells on the expression of *fshr* and *lhcgr* in the follicle cells. zfBMP2b and zfBMP4 dose-dependently down-regulated *fshr* at 48 h. zfBMP2b down-regulated *lhcgr* at 48 h as well, though zfBMP4 did not have significant effect on *lhcgr* at 48 h The values are the mean ± SEM (n=6) from a representative experiment. Asterisk (*) indicates statistical significance (*, P<0.05; **, P<0.01; ***, P<0.001 vs. control).

Chapter 6

General Discussion

As one of the most dynamic organs in teleosts, the ovary and its development have attracted the interest of many scientists. It is well known that ovarian development is subjected to hormonal control by the pituitary hormones, namely the gonadotropins, FSH and LH. However, there are also various intraovarian factors that act locally in the ovary to control its own development (227). Such local factors include epidermal growth factor (EGF) family (178, 228), transforming growth factor-β (TGF-β) superfamily (229) and the activin-inhibin-follistatin system (34, 173). BMPs, belonging to the TGF-β superfamily, have also aroused the interest of scientists to investigate their roles in reproduction. BMPs have been found to modulate ovarian development in addition to their roles in growth and differentiation (71). So far, only BMP15 and GDF9 have been characterized in the zebrafish (166-168). Other BMP family members remain unknown about their roles in the zebrafish ovary. Hence in this study, we aim to explore the rest of the BMP family by characterizing *bmp2a*, *bmp2b*, *bmp4*, *bmp6*, *bmp7a*, as well as the two type II receptors, *bmpr2a* and *bmpr2b*. Their spatiotemporal expression profiles were first investigated. Recombinant zebrafish BMPs (zfBMP2b and zfBMP4) were produced and their regulatory roles in controlling the activin-inhibin-follistatin system and the gonadotropin receptors were investigated.

6.1 Characterization of BMPs in the zebrafish ovary

The current study started off with characterizing the BMP ligands, *bmp2a*, *bmp2b*, *bmp4*, *bmp6*, *bmp7a* and the two BMP type II receptors, *bmpr2a* and *bmpr2b*. These BMP ligands and receptors were found to be expressed ubiquitously in different tissues in the zebrafish. This is not surprising as BMPs are well known to be important for growth and development of different organs. Their expression in the gonads (ovary and testis) indicated their potential functional roles in reproduction. Following the tissue distribution studies, we investigated the spatiotemporal expression patterns of these proteins. The zebrafish ovarian follicle consists of an oocyte surrounded by the somatic follicle layer which is made up of granulosa cells and theca

cells. To understand how BMPs signal in the ovarian follicle, we localized the BMP ligands and receptors within the follicle. In recent years, our lab has developed a novel approach to mechanically separate the follicle layer away from the oocyte (168), making localization analysis more sensitive and convincing. Our results showed that the BMP ligands were located exclusively in the oocyte (except for *bmp4* which was found in both compartments), while the two forms of BMP type II receptors were located exclusively in the follicle layer, indicating a potential paracrine signaling from the oocyte towards the follicle layer. This is opposite to the activin system in which the ligands were exclusively in the follicle layer while the receptors were abundant in the oocyte (171). Also, our findings are different from that in mammals, in which the distribution patterns of the ligands and receptors are more diverse among different species (151, 152, 174, 175). Thus, this study represents the first to identify BMPs as oocyte-derived factors in the ovary of teleosts.

In addition to the localization studies, we also examined the temporal expression changes of the BMPs and receptors during folliculogenesis. Unlike the situation in mammals in which the growth of the follicle is mainly due to proliferation of follicle cells, the growth of zebrafish follicle is mainly caused by the growth of the oocyte, due to the formation of cortical alveoli and incorporation of vitellogenin. During folliculogenesis, the BMP ligands increased their expression from PG to PV stage, followed by a drop as folliculogenesis continued. The increase was most drastic for *bmp2b*, having a 10-fold increase from PG to PV stage. This led us to hypothesize its potential role in promoting early folliculogenesis. As documented in mammalian studies, BMPs also promote primordial follicle development (57, 150) . Thus, the role of BMPs in early folliculogenesis is worth investigating in the future.

Interestingly, we observed a steady increase in expression of the BMP receptors during folliculogenesis, and finally a surge at the full-grown stage. This signifies that BMP signaling is the most prominent at the final oocyte maturation stage, paralleling that of LH receptor, suggesting a potential role for BMPs at final oocyte maturation and/or ovulation.

6.2 Production of recombinant zfBMPs

With the BMPs characterized in the zebrafish, we want to find out their functional roles. The availability of homologous BMPs from the zebrafish would be essential tools for functional characterization. We decided to use CHO cells as the bioreactors to produce the zebrafish

BMPs. Our lab has previously used CHO cells to establish stable cell lines for producing recombinant zebrafish gonadotropins (197), goldfish activin (204) and follistatin (230). We used CHO cells because it is a mammalian cell line that can perform post-translational modifications and refolding processes. Also, they are easy to maintain. They do not have the problem of forming inclusion bodies as the prokaryotes do. After we have established the CHO cell lines for recombinant zfBMP2b and zfBMP4, some zebrafish BMPs, namely zfBMP2a and zfBMP4, became commercially available (R&D). They are made from *E. coli* with unexpectedly high ED_{50} values (zfBMP4, Cat no. 1128-BM, ED_{50}: 0.2-1 μg/ml; zfBMP2a, Cat no. 111-BM, ED_{50}: 100-400 ng/ml). When tested in our experiments, our recombinant BMPs (zfBMP2b and zfBMP4) from CHO cells actually out-performed these commercial ones although they showed similar effects. This indicates that the recombinant proteins produced from mammalian cell lines are more potent with better bioactivity.

After producing the recombinant proteins, we tested their biological activity. Earlier, we have established an assay using zebrafish primary follicle cell culture and Western blotting. We found that human BMP2 induced phosphorylation of Smad1/5/8, which are downstream mediators in BMP signaling, in cultured follicle cells starting from 30 min of incubation. Smad phosphorylation is an indication that active BMP signaling occurred in the zebrafish follicle cells, and that the BMPs being administered had biological activity. When we administered the CHO cell-derived recombinant zfBMPs onto the cultured follicle cells, the BMPs could successfully induce Smad phosphorylation, indicating that they were biologically active. Also, this further confirmed the paracrine mode of BMP actions in the zebrafish follicle.

Previously, the two type II receptors, *bmpr2a* and *bmpr2b*, were found to establish left-right asymmetry in the zebrafish (169). Yet, their roles in reproduction remain unknown. In view of this, we have also produced the ectodomains of the two type II receptors using CHO cells. Our data showed that the truncated forms of the receptors could attenuate BMP signaling induced by the CHO cell-derived recombinant zfBMPs, as we observed a decrease in Smad signaling. Thus, the involvement of the two receptors in BMP signaling of the zebrafish ovary is confirmed.

6.3 Establishment of co-culture system

In this study, we have developed a novel approach to conduct regulation studies. We co-incubated the recombinant BMP-producing CHO cells and the zebrafish follicle cells. Interestingly, the CHO cells grew with the follicle cells in a healthy manner. The morphology of both kinds of cells appeared to be the same as if they were cultured alone. In addition, the observed survival rate of both kinds of cells was reasonable high. The BMPs that were secreted from the CHO cells would act directly on the follicle cells in a paracrine manner, mimicking the environment in the follicle, in which the BMPs from the oocyte would act directly on the follicle cells in a paracrine manner.

The CHO cells enter high production phase at 28°C (rather than at 37°C during proliferation phase), which is coincidently the optimal temperature at which the follicle cells are usually incubated at, making this approach feasible. There are several advantages to this approach. By using the co-culture system, we eliminated the need to concentrate and purify the recombinant proteins. Also, sometimes even the concentrated control medium made from CHO cells transfected with empty vector would have unwanted effects on some target genes due to factors such as increased osmolality, making it hard to interpret the results. Using the co-culture system would reduce this problem. In addition, the CHO cells can be easily transfected, allowing easy manipulation of target gene expression. For example, in this study, we used siRNA approach to knock down zfBMP4 expression in the CHO cells followed by examining the changes in target gene expression in the co-cultured follicle cells. Most importantly, our results obtained from the co-culture experiment were validated by the commercial recombinant zfBMPs made from prokaryotic cells. This definitely adds credibility to this novel approach and increases its potential to become a research platform in the future.

There is still room for improvement for the design of the co-culture system. In the follicle, the factors are released from within the oocyte and target on the somatic cells outside. Our co-culture system can only place the recombinant CHO cells and follicle cells side by side, without the "inside-outside" arrangement. We can try to improve the design of the co-culture system in a more three-dimensional way in order to further mimick what happens in the follicle.

Our laboratory has previously established several cell lines, such as the gonadotropins-producing cell lines (FSH and LH) (197). In the future, we can possibly incubate a mix of the BMP-producing cell lines with the gonadotropins-producing cell lines to observe their interactive effects on the target genes in the follicle cells. Furthermore, it will be an interesting idea to co-

incubate the recombinant BMP cell lines directly with the zebrafish follicles. The zebrafish follicles, sitting on top of the "carpet" of BMP-producing CHO cells, will possibly be subjected to regulation by the BMPs secreting from the CHO cells in a paracrine manner. More manipulations on this novel co-culture approach will be possible, and hopefully this can bring more insights on the regulatory role of BMPs in ovarian development.

6.4 Regulation studies

In this study, using the co-culture approach, we found out that BMPs exert regulatory roles on the activin-inhibin-follistatin system as well as the gonadotropin receptors. Zebrafish BMP2b and BMP4 slightly but significantly down-regulated the expression of the three activin β subunits (*inhbaa*, *inhbab*, *inhbb*) but dramatically up-regulated the antagonist of activin, inhibin α (*inha*), as well as the activin-binding protein, follistatin (*fst*). BMP signaling reaches the highest at the FG stage. We hypothesize that BMPs from the oocyte may signal the upper level of the brain-pituitary-gonadal axis (HPG) by pushing up the expression of inhibin in the follicle cells, which may travel to the pituitary through circulation as a hormone to shut off activin action in the pituitary, resulting in a decrease in FSH, but an increase in LH production, triggering oocyte maturation. By down-regulating the expression of activin subunits, BMPs may hinder the process of oocyte maturation as activin has been shown to promote maturational competency (208) and final oocyte maturation in the zebrafish (231). This was supported by our evidence that zfBMP4 slightly but significantly reduced spontaneous maturation rate of FG follicles *in vitro*. The increased inhibin α expression also helps to divert the synthesis from activins to inhibins in the follicle cells. Also, zebrafish BMP2b and BMP4 significantly up-regulated follistatin, the activin-binding protein, to modulate the activity of activin, which may represent another mechanism to down-regulate activin output, leading to the suppression of oocyte maturation.

Prior to and during oocyte maturation, there is a decrease in FSH signaling and an increase in LH signaling, as suggested by the expression of their receptors, *fshr* and *lhcgr*. BMPs modulated the gonadotropin receptors by down-regulating *fshr* and up-regulating *lhcgr*, helping to promote oocyte maturation (Fig. 6.1). As the activin and inhibin subunits and gonadotropin receptors are all located on the follicle cells, the BMPs action on these molecules may represent a mechanism by which the oocyte regulates the function of the follicle cells and

the entire HPG axis in general (Fig. 6.2). This hypothesis is truly exciting and it will be further tested in the future.

With BMP signaling reaching the peak at FG stage as well as its action in pushing up inhibin α and leading to LH production to trigger oocyte maturation, BMPs apparently helps to trigger oocyte maturation. However, our studies indicated that the CHO-recombinant zfBMP4 suppressed spontaneous oocyte maturation. Our speculation is that the seemingly contradictory roles of BMPs may be physiologically relevant in that while they help to trigger or activate the HPG axis for final oocyte maturation and ovulation, these molecules also exert an inhibitory tone locally on the FG follicles to prevent precocious maturation before the LH from the pituitary and DHP from the follicle arrive. This would ensure a synchronous maturation of the follicles, which is believed to benefit the reproduction. In the isolated full-grown follicles, BMPs suppressed activin but increased inhibin α and follistatin expression (as demonstrated in chapter 4), thus decreasing the stimulatory effect of activin on oocyte maturation and leading to a decrease in the maturation rate.

In our study, we tried to demonstrate that knockdown of BMP2b in the oocyte can lead to a decreased expression of inhibin α. There was a trend that expression of inhibin α decreased when morpholino targeting BMP2b was injected into the follicle. However, the result was not significant. This is probably due to the presence of other oocyte factors, including the BMPs, that might compensate the regulatory role of BMP2b. Due to this possible redundant role of the BMPs, we cannot conclude yet BMPs are indeed signaling from the oocyte to the follicle cells using this *in vivo* model. Other approaches are needed to validate our hypothesis. An alternative approach would be to over-express BMP2b in the follicle by microinjection, although there might be a possibility that the presence of abundant mRNA binding proteins in the oocyte might bind to the injected mRNA and make translation inefficient.

Though our understanding of the regulatory roles of BMPs as potential modulators in ovarian development is still limited, our study opens the doorway to further explore the physiological significance of BMPs in ovarian development.

6.5 Significance of the study

Besides the pituitary hormones that govern the ovarian development, there also exist the intraovarian growth factors that modulate ovarian development. The identified intraovarian

factors include the epidermal growth factor family, activin-inhibin-follistatin system, transforming growth factor-β, growth differentiation factor 9, bone morphogenetic protein 15 and insulin-like growth factor (227). The current study has identified the BMP family, a group of the transforming growth factor-β superfamily, as one of the intraovarian growth factors as well. More importantly, we have identified the BMPs as oocyte-derived factors, signaling from the oocyte towards the follicle layer in a paracrine manner. This added on top of the EGF family and GDF9 another group of growth factors derived from the oocyte in the zebrafish.

Besides growth and differentiation, we have identified the potential roles of BMPs in zebrafish female reproduction through their modulation on other important regulators, the activin-inhibin-follistatin system and the gonadotropin receptors. We have established stable cell lines of BMP-producing CHO cells, and have established a co-culture platform for future studies. Further understanding on the BMPs could help to improve fertility of livestock, endangered species and human beings. BMPs could potentially be used as therapeutic drugs for fertility problems. The identification of BMPs as oocyte-derived factors and their regulatory roles in the zebrafish ovary has deepened our understanding of vertebrate ovarian physiology.

6.6 Perspectives of the project

The current study has investigated the spatiotemporal expression profile of the BMP family, identifying BMPs as oocyte-derived factors signaling the somatic follicle cells. Recombinant BMP proteins have been produced and their biological activity has been confirmed. Furthermore, BMPs were found to exert regulatory effects on the activin-inhibin-follistatin system as well as the two gonadotropin receptors. Yet, our understanding of the BMP system in zebrafish ovary is just the tip of an iceberg. Also, the expression pattern of the BMP family as well as their regulation on the activin-inhibin-follistatin system and the gonadotropin receptors are mainly based on mRNA levels. More future work on the protein expression pattern and functional studies are needed to explore the role of BMPs in zebrafish ovary.

6.6.1 Early follicle development

Based on the temporal expression profile data, all the BMP ligands (except *bmp7a*) exhibited an increase in expression from PG to PV stage. It would be interesting to investigate if BMPs play a role in promoting early folliculogenesis. In fact, in mammals, it has been

demonstrated that exogenous BMP4 promoted early follicle development in the whole ovary organ culture from 4-day old rats for 2 weeks. It was found that the BMP4-treated ovaries had a significantly higher proportion of developing primary follicles and fewer arrested primordial follicles than untreated controls, suggesting that BMP4 promoted primordial follicle development and the primordial-to-primary follicle transition (150). Another study also proposed a role for BMP7 in promoting primordial-to-primary follicle transition, based on the effect of BMP7 on cultured neonatal mouse ovaries (57). In the zebrafish, we can try to develop an organ culture system, incubating the zebrafish ovaries with the recombinant BMPs and observe their morphological and histological changes after long-term treatment. This will help us to find out whether BMPs could promote early folliculogenesis. Limited studies have been done to investigate the role of local growth factors in promoting early folliculogenesis in teleosts as compared to those in mammals.

6.6.2 Oocyte maturation

Studies have been done to investigate the roles of activin, EGF and BMP 15 in oocyte maturation in the zebrafish ovary. Activin and EGF both promote oocyte maturation (178, 208). Yet, BMP15 has been found to suppress hCG-induced oocyte maturation (167). The role of other BMP family members in this event remains unknown. The significant increase in the expression of BMP receptors at FG stage strongly implicates BMP family in the control of final oocyte maturation and ovulation. In our study, recombinant zfBMP4 was found to reduce the spontaneous maturation; however, its effect was mild, suggesting that the BMP system may play other roles in the final stage of follicle development. This would be an interesting issue to explore in future studies.

6.6.3 Regulation of BMP receptors

In contrast to the BMP ligands which exhibited an increase followed by a decrease in expression during folliculogenesis, the BMP receptors steadily increase in expression throughout folliculogenesis, reaching the peak at FG stage. We have attempted to find out what factors could possibly push up the expression of the two receptors during folliculogenesis. We have used the follicle cell culture system trying to search for the factors that possibly up-regulate the two BMP receptors, yet we still cannot identify any yet. More searches on this will be needed as

it will provide important insights on how the BMP system is being regulated in the zebrafish ovary.

6.6.4 Steroidogenesis

Steroidogenesis, the production of steroids in the zebrafish ovary, is an important process in follicle development. Steroids that are produced in the follicle are essential for its growth and maturation. For example, vitellogenins are synthesized in the liver under the stimulation of estrogen. Vitellogenins are then sequestered by the follicles to be incorporated into the yolk in the oocyte, which provides nutrients for the developing embryo after fertilization.

It has been reported in mammalian studies that BMPs modulate FSH-induced steroidogenesis. It was demonstrated in primary cultures of rat granulosa cells that FSH increased estrogen and progesterone production in dose-dependent manner, while BMP4 and BMP7 could increase FSH-induced estrogen production and decrease FSH-induced progesterone production. Yet, treatment with BMP4 and BMP7 alone had no effect on basal estrogen and progesterone production (149). We can make use of the primary follicle cell culture system to investigate the effects of BMPs in steroid production. We can incubate the cultured primary follicle cells with BMPs, either with or without gonadotropins, followed by collecting the culture media and measuring steroid production, for example, estradiol or DHP, by ELISA. Also, we can examine the effects of BMPs on the steroidogenic enzymes, for example, aromatase, to see whether BMPs exert regulatory effects on steroidogenesis.

6.6.5 Interaction with gonadotropins

So far we have seen that BMPs, as intraovarian local factors, may play important regulatory roles in the zebrafish ovary. Yet, it is important to bear in mind that the pituitary hormones, mainly the gonadotropins, play an indispensable role in regulating ovarian development. The interaction of the BMP system and the gonadotropins is worth investigating. With the co-culture platform established, as well as the availability of the gonadotropin-producing cell lines, we can co-incubate the gonadotropin-producing cell lines and BMP-producing cell lines to investigate their interactive effects on important regulatory genes in the ovary. The gonadotropins from pituitary and the oocyte-derived BMPs are expected to converge

their signals at the follicle cells. How the target genes in the follicle cells are subjected to the co-regulation by the pituitary hormones and BMPs from the oocyte will be interesting to explore.

6.6.6 Target genes of BMPs

It is well established that downstream of BMP signaling are the Smads. Our current study has demonstrated the biological activity of the recombinant proteins by their ability to induce Smad signaling in cultured follicle cells. However, less is known about their target genes in the zebrafish ovary. Another class of genes that are targeted by BMP signaling is the Id family (inhibitors of DNA binding/differentiation) that signals in various types of cells. In mammals, there are four members of the family, namely Id1 to Id4. They are negative regulators of basic helix-loop-helix transcription factors, being important in cell fate determination and embryonic stem cell self-renewal (232). However, apparently, the Id genes have not been studied in the zebrafish ovary yet. Characterization of them would help us to further understand BMP signaling in the zebrafish ovary, so as to get a more complete picture.

6.6.7 Transgenic zebrafish of BMPs

Knockout or mutational studies of BMPs in mammalian models have provided convincing evidence that BMPs are important in reproduction. For example, BMP15 knockout mice were proven to be subfertile (46). A mutation in BMPR-IB in ewes is associated with increased ovulation (160). In fact, zebrafish with mutations in BMPs have been generated by crossing two heterozygotes to result in homozygous offspring lacking BMPs. For example, zebrafish defective in *bmp2b* (*swirl*) were generated, and could be rescued by mRNA injection at early embryonic stage to survive to adulthood. Yet, they did not have enough *bmp2b* during their subsequent development and exhibited development defects, for example in balancing (83). We could try to produce these homozygous zebrafish embryos lacking BMPs, rescue them with mRNA injection at early stages and let them survive to adulthood until they are sexually mature. In this way, we can investigate the development and function of the zebrafish ovary without specific BMPs, which promises to provide insights into the role of specific BMPs in the ovary. Yet, the difficulty lies in that the BMP-defective zebrafish might exhibit developmental defects in other organs as BMPs are essential for many growth and differentiation processes, and the individuals may not be able to survive to sexually mature stage.

6.7 Conclusions

In conclusion, we have found from the current study that BMPs (*bmp2a, bmp2b, bmp4, bmp6, bmp7a*) are oocyte-derived factors that potentially signal to the follicle cells in a paracrine manner. *bmp4*, as an exception, was found in both the oocyte compartment and the follicle cell compartment. We are unsure of the reason behind its different localization pattern with other BMP ligands. Therefore we chose to produce zfBMP4 as one of the recombinant BMP proteins using CHO cells. Our results from the co-culture experiment indicated that the actions of both zfBMP2b and zfBMP4 were similar. The actions of zfBMP4, which expresses in both compartments, were not different from those of zfBMP2b, which was identified as an oocyte-specific factor. This might point out the redundant role of the BMPs in the follicle. It will be exciting to investigate the reason behind the localization of *bmp4* in the follicle cell compartment.

Although we have studied the regulatory role of BMPs and their signaling in the zebrafish ovary, our understanding of the system is still very limited. Many questions on the BMP system in the zebrafish ovary remain unanswered and it is worth further investigation. We have proposed some possible experiments in the last chapter to further explore the BMP system. More platforms, for example, the ovary organ culture, measurement of steroid production, or transgenic zebrafish generation, will be useful to facilitate our study. With more platforms being established, our understanding on the BMPs as important regulators in ovarian development and function will definitely be enhanced and deepened.

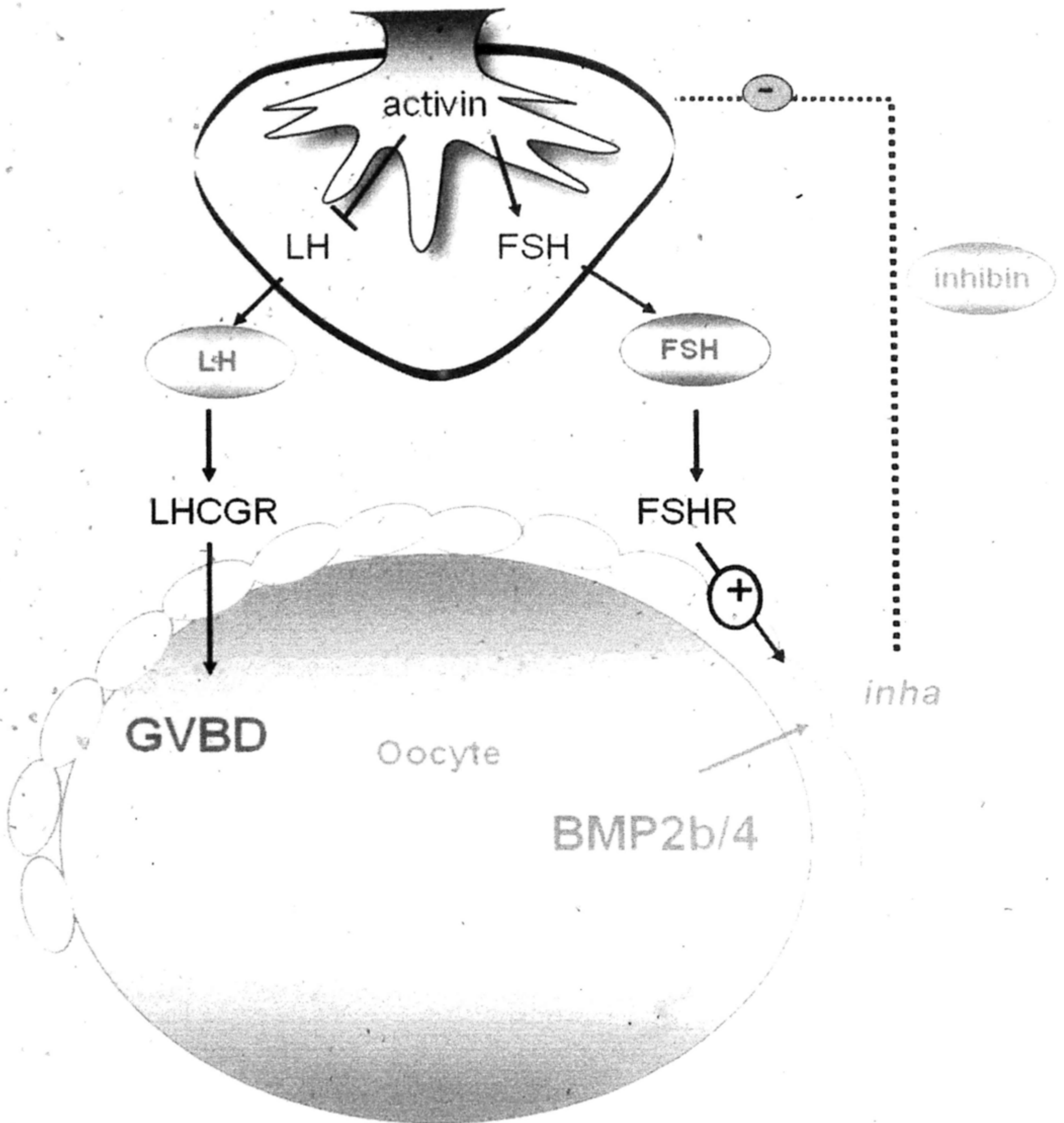


Fig. 6.1 Hypothesized working model for BMPs in the zebrafish ovary. Activin from the pituitary leads to production of FSH, which acts on FSHR for follicle growth. When the follicle is ready to mature, BMPs from the oocyte up-regulate inhibin α on the follicle cells, increasing the signal of inhibin traveling to the pituitary to inform the pituitary for its readiness to mature. Inhibin antagonizes activin in the pituitary, reducing the production of FSH but increasing the production of LH. LH in turn acts on LHCGR in the ovary to trigger oocyte maturation.

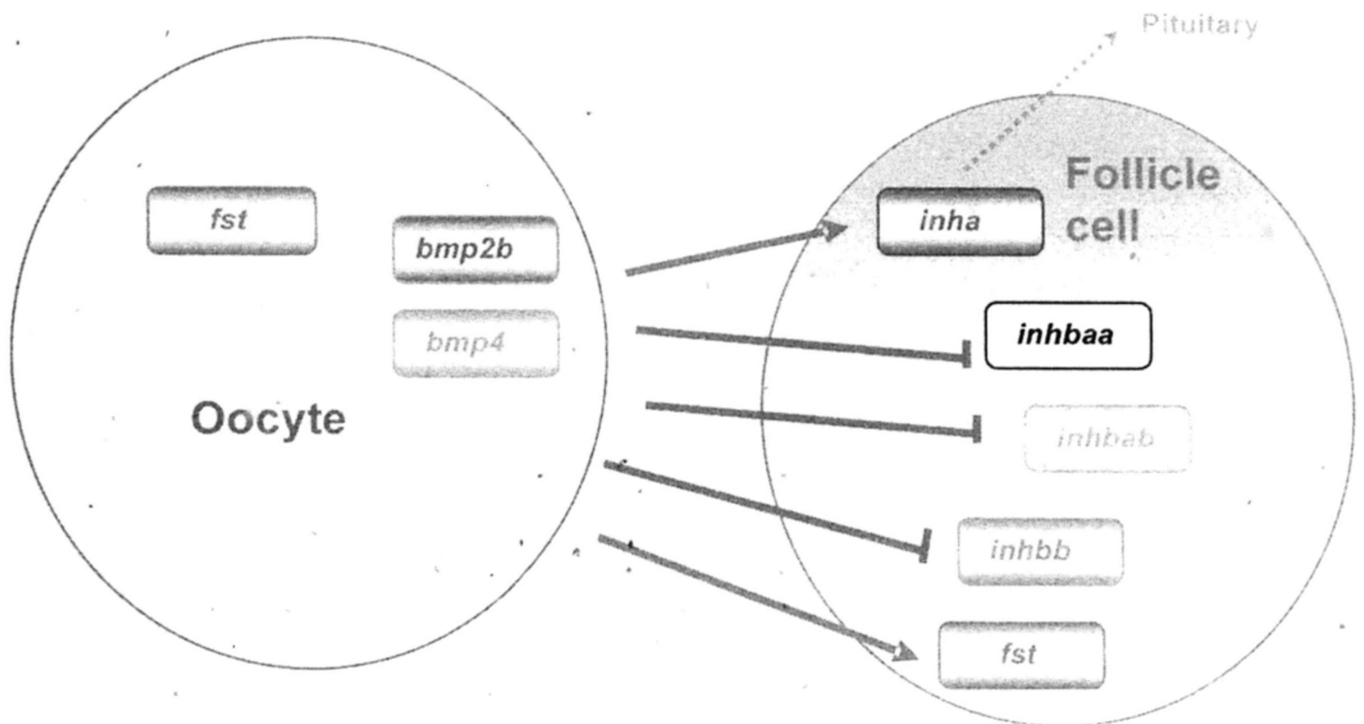


Fig. 6.2 Paracrine regulation of activin-inhibin-follistatin system in the follicle cells by BMPs from the oocyte. BMPs from the oocyte down-regulate activin and up-regulate its antagonist inhibin and binding protein follistatin in the follicle cells, leading to reduced activin output in the follicle. This may help keeping the oocyte in check to prevent it from precocious maturation. Meanwhile, the increased inhibin output would signal the pituitary for the readiness of the oocytes to mature and ovulate. Inhibin is expected to antagonize activin in the pituitary, leading to reduced FSH secretion but increased LH secretion. The increased LH in turn acts on LHCGR, which reaches its highest expression level in the FG follicles, will drive the follicles to mature and ovulate.

References

1. **Voutilainen R** 1992 Differentiation of the fetal gonad. Horm Res 38 Suppl 2:66-71
2. **Yao HH, Matzuk MM, Jorgez CJ, Menke DB, Page DC, Swain A, Capel B** 2004 *Follistatin* operates downstream of *Wnt4* in mammalian ovary organogenesis. Dev Dyn 230:210-215
3. **Merchant-Larios H, Centeno B** 1981 Morphogenesis of the ovary from the sterile W/Wv mouse. Prog Clin Biol Res 59B:383-392
4. **Oktem O, Urman B** 2010 Understanding follicle growth *in vivo*. Hum Reprod 25:2944-2954
5. **Zou K, Yuan Z, Yang Z, Luo H, Sun K, Zhou L, Xiang J, Shi L, Yu Q, Zhang Y, Hou R, Wu J** 2009 Production of offspring from a germline stem cell line derived from neonatal ovaries. Nat Cell Biol 11:631-636
6. **Oktem O, Oktay K** 2008 The ovary: anatomy and function throughout human life. Ann N Y Acad Sci 1127:1-9
7. **Wandji SA, Srsen V, Voss AK, Eppig JJ, Fortune JE** 1996 Initiation *in vitro* of growth of bovine primordial follicles. Biol Reprod 55:942-948
8. **Oktay K, Briggs D, Gosden RG** 1997 Ontogeny of follicle-stimulating hormone receptor gene expression in isolated human ovarian follicles. J Clin Endocrinol Metab 82:3748-3751
9. **Packer AI, Hsu YC, Besmer P, Bachvarova RF** 1994 The ligand of the c-kit receptor promotes oocyte growth. Dev Biol 161:194-205
10. **Parrott JA, Skinner MK** 1999 Kit-ligand/stem cell factor induces primordial follicle development and initiates folliculogenesis. Endocrinology 140:4262-4271
11. **Yoshida H, Takakura N, Kataoka H, Kunisada T, Okamura H, Nishikawa SI** 1997 Stepwise requirement of c-kit tyrosine kinase in mouse ovarian follicle development. Dev Biol 184:122-137
12. **Liu K, Rajareddy S, Liu L, Jagarlamudi K, Boman K, Selstam G, Reddy P** 2006 Control of mammalian oocyte growth and early follicular development by the oocyte PI3 kinase pathway: new roles for an old timer. Dev Biol 299:1-11
13. **Reddy P, Shen L, Ren C, Boman K, Lundin E, Ottander U, Lindgren P, Liu YX, Sun QY, Liu K** 2005 Activation of Akt (PKB) and suppression of FKHRL1 in mouse and rat oocytes by stem cell factor during follicular activation and development. Dev Biol 281:160-170
14. **Liu L, Rajareddy S, Reddy P, Jagarlamudi K, Du C, Shen Y, Guo Y, Boman K, Lundin E, Ottander U, Selstam G, Liu K** 2007 Phosphorylation and inactivation of glycogen synthase kinase-3 by soluble kit ligand in mouse oocytes during early follicular development. J Mol Endocrinol 38:137-146
15. **Reddy P, Liu L, Adhikari D, Jagarlamudi K, Rajareddy S, Shen Y, Du C, Tang W, Hamalainen T, Peng SL, Lan ZJ, Cooney AJ, Huhtaniemi I, Liu K** 2008 Oocyte-specific deletion of *Pten* causes premature activation of the primordial follicle pool. Science 319:611-613
16. **Castrillon DH, Miao L, Kollipara R, Horner JW, DePinho RA** 2003 Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. Science 301:215-218

17. **Rankin T, Familari M, Lee E, Ginsberg A, Dwyer N, Blanchette-Mackie J, Drago J, Westphal H, Dean J** 1996 Mice homozygous for an insertional mutation in the *Zp3* gene lack a zona pellucida and are infertile. Development 122:2903-2910
18. **Knight PG, Glister C** 2006 TGF-β superfamily members and ovarian follicle development. Reproduction 132:191-206
19. **Hayashi M, McGee EA, Min G, Klein C, Rose UM, van Duin M, Hsueh AJ** 1999 Recombinant growth differentiation factor-9 (GDF-9) enhances growth and differentiation of cultured early ovarian follicles. Endocrinology 140:1236-1244
20. **Hreinsson JG, Scott JE, Rasmussen C, Swahn ML, Hsueh AJ, Hovatta O** 2002 Growth differentiation factor-9 promotes the growth, development, and survival of human ovarian follicles in organ culture. J Clin Endocrinol Metab 87:316-321
21. **Dong J, Albertini DF, Nishimori K, Kumar TR, Lu N, Matzuk MM** 1996 Growth differentiation factor-9 is required during early ovarian folliculogenesis. Nature 383:531-535
22. **Juengel JL, Hudson NL, Heath DA, Smith P, Reader KL, Lawrence SB, O'Connell AR, Laitinen MP, Cranfield M, Groome NP, Ritvos O, McNatty KP** 2002 Growth differentiation factor 9 and bone morphogenetic protein 15 are essential for ovarian follicular development in sheep. Biol Reprod 67:1777-1789
23. **Otsuka F, Yao Z, Lee T, Yamamoto S, Erickson GF, Shimasaki S** 2000 Bone morphogenetic protein-15. Identification of target cells and biological functions. J Biol Chem 275:39523-39528
24. **Dodson WC, Schomberg DW** 1987 The effect of transforming growth factor-β on follicle-stimulating hormone-induced differentiation of cultured rat granulosa cells. Endocrinology 120:512-516
25. **Zhao J, Taverne MA, van der Weijden GC, Bevers MM, van den Hurk R** 2001 Effect of activin A on In Vitro development of rat preantral follicles and localization of activin A and activin receptor II. Biol Reprod 65:967-977
26. **Durlinger AL, Gruijters MJ, Kramer P, Karels B, Kumar TR, Matzuk MM, Rose UM, de Jong FH, Uilenbroek JT, Grootegoed JA, Themmen AP** 2001 Anti-Mullerian hormone attenuates the effects of FSH on follicle development in the mouse ovary. Endocrinology 142:4891-4899
27. **Findlay JK** 1993 An update on the roles of inhibin, activin, and follistatin as local regulators of folliculogenesis. Biol Reprod 48:15-23
28. **Durlinger AL, Kramer P, Karels B, de Jong FH, Uilenbroek JT, Grootegoed JA, Themmen AP** 1999 Control of primordial follicle recruitment by Anti-Mullerian hormone in the mouse ovary. Endocrinology 140:5789-5796
29. **Durlinger AL, Gruijters MJ, Kramer P, Karels B, Ingraham HA, Nachtigal MW, Uilenbroek JT, Grootegoed JA, Themmen AP** 2002 Anti-Mullerian hormone inhibits initiation of primordial follicle growth in the mouse ovary. Endocrinology 143:1076-1084
30. **Yamoto M, Minami S, Nakano R, Kobayashi M** 1992 Immunohistochemical localization of inhibin/activin subunits in human ovarian follicles during the menstrual cycle. J Clin Endocrinol Metab 74:989-993
31. **Richards JS** 2007 Genetics of ovulation. Semin Reprod Med 25:235-242
32. **Nagahama Y, Yoshikuni M, Yamashita M, Tokumoto T, Katsu Y** 1995 Regulation of oocyte growth and maturation in fish. Curr Top Dev Biol 30:103-145

33. **Selman K, Wallace RA, Sarka A, Qi XP** 1993 Stages of Oocyte Development in the Zebrafish, *Brachydanio-Rerio*. J Morphol 218:203-224
34. **Wang Y, Ge W** 2004 Developmental profiles of activin βA, βB, and follistatin expression in the zebrafish ovary: evidence for their differential roles during sexual maturation and ovulatory cycle. Biol Reprod 71:2056-2064
35. **Eppig JJ** 2001 Oocyte control of ovarian follicular development and function in mammals. Reproduction 122:829-838
36. **Buccione R, Schroeder AC, Eppig JJ** 1990 Interactions between somatic cells and germ cells throughout mammalian oogenesis. Biol Reprod 43:543-547
37. **Soyal SM, Amleh A, Dean J** 2000 FIGα, a germ cell-specific transcription factor required for ovarian follicle formation. Development 127:4645-4654
38. **Anderson E, Albertini DF** 1976 Gap junctions between the oocyte and companion follicle cells in the mammalian ovary. J Cell Biol 71:680-686
39. **Su YQ, Sugiura K, Eppig JJ** 2009 Mouse oocyte control of granulosa cell development and function: paracrine regulation of cumulus cell metabolism. Semin Reprod Med 27:32-42
40. **Biggers JD, Whittingham DG, Donahue RP** 1967 The pattern of energy metabolism in the mouse oocyte and zygote. Proc Natl Acad Sci U S A 58:560-567
41. **Leese HJ, Barton AM** 1985 Production of pyruvate by isolated mouse cumulus cells. J Exp Zool 234:231-236
42. **Sugiura K, Pendola FL, Eppig JJ** 2005 Oocyte control of metabolic cooperativity between oocytes and companion granulosa cells: energy metabolism. Dev Biol 279:20-30
43. **Colonna R, Mangia F** 1983 Mechanisms of amino acid uptake in cumulus-enclosed mouse oocytes. Biol Reprod 28:797-803
44. **Eppig JJ, Pendola FL, Wigglesworth K, Pendola JK** 2005 Mouse oocytes regulate metabolic cooperativity between granulosa cells and oocytes: amino acid transport. Biol Reprod 73:351-357
45. **Su YQ, Sugiura K, Wigglesworth K, O'Brien MJ, Affourtit JP, Pangas SA, Matzuk MM, Eppig JJ** 2008 Oocyte regulation of metabolic cooperativity between mouse cumulus cells and oocytes: BMP15 and GDF9 control cholesterol biosynthesis in cumulus cells. Development 135:111-121
46. **Yan C, Wang P, DeMayo J, DeMayo FJ, Elvin JA, Carino C, Prasad SV, Skinner SS, Dunbar BS, Dube JL, Celeste AJ, Matzuk MM** 2001 Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. Mol Endocrinol 15:854-866
47. **Su YQ, Wu X, O'Brien MJ, Pendola FL, Denegre JN, Matzuk MM, Eppig JJ** 2004 Synergistic roles of BMP15 and GDF9 in the development and function of the oocyte-cumulus cell complex in mice: genetic evidence for an oocyte-granulosa cell regulatory loop. Dev Biol 276:64-73
48. **Eppig JJ, Wigglesworth K, Pendola FL** 2002 The mammalian oocyte orchestrates the rate of ovarian follicular development. Proc Natl Acad Sci U S A 99:2890-2894
49. **Urist MR** 1965 Bone: formation by autoinduction. Science 150:893-899
50. **Hogan BL** 1996 Bone morphogenetic proteins: multifunctional regulators of vertebrate development. Genes Dev 10:1580-1594
51. **Wozney JM** 1992 The bone morphogenetic protein family and osteogenesis. Mol Reprod Dev 32:160-167

52. **Sampath TK, Reddi AH** 1981 Dissociative extraction and reconstitution of extracellular matrix components involved in local bone differentiation. Proc Natl Acad Sci U S A 78:7599-7603
53. **Wang EA, Rosen V, Cordes P, Hewick RM, Kriz MJ, Luxenberg DP, Sibley BS, Wozney JM** 1988 Purification and characterization of other distinct bone-inducing factors. Proc Natl Acad Sci U S A 85:9484-9488
54. **Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA** 1988 Novel regulators of bone formation: molecular clones and activities. Science 242:1528-1534
55. **Celeste AJ, Iannazzi JA, Taylor RC, Hewick RM, Rosen V, Wang EA, Wozney JM** 1990 Identification of transforming growth factor β family members present in bone-inductive protein purified from bovine bone. Proc Natl Acad Sci U S A 87:9843-9847
56. **Toriumi DM, Kotler HS, Luxenberg DP, Holtrop ME, Wang EA** 1991 Mandibular reconstruction with a recombinant bone-inducing factor. Functional, histologic, and biomechanical evaluation. Arch Otolaryngol Head Neck Surg 117:1101-1112
57. **Lee WS, Yoon SJ, Yoon TK, Cha KY, Lee SH, Shimasaki S, Lee S, Lee KA** 2004 Effects of bone morphogenetic protein-7 (BMP-7) on primordial follicular growth in the mouse ovary. Mol Reprod Dev 69:159-163
58. **Martinez-Barbera JP, Toresson H, Da Rocha S, Krauss S** 1997 Cloning and expression of three members of the zebrafish Bmp family: *Bmp2a*, *Bmp2b* and *Bmp4*. Gene 198:53-59
59. **Wilson PA, Lagna G, Suzuki A, Hemmati-Brivanlou A** 1997 Concentration-dependent patterning of the *Xenopus* ectoderm by BMP4 and its signal transducer Smad1. Development 124:3177-3184
60. **Dale L, Wardle FC** 1999 A gradient of BMP activity specifies dorsal-ventral fates in early *Xenopus* embryos. Semin Cell Dev Biol 10:319-326
61. **Chang H, Brown CW, Matzuk MM** 2002 Genetic analysis of the mammalian transforming growth factor-β superfamily. Endocr Rev 23:787-823
62. **Griffith DL, Keck PC, Sampath TK, Rueger DC, Carlson WD** 1996 Three-dimensional structure of recombinant human osteogenic protein 1: structural paradigm for the transforming growth factor β superfamily. Proc Natl Acad Sci U S A 93:878-883
63. **Vitt UA, Hsu SY, Hsueh AJ** 2001 Evolution and classification of cystine knot-containing hormones and related extracellular signaling molecules. Mol Endocrinol 15:681-694
64. **Israel DI, Nove J, Kerns KM, Moutsatsos IK, Kaufman RJ** 1992 Expression and characterization of bone morphogenetic protein-2 in Chinese hamster ovary cells. Growth Factors 7:139-150
65. **Jones WK, Richmond EA, White K, Sasak H, Kusmik W, Smart J, Oppermann H, Rueger DC, Tucker RF** 1994 Osteogenic protein-1 (OP-1) expression and processing in Chinese hamster ovary cells: isolation of a soluble complex containing the mature and pro-domains of OP-1. Growth Factors 11:215-225
66. **Israel DI, Nove J, Kerns KM, Kaufman RJ, Rosen V, Cox KA, Wozney JM** 1996 Heterodimeric bone morphogenetic proteins show enhanced activity *in vitro* and *in vivo*. Growth Factors 13:291-300

67. **McPherron AC, Lee SJ** 1993 GDF-3 and GDF-9: two new members of the transforming growth factor-β superfamily containing a novel pattern of cysteines. J Biol Chem 268:3444-3449
68. **Dube JL, Wang P, Elvin J, Lyons KM, Celeste AJ, Matzuk MM** 1998 The bone morphogenetic protein 15 gene is X-linked and expressed in oocytes. Mol Endocrinol 12:1809-1817
69. **Liao WX, Moore RK, Otsuka F, Shimasaki S** 2003 Effect of intracellular interactions on the processing and secretion of bone morphogenetic protein-15 (BMP-15) and growth and differentiation factor-9. Implication of the aberrant ovarian phenotype of BMP-15 mutant sheep. J Biol Chem 278:3713-3719
70. **Kessler E, Takahara K, Biniaminov L, Brusel M, Greenspan DS** 1996 Bone morphogenetic protein-1: the type I procollagen C-proteinase. Science 271:360-362
71. **Shimasaki S, Moore RK, Otsuka F, Erickson GF** 2004 The bone morphogenetic protein system in mammalian reproduction. Endocr Rev 25:72-101
72. **Chen D, Zhao M, Mundy GR** 2004 Bone morphogenetic proteins. Growth Factors 22:233-241
73. **Dale L, Jones CM** 1999 BMP signalling in early *Xenopus* development. Bioessays 21:751-760
74. **Yamaguchi A, Katagiri T, Ikeda T, Wozney JM, Rosen V, Wang EA, Kahn AJ, Suda T, Yoshiki S** 1991 Recombinant human bone morphogenetic protein-2 stimulates osteoblastic maturation and inhibits myogenic differentiation *in vitro*. J Cell Biol 113:681-687
75. **Gimble JM, Morgan C, Kelly K, Wu X, Dandapani V, Wang CS, Rosen V** 1995 Bone morphogenetic proteins inhibit adipocyte differentiation by bone marrow stromal cells. J Cell Biochem 58:393-402
76. **Katagiri T, Yamaguchi A, Komaki M, Abe E, Takahashi N, Ikeda T, Rosen V, Wozney JM, Fujisawa-Sehara A, Suda T** 1994 Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. J Cell Biol 127:1755-1766
77. **Robert B** 2007 Bone morphogenetic protein signaling in limb outgrowth and patterning. Dev Growth Differ 49:455-468
78. **King JA, Marker PC, Seung KJ, Kingsley DM** 1994 BMP5 and the molecular, skeletal, and soft-tissue alterations in *short ear* mice. Dev Biol 166:112-122
79. **Dudley AT, Lyons KM, Robertson EJ** 1995 A requirement for bone morphogenetic protein-7 during development of the mammalian kidney and eye. Genes Dev 9:2795-2807
80. **Vainio S, Karavanova I, Jowett A, Thesleff I** 1993 Identification of BMP-4 as a signal mediating secondary induction between epithelial and mesenchymal tissues during early tooth development. Cell 75:45-58
81. **Crotwell PL, Sommervold AR, Mabee PM** 2004 Expression of *bmp2a* and *bmp2b* in late-stage zebrafish median fin development. Gene Expr Patterns 5:291-296
82. **Smith A, Avaron F, Guay D, Padhi BK, Akimenko MA** 2006 Inhibition of BMP signaling during zebrafish fin regeneration disrupts fin growth and scleroblasts differentiation and function. Dev Biol 299:438-454

83. **Hammond KL, Loynes HE, Mowbray C, Runke G, Hammerschmidt M, Mullins MC, Hildreth V, Chaudhry B, Whitfield TT** 2009 A late role for *bmp2b* in the morphogenesis of semicircular canal ducts in the zebrafish inner ear. PLoS One 4:e4368
84. **Chocron S, Verhoeven MC, Rentzsch F, Hammerschmidt M, Bakkers J** 2007 Zebrafish Bmp4 regulates left-right asymmetry at two distinct developmental time points. Dev Biol 305:577-588
85. **Dick A, Hild M, Bauer H, Imai Y, Maifeld H, Schier AF, Talbot WS, Bouwmeester T, Hammerschmidt M** 2000 Essential role of Bmp7 (*snailhouse*) and its prodomain in dorsoventral patterning of the zebrafish embryo. Development 127:343-354
86. **Schmid B, Furthauer M, Connors SA, Trout J, Thisse B, Thisse C, Mullins MC** 2000 Equivalent genetic roles for *bmp7/snailhouse* and *bmp2b/swirl* in dorsoventral pattern formation. Development 127:957-967
87. **Shawi M, Serluca FC** 2008 Identification of a BMP7 homolog in zebrafish expressed in developing organ systems. Gene Expr Patterns 8:369-375
88. **Yamashita H, Ten Dijke P, Heldin CH, Miyazono K** 1996 Bone morphogenetic protein receptors. Bone 19:569-574
89. **Mathews LS, Vale WW** 1991 Expression cloning of an activin receptor, a predicted transmembrane serine kinase. Cell 65:973-982
90. **Attisano L, Wrana JL, Cheifetz S, Massague J** 1992 Novel activin receptors: distinct genes and alternative mRNA splicing generate a repertoire of serine/threonine kinase receptors. Cell 68:97-108
91. **Baarends WM, van Helmond MJ, Post M, van der Schoot PJ, Hoogerbrugge JW, de Winter JP, Uilenbroek JT, Karels B, Wilming LG, Meijers JH, et al.** 1994 A novel member of the transmembrane serine/threonine kinase receptor family is specifically expressed in the gonads and in mesenchymal cells adjacent to the mullerian duct. Development 120:189-197
92. **di Clemente N, Wilson C, Faure E, Boussin L, Carmillo P, Tizard R, Picard JY, Vigier B, Josso N, Cate R** 1994 Cloning, expression, and alternative splicing of the receptor for anti-Mullerian hormone. Mol Endocrinol 8:1006-1020
93. **Rosenzweig BL, Imamura T, Okadome T, Cox GN, Yamashita H, ten Dijke P, Heldin CH, Miyazono K** 1995 Cloning and characterization of a human type II receptor for bone morphogenetic proteins. Proc Natl Acad Sci U S A 92:7632-7636
94. **Lin HY, Wang XF, Ng-Eaton E, Weinberg RA, Lodish HF** 1992 Expression cloning of the TGF-β type II receptor, a functional transmembrane serine/threonine kinase. Cell 68:775-785
95. **ten Dijke P, Ichijo H, Franzen P, Schulz P, Saras J, Toyoshima H, Heldin CH, Miyazono K** 1993 Activin receptor-like kinases: a novel subclass of cell-surface receptors with predicted serine/threonine kinase activity. Oncogene 8:2879-2887
96. **Franzen P, ten Dijke P, Ichijo H, Yamashita H, Schulz P, Heldin CH, Miyazono K** 1993 Cloning of a TGFβ type I receptor that forms a heteromeric complex with the TGFβ type II receptor. Cell 75:681-692
97. **Tsuchida K, Mathews LS, Vale WW** 1993 Cloning and characterization of a transmembrane serine kinase that acts as an activin type I receptor. Proc Natl Acad Sci U S A 90:11242-11246

98. **ten Dijke P, Yamashita H, Ichijo H, Franzen P, Laiho M, Miyazono K, Heldin CH** 1994 Characterization of type I receptors for transforming growth factor-β and activin. Science 264:101-104
99. **Tsuchida K, Sawchenko PE, Nishikawa S, Vale WW** 1996 Molecular cloning of a novel type I receptor serine/threonine kinase for the TGF β superfamily from rat brain. Mol Cell Neurosci 7:467-478
100. **Mintzer KA, Lee MA, Runke G, Trout J, Whitman M, Mullins MC** 2001 *Lost-a-fin* encodes a type I BMP receptor, Alk8, acting maternally and zygotically in dorsoventral pattern formation. Development 128:859-869
101. **Liu F, Ventura F, Doody J, Massague J** 1995 Human type II receptor for bone morphogenic proteins (BMPs): extension of the two-kinase receptor model to the BMPs. Mol Cell Biol 15:3479-3486
102. **Nohno T, Ishikawa T, Saito T, Hosokawa K, Noji S, Wolsing DH, Rosenbaum JS** 1995 Identification of a human type II receptor for bone morphogenetic protein-4 that forms differential heteromeric complexes with bone morphogenetic protein type I receptors. J Biol Chem 270:22522-22526
103. **Ebisawa T, Tada K, Kitajima I, Tojo K, Sampath TK, Kawabata M, Miyazono K, Imamura T** 1999 Characterization of bone morphogenetic protein-6 signaling pathways in osteoblast differentiation. J Cell Sci 112 (Pt 20):3519-3527
104. **Moore RK, Otsuka F, Shimasaki S** 2003 Molecular basis of bone morphogenetic protein-15 signaling in granulosa cells. J Biol Chem 278:304-310
105. **Vitt UA, Mazerbourg S, Klein C, Hsueh AJ** 2002 Bone morphogenetic protein receptor type II is a receptor for growth differentiation factor-9. Biol Reprod 67:473-480
106. **Yamashita H, ten Dijke P, Huylebroeck D, Sampath TK, Andries M, Smith JC, Heldin CH, Miyazono K** 1995 Osteogenic protein-1 binds to activin type II receptors and induces certain activin-like effects. J Cell Biol 130:217-226
107. **Nishitoh H, Ichijo H, Kimura M, Matsumoto T, Makishima F, Yamaguchi A, Yamashita H, Enomoto S, Miyazono K** 1996 Identification of type I and type II serine/threonine kinase receptors for growth/differentiation factor-5. J Biol Chem 271:21345-21352
108. **ten Dijke P, Yamashita H, Sampath TK, Reddi AH, Estevez M, Riddle DL, Ichijo H, Heldin CH, Miyazono K** 1994 Identification of type I receptors for osteogenic protein-1 and bone morphogenetic protein-4. J Biol Chem 269:16985-16988
109. **Natsume T, Tomita S, Iemura S, Kinto N, Yamaguchi A, Ueno N** 1997 Interaction between soluble type I receptor for bone morphogenetic protein and bone morphogenetic protein-4. J Biol Chem 272:11535-11540
110. **Macias-Silva M, Hoodless PA, Tang SJ, Buchwald M, Wrana JL** 1998 Specific activation of Smad1 signaling pathways by the BMP7 type I receptor, ALK2. J Biol Chem 273:25628-25636
111. **Aoki H, Fujii M, Imamura T, Yagi K, Takehara K, Kato M, Miyazono K** 2001 Synergistic effects of different bone morphogenetic protein type I receptors on alkaline phosphatase induction. J Cell Sci 114:1483-1489
112. **Nohe A, Hassel S, Ehrlich M, Neubauer F, Sebald W, Henis YI, Knaus P** 2002 The mode of bone morphogenetic protein (BMP) receptor oligomerization determines different BMP-2 signaling pathways. J Biol Chem 277:5330-5338

113. **Graff JM, Thies RS, Song JJ, Celeste AJ, Melton DA** 1994 Studies with a *Xenopus* BMP receptor suggest that ventral mesoderm-inducing signals override dorsal signals *in vivo*. Cell 79:169-179

114. **Suzuki A, Thies RS, Yamaji N, Song JJ, Wozney JM, Murakami K, Ueno N** 1994 A truncated bone morphogenetic protein receptor affects dorsal-ventral patterning in the early *Xenopus* embryo. Proc Natl Acad Sci U S A 91:10255-10259

115. **Zou H, Niswander L** 1996 Requirement for BMP signaling in interdigital apoptosis and scale formation. Science 272:738-741

116. **Dewulf N, Verschueren K, Lonnoy O, Moren A, Grimsby S, Vande Spiegle K, Miyazono K, Huylebroeck D, Ten Dijke P** 1995 Distinct spatial and temporal expression patterns of two type I receptors for bone morphogenetic proteins during mouse embryogenesis. Endocrinology 136:2652-2663

117. **Ishidou Y, Kitajima I, Obama H, Maruyama I, Murata F, Imamura T, Yamada N, ten Dijke P, Miyazono K, Sakou T** 1995 Enhanced expression of type I receptors for bone morphogenetic proteins during bone formation. J Bone Miner Res 10:1651-1659

118. **Heldin CH, Miyazono K, ten Dijke P** 1997 TGF-β signalling from cell membrane to nucleus through SMAD proteins. Nature 390:465-471

119. **Raftery LA, Twombly V, Wharton K, Gelbart WM** 1995 Genetic screens to identify elements of the decapentaplegic signaling pathway in *Drosophila*. Genetics 139:241-254

120. **Sekelsky JJ, Newfeld SJ, Raftery LA, Chartoff EH, Gelbart WM** 1995 Genetic characterization and cloning of mothers against *dpp*, a gene required for decapentaplegic function in *Drosophila melanogaster*. Genetics 139:1347-1358

121. **Wiersdorff V, Lecuit T, Cohen SM, Mlodzik M** 1996 *Mad* acts downstream of Dpp receptors, revealing a differential requirement for *dpp* signaling in initiation and propagation of morphogenesis in the *Drosophila* eye. Development 122:2153-2162

122. **Savage C, Das P, Finelli AL, Townsend SR, Sun CY, Baird SE, Padgett RW** 1996 *Caenorhabditis elegans* genes *sma-2*, *sma-3*, and *sma-4* define a conserved family of transforming growth factor β pathway components. Proc Natl Acad Sci U S A 93:790-794

123. **Graff JM, Bansal A, Melton DA** 1996 *Xenopus* Mad proteins transduce distinct subsets of signals for the TGFβ superfamily. Cell 85:479-487

124. **Hahn SA, Schutte M, Hoque AT, Moskaluk CA, da Costa LT, Rozenblum E, Weinstein CL, Fischer A, Yeo CJ, Hruban RH, Kern SE** 1996 DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science 271:350-353

125. **Hoodless PA, Haerry T, Abdollah S, Stapleton M, O'Connor MB, Attisano L, Wrana JL** 1996 MADR1, a MAD-related protein that functions in BMP2 signaling pathways. Cell 85:489-500

126. **Liu F, Hata A, Baker JC, Doody J, Carcamo J, Harland RM, Massague J** 1996 A human Mad protein acting as a BMP-regulated transcriptional activator. Nature 381:620-623

127. **Wrana JL, Attisano L, Carcamo J, Zentella A, Doody J, Laiho M, Wang XF, Massague J** 1992 TGFβ signals through a heteromeric protein kinase receptor complex. Cell 71:1003-1014

128. **Yamamoto N, Akiyama S, Katagiri T, Namiki M, Kurokawa T, Suda T** 1997 Smad1 and Smad5 act downstream of intracellular signalings of BMP-2 that inhibits myogenic

differentiation and induces osteoblast differentiation in C2C12 myoblasts. Biochem Biophys Res Commun 238:574-580

129. **Chen Y, Bhushan A, Vale W** 1997 Smad8 mediates the signaling of the ALK-2 [corrected] receptor serine kinase. Proc Natl Acad Sci U S A 94:12938-12943
130. **Lagna G, Hata A, Hemmati-Brivanlou A, Massague J** 1996 Partnership between DPC4 and SMAD proteins in TGF-β signalling pathways. Nature 383:832-836
131. **Imamura T, Takase M, Nishihara A, Oeda E, Hanai J, Kawabata M, Miyazono K** 1997 Smad6 inhibits signalling by the TGF-β superfamily. Nature 389:622-626
132. **Hayashi H, Abdollah S, Qiu Y, Cai J, Xu YY, Grinnell BW, Richardson MA, Topper JN, Gimbrone MA, Jr., Wrana JL, Falb D** 1997 The MAD-related protein Smad7 associates with the TGFβ receptor and functions as an antagonist of TGFβ signaling. Cell 89:1165-1173
133. **Iemura S, Yamamoto TS, Takagi C, Uchiyama H, Natsume T, Shimasaki S, Sugino H, Ueno N** 1998 Direct binding of follistatin to a complex of bone-morphogenetic protein and its receptor inhibits ventral and epidermal cell fates in early *Xenopus* embryo. Proc Natl Acad Sci U S A 95:9337-9342
134. **Otsuka F, Moore RK, Iemura S, Ueno N, Shimasaki S** 2001 Follistatin inhibits the function of the oocyte-derived factor BMP-15. Biochem Biophys Res Commun 289:961-966
135. **Guo Q, Kumar TR, Woodruff T, Hadsell LA, DeMayo FJ, Matzuk MM** 1998 Overexpression of mouse follistatin causes reproductive defects in transgenic mice. Mol Endocrinol 12:96-106
136. **Abreu JG, Ketpura NI, Reversade B, De Robertis EM** 2002 Connective-tissue growth factor (CTGF) modulates cell signalling by BMP and TGF-β. Nat Cell Biol 4:599-604
137. **Zimmerman LB, De Jesus-Escobar JM, Harland RM** 1996 The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. Cell 86:599-606
138. **Merino R, Macias D, Ganan Y, Economides AN, Wang X, Wu Q, Stahl N, Sampath KT, Varona P, Hurle JM** 1999 Expression and function of *Gdf-5* during digit skeletogenesis in the embryonic chick leg bud. Dev Biol 206:33-45
139. **Groppe J, Greenwald J, Wiater E, Rodriguez-Leon J, Economides AN, Kwiatkowski W, Affolter M, Vale WW, Belmonte JC, Choe S** 2002 Structural basis of BMP signalling inhibition by the cystine knot protein Noggin. Nature 420:636-642
140. **Marcelino J, Sciortino CM, Romero MF, Ulatowski LM, Ballock RT, Economides AN, Eimon PM, Harland RM, Warman ML** 2001 Human disease-causing NOG missense mutations: effects on noggin secretion, dimer formation, and bone morphogenetic protein binding. Proc Natl Acad Sci U S A 98:11353-11358
141. **Sasai Y, Lu B, Steinbeisser H, Geissert D, Gont LK, De Robertis EM** 1994 Xenopus *chordin*: a novel dorsalizing factor activated by organizer-specific homeobox genes. Cell 79:779-790
142. **Piccolo S, Sasai Y, Lu B, De Robertis EM** 1996 Dorsoventral patterning in *Xenopus*: inhibition of ventral signals by direct binding of chordin to BMP-4. Cell 86:589-598
143. **Lewis KA, Gray PC, Blount AL, MacConell LA, Wiater E, Bilezikjian LM, Vale W** 2000 Betaglycan binds inhibin and can mediate functional antagonism of activin signalling. Nature 404:411-414
144. **Wiater E, Vale W** 2003 Inhibin is an antagonist of bone morphogenetic protein signaling. J Biol Chem 278:7934-7941

145. **Miyazono K, Kusanagi K, Inoue H** 2001 Divergence and convergence of TGF-β/BMP signaling. J Cell Physiol 187:265-276
146. **Onichtchouk D, Chen YG, Dosch R, Gawantka V, Delius H, Massague J, Niehrs C** 1999 Silencing of TGF-β signalling by the pseudoreceptor BAMBI. Nature 401:480-485
147. **Zhu H, Kavsak P, Abdollah S, Wrana JL, Thomsen GH** 1999 A SMAD ubiquitin ligase targets the BMP pathway and affects embryonic pattern formation. Nature 400:687-693
148. **Xu RH, Kim J, Taira M, Zhan S, Sredni D, Kung HF** 1995 A dominant negative bone morphogenetic protein 4 receptor causes neuralization in *Xenopus* ectoderm. Biochem Biophys Res Commun 212:212-219
149. **Shimasaki S, Zachow RJ, Li D, Kim H, Iemura S, Ueno N, Sampath K, Chang RJ, Erickson GF** 1999 A functional bone morphogenetic protein system in the ovary. Proc Natl Acad Sci U S A 96:7282-7287
150. **Nilsson EE, Skinner MK** 2003 Bone morphogenetic protein-4 acts as an ovarian follicle survival factor and promotes primordial follicle development. Biol Reprod 69:1265-1272
151. **Pierre A, Pisselet C, Dupont J, Bontoux M, Monget P** 2005 Bone morphogenetic protein 5 expression in the rat ovary: biological effects on granulosa cell proliferation and steroidogenesis. Biol Reprod 73:1102-1108
152. **Souza CJ, Campbell BK, McNeilly AS, Baird DT** 2002 Effect of bone morphogenetic protein 2 (BMP2) on oestradiol and inhibin A production by sheep granulosa cells, and localization of BMP receptors in the ovary by immunohistochemistry. Reproduction 123:363-369
153. **Otsuka F, Moore RK, Shimasaki S** 2001 Biological function and cellular mechanism of bone morphogenetic protein-6 in the ovary. J Biol Chem 276:32889-32895
154. **Otsuka F, Yamamoto S, Erickson GF, Shimasaki S** 2001 Bone morphogenetic protein-15 inhibits follicle-stimulating hormone (FSH) action by suppressing FSH receptor expression. J Biol Chem 276:11387-11392
155. **Davis GH, McEwan JC, Fennessy PF, Dodds KG, Farquhar PA** 1991 Evidence for the presence of a major gene influencing ovulation rate on the X chromosome of sheep. Biol Reprod 44:620-624
156. **Davis GH, McEwan JC, Fennessy PF, Dodds KG, McNatty KP, O WS** 1992 Infertility due to bilateral ovarian hypoplasia in sheep homozygous ($FecX^I$ $FecX^I$) for the Inverdale prolificacy gene located on the X chromosome. Biol Reprod 46:636-640
157. **Braw-Tal R, McNatty KP, Smith P, Heath DA, Hudson NL, Phillips DJ, McLeod BJ, Davis GH** 1993 Ovaries of ewes homozygous for the X-linked Inverdale gene ($FecX^I$) are devoid of secondary and tertiary follicles but contain many abnormal structures. Biol Reprod 49:895-907
158. **Elvin JA, Yan C, Wang P, Nishimori K, Matzuk MM** 1999 Molecular characterization of the follicle defects in the growth differentiation factor 9-deficient ovary. Mol Endocrinol 13:1018-1034
159. **Carabatsos MJ, Elvin J, Matzuk MM, Albertini DF** 1998 Characterization of oocyte and follicle development in growth differentiation factor-9-deficient mice. Dev Biol 204:373-384
160. **Mulsant P, Lecerf F, Fabre S, Schibler L, Monget P, Lanneluc I, Pisselet C, Riquet J, Monniaux D, Callebaut I, Cribiu E, Thimonier J, Teyssier J, Bodin L, Cognie Y, Chitour N, Elsen JM** 2001 Mutation in bone morphogenetic protein receptor-IB is

associated with increased ovulation rate in Booroola Merino ewes. Proc Natl Acad Sci U S A 98:5104-5109

161. **Yi SE, LaPolt PS, Yoon BS, Chen JY, Lu JK, Lyons KM** 2001 The type I BMP receptor *BmprIB* is essential for female reproductive function. Proc Natl Acad Sci U S A 98:7994-7999

162. **Bobe J, Nguyen T, Jalabert B** 2004 Targeted gene expression profiling in the rainbow trout (*Oncorhynchus mykiss*) ovary during maturational competence acquisition and oocyte maturation. Biol Reprod 71:73-82

163. **Baron D, Houlgatte R, Fostier A, Guiguen Y** 2005 Large-scale temporal gene expression profiling during gonadal differentiation and early gametogenesis in rainbow trout. Biol Reprod 73:959-966

164. **Lankford SE, Weber GM** 2010 Temporal mRNA expression of transforming growth factor-beta superfamily members and inhibitors in the developing rainbow trout ovary. Gen Comp Endocrinol 166:250-258

165. **Halm S, Ibanez AJ, Tyler CR, Prat F** 2008 Molecular characterisation of growth differentiation factor 9 (*gdf9*) and bone morphogenetic protein 15 (*bmp15*) and their patterns of gene expression during the ovarian reproductive cycle in the European sea bass. Mol Cell Endocrinol 291:95-103

166. **Clelland E, Kohli G, Campbell RK, Sharma S, Shimasaki S, Peng C** 2006 Bone morphogenetic protein-15 in the zebrafish ovary: complementary deoxyribonucleic acid cloning, genomic organization, tissue distribution, and role in oocyte maturation. Endocrinology 147:201-209

167. **Clelland ES, Tan Q, Balofsky A, Lacivita R, Peng C** 2007 Inhibition of premature oocyte maturation: a role for bone morphogenetic protein 15 in zebrafish ovarian follicles. Endocrinology 148:5451-5458

168. **Liu L, Ge W** 2007 Growth differentiation factor 9 and its spatiotemporal expression and regulation in the zebrafish ovary. Biol Reprod 76:294-302

169. **Monteiro R, van Dinther M, Bakkers J, Wilkinson R, Patient R, ten Dijke P, Mummery C** 2008 Two novel type II receptors mediate BMP signalling and are required to establish left-right asymmetry in zebrafish. Dev Biol 315:55-71

170. **Aaltonen J, Laitinen MP, Vuojolainen K, Jaatinen R, Horelli-Kuitunen N, Seppa L, Louhio H, Tuuri T, Sjoberg J, Butzow R, Hovata O, Dale L, Ritvos O** 1999 Human growth differentiation factor 9 (GDF-9) and its novel homolog GDF-9B are expressed in oocytes during early folliculogenesis. J Clin Endocrinol Metab 84:2744-2750

171. **Tse AC, Ge W** 2010 Spatial localization of EGF family ligands and receptors in the zebrafish ovarian follicle and their expression profiles during folliculogenesis. Gen Comp Endocrinol 167:397-407

172. **Kwok HF, So WK, Wang Y, Ge W** 2005 Zebrafish gonadotropins and their receptors: I. Cloning and characterization of zebrafish follicle-stimulating hormone and luteinizing hormone receptors--evidence for their distinct functions in follicle development. Biol Reprod 72:1370-1381

173. **Poon SK, So WK, Yu X, Liu L, Ge W** 2009 Characterization of inhibin α subunit (*inha*) in the zebrafish: evidence for a potential feedback loop between the pituitary and ovary. Reproduction 138:709-719

174. **Erickson GF, Shimasaki S** 2003 The spatiotemporal expression pattern of the bone morphogenetic protein family in rat ovary cell types during the estrous cycle. Reprod Biol Endocrinol 1:9

175. **Jaatinen R, Rosen V, Tuuri T, Ritvos O** 1996 Identification of ovarian granulosa cells as a novel site of expression for bone morphogenetic protein-3 (BMP-3/osteogenin) and regulation of BMP-3 messenger ribonucleic acids by chorionic gonadotropin in cultured human granulosa-luteal cells. J Clin Endocrinol Metab 81:3877-3882

176. **Wilson T, Wu XY, Juengel JL, Ross IK, Lumsden JM, Lord EA, Dodds KG, Walling GA, McEwan JC, O'Connell AR, McNatty KP, Montgomery GW** 2001 Highly prolific Booroola sheep have a mutation in the intracellular kinase domain of bone morphogenetic protein IB receptor (ALK-6) that is expressed in both oocytes and granulosa cells. Biol Reprod 64:1225-1235

177. **Ashkenazi H, Cao X, Motola S, Popliker M, Conti M, Tsafriri A** 2005 Epidermal growth factor family members: endogenous mediators of the ovulatory response. Endocrinology 146:77-84

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

179. **Wang C, Roy SK** 2009 Expression of bone morphogenetic protein receptor (BMPR) during perinatal ovary development and primordial follicle formation in the hamster: possible regulation by FSH. Endocrinology 150:1886-1896

180. **Wang EA, Rosen V, D'Alessandro JS, Bauduy M, Cordes P, Harada T, Israel DI, Hewick RM, Kerns KM, LaPan P, et al.** 1990 Recombinant human bone morphogenetic protein induces bone formation. Proc Natl Acad Sci U S A 87:2220-2224

181. **Vallejo LF, Brokelmann M, Marten S, Trappe S, Cabrera-Crespo J, Hoffmann A, Gross G, Weich HA, Rinas U** 2002 Renaturation and purification of bone morphogenetic protein-2 produced as inclusion bodies in high-cell-density cultures of recombinant *Escherichia coli*. J Biotechnol 94:185-194

182. **Long S, Truong L, Bennett K, Phillips A, Wong-Staal F, Ma H** 2006 Expression, purification, and renaturation of bone morphogenetic protein-2 from *Escherichia coli*. Protein Expr Purif 46:374-378

183. **Bessa PC, Pedro AJ, Klosch B, Nobre A, van Griensven M, Reis RL, Casal M** 2008 Osteoinduction in human fat-derived stem cells by recombinant human bone morphogenetic protein-2 produced in *Escherichia coli*. Biotechnol Lett 30:15-21

184. **Ihm HJ, Yang SJ, Huh JW, Choi SY, Cho SW** 2008 Soluble expression and purification of synthetic human bone morphogenetic protein-2 in *Escherichia coli*. BMB Rep 41:404-407

185. **Mace PD, Cutfield JF, Cutfield SM** 2007 Bacterial expression and purification of the ovine type II bone morphogenetic protein receptor ectodomain. Protein Expr Purif 52:40-49

186. **Hazama M, Aono A, Ueno N, Fujisawa Y** 1995 Efficient expression of a heterodimer of bone morphogenetic protein subunits using a baculovirus expression system. Biochem Biophys Res Commun 209:859-866

187. **Aono A, Hazama M, Notoya K, Taketomi S, Yamasaki H, Tsukuda R, Sasaki S, Fujisawa Y** 1995 Potent ectopic bone-inducing activity of bone morphogenetic protein-4/7 heterodimer. Biochem Biophys Res Commun 210:670-677

188. **Suzuki A, Nishimatsu S, Shoda A, Takebayashi K, Murakami K, Ueno N** 1993 Biochemical properties of amphibian bone morphogenetic protein-4 expressed in CHO cells. Biochem J 291 (Pt 2):413-417
189. **Bollati-Fogolin M, Forno G, Nimtz M, Conradt HS, Etcheverrigaray M, Kratje R** 2005 Temperature reduction in cultures of hGM-CSF-expressing CHO cells: effect on productivity and product quality. Biotechnol Prog 21:17-21
190. **Yoon SK, Song JY, Lee GM** 2003 Effect of low culture temperature on specific productivity, transcription level, and heterogeneity of erythropoietin in Chinese hamster ovary cells. Biotechnol Bioeng 82:289-298
191. **Reuveny S, Velez D, Macmillan JD, Miller L** 1986 Factors affecting cell growth and monoclonal antibody production in stirred reactors. J Immunol Methods 86:53-59
192. **Barnabe N, Butler M** 1994 Effect of temperature on nucleotide pools and monoclonal antibody production in a mouse hybridoma. Biotechnol Bioeng 44:1235-1245
193. **Weidemann R, Ludwig A, Kretzmer G** 1994 Low temperature cultivation--a step towards process optimisation. Cytotechnology 15:111-116
194. **Chuppa S, Tsai YS, Yoon S, Shackleford S, Rozales C, Bhat R, Tsay G, Matanguihan C, Konstantinov K, Naveh D** 1997 Fermentor temperature as a tool for control of high-density perfusion cultures of mammalian cells. Biotechnol Bioeng 55:328-338
195. **Ludwig A, Tomeczkowski J, Kretzmer G** 1992 Influence of the temperature on the shear stress sensitivity of adherent BHK 21 cells. Appl Microbiol Biotechnol 38:323-327
196. **Fox SR, Patel UA, Yap MG, Wang DI** 2004 Maximizing interferon-γ production by Chinese hamster ovary cells through temperature shift optimization: experimental and modeling. Biotechnol Bioeng 85:177-184
197. **So WK, Kwok HF, Ge W** 2005 Zebrafish gonadotropins and their receptors: II. Cloning and characterization of zebrafish follicle-stimulating hormone and luteinizing hormone subunits--their spatial-temporal expression patterns and receptor specificity. Biol Reprod 72:1382-1396
198. **Wang Y, Ge W** 2003 Spatial expression patterns of activin and its signaling system in the zebrafish ovarian follicle: evidence for paracrine action of activin on the oocytes. Biol Reprod 69:1998-2006
199. **Wang Y, Ge W** 2003 Gonadotropin regulation of follistatin expression in the cultured ovarian follicle cells of zebrafish, *Danio rerio*. Gen Comp Endocrinol 134:308-315
200. **Al-Musawi SL, Gladwell RT, Knight PG** 2007 Bone morphogenetic protein-6 enhances gonadotrophin-dependent progesterone and inhibin secretion and expression of mRNA transcripts encoding gonadotrophin receptors and inhibin/activin subunits in chicken granulosa cells. Reproduction 134:293-306
201. **Shi J, Yoshino O, Osuga Y, Koga K, Hirota Y, Hirata T, Yano T, Nishii O, Taketani Y** 2009 Bone morphogenetic protein-6 stimulates gene expression of follicle-stimulating hormone receptor, inhibin/activin β subunits, and anti-Mullerian hormone in human granulosa cells. Fertil Steril 92:1794-1798
202. **Glister C, Kemp CF, Knight PG** 2004 Bone morphogenetic protein (BMP) ligands and receptors in bovine ovarian follicle cells: actions of BMP-4, -6 and -7 on granulosa cells and differential modulation of Smad-1 phosphorylation by follistatin. Reproduction 127:239-254

203. **Lin SW, Ge W** 2009 Differential regulation of gonadotropins (FSH and LH) and growth hormone (GH) by neuroendocrine, endocrine, and paracrine factors in the zebrafish--an *in vitro* approach. Gen Comp Endocrinol 160:183-193
204. **Yam KM, Yu KL, Ge W** 1999 Cloning and characterization of goldfish activin β_A subunit. Mol Cell Endocrinol 154:45-54
205. **Yuen CW, Ge W** 2004 Follistatin suppresses FSHβ but increases LHβ expression in the goldfish - evidence for an activin-mediated autocrine/paracrine system in fish pituitary. Gen Comp Endocrinol 135:108-115
206. **Woodruff TK, Besecke LM, Groome N, Draper LB, Schwartz NB, Weiss J** 1996 Inhibin A and inhibin B are inversely correlated to follicle-stimulating hormone, yet are discordant during the follicular phase of the rat estrous cycle, and inhibin A is expressed in a sexually dimorphic manner. Endocrinology 137:5463-5467
207. **Welt CK, Martin KA, Taylor AE, Lambert-Messerlian GM, Crowley WF, Jr., Smith JA, Schoenfeld DA, Hall JE** 1997 Frequency modulation of follicle-stimulating hormone (FSH) during the luteal-follicular transition: evidence for FSH control of inhibin B in normal women. J Clin Endocrinol Metab 82:2645-2652
208. **Pang Y, Ge W** 2002 Gonadotropin and activin enhance maturational competence of oocytes in the zebrafish (*Danio rerio*). Biol Reprod 66:259-265
209. **Levavi-Sivan B, Bogerd J, Mananos EL, Gomez A, Lareyre JJ** 2010 Perspectives on fish gonadotropins and their receptors. Gen Comp Endocrinol 165:412-437
210. **Yan L, Swanson P, Dickhoff WW** 1992 A two-receptor model for salmon gonadotropins (GTH I and GTH II). Biol Reprod 47:418-427
211. **Laan M, Richmond H, He C, Campbell RK** 2002 Zebrafish as a model for vertebrate reproduction: characterization of the first functional zebrafish (*Danio rerio*) gonadotropin receptor. Gen Comp Endocrinol 125:349-364
212. **Oba Y, Hirai T, Yoshiura Y, Yoshikuni M, Kawauchi H, Nagahama Y** 1999 The duality of fish gonadotropin receptors: cloning and functional characterization of a second gonadotropin receptor cDNA expressed in the ovary and testis of amago salmon (*Oncorhynchus rhodurus*). Biochem Biophys Res Commun 265:366-371
213. **Bogerd J, Blomenrohr M, Andersson E, van der Putten HH, Tensen CP, Vischer HF, Granneman JC, Janssen-Dommerholt C, Goos HJ, Schulz RW** 2001 Discrepancy between molecular structure and ligand selectivity of a testicular follicle-stimulating hormone receptor of the African catfish (*Clarias gariepinus*). Biol Reprod 64:1633-1643
214. **Richards JS, Ireland JJ, Rao MC, Bernath GA, Midgley AR, Jr., Reichert LE, Jr.** 1976 Ovarian follicular development in the rat: hormone receptor regulation by estradiol, follicle stimulating hormone and luteinizing hormone. Endocrinology 99:1562-1570
215. **Rao MC, Richards JS, Midgley AR, Jr., Reichert LE, Jr.** 1977 Regulation of gonadotropin receptors by luteinizing hormone in granulosa cells. Endocrinology 101:512-523
216. **Jia XC, Hsueh AJ** 1984 Homologous regulation of hormone receptors: luteinizing hormone increases its own receptors in cultured rat granulosa cells. Endocrinology 115:2433-2439
217. **Luo W, Wiltbank MC** 2006 Distinct regulation by steroids of messenger RNAs for FSHR and CYP19A1 in bovine granulosa cells. Biol Reprod 75:217-225

218. **Minegishi T, Kishi H, Tano M, Kameda T, Hirakawa T, Miyamoto K** 1999 Control of FSH receptor mRNA expression in rat granulosa cells by 3',5'-cyclic adenosine monophosphate, activin, and follistatin. Mol Cell Endocrinol 149:71-77
219. **Hirakawa T, Minegishi T, Abe K, Kishi H, Ibuki Y, Miyamoto K** 1999 A role of insulin-like growth factor I in luteinizing hormone receptor expression in granulosa cells. Endocrinology 140:4965-4971
220. **Nakamura K, Nakamura M, Igarashi S, Miyamoto K, Eto Y, Ibuki Y, Minegishi T** 1994 Effect of activin on luteinizing hormone-human chorionic gonadotropin receptor messenger ribonucleic acid in granulosa cells. Endocrinology 134:2329-2335
221. **Kishi H, Minegishi T, Tano M, Abe Y, Ibuki Y, Miyamoto K** 1997 Down-regulation of LH/hCG receptor in rat cultured granulosa cells. FEBS Lett 402:198-202
222. **Xiao S, Robertson DM, Findlay JK** 1992 Effects of activin and follicle-stimulating hormone (FSH)-suppressing protein/follistatin on FSH receptors and differentiation of cultured rat granulosa cells. Endocrinology 131:1009-1016
223. **Weil C, Bougoussa-Houadec M, Gallais C, Itoh S, Sekine S, Valotaire Y** 1995 Preliminary evidence suggesting variations of GtH 1 and GtH2 mRNA levels at different stages of gonadal development in rainbow trout, *Oncorhynchus mykiss*. Gen Comp Endocrinol 100:327-333
224. **Prat F, Sumpter JP, Tyler CR** 1996 Validation of radioimmunoassays for two salmon gonadotropins (GTH I and GTH II) and their plasma concentrations throughout the reproductivecycle in male and female rainbow trout (*Oncorhynchus mykiss*). Biol Reprod 54:1375-1382
225. **Yoshiura Y, Kobayashi M, Kato Y, Aida K** 1997 Molecular cloning of the cDNAs encoding two gonadotropin β subunits (GTH-Iβ and -IIβ) from the goldfish, *Carassius auratus*. Gen Comp Endocrinol 105:379-389
226. **Shi J, Yoshino O, Osuga Y, Nishii O, Yano T, Taketani Y** 2008 Bone morphogenetic protein 7 (BMP-7) increases the expression of follicle-stimulating hormone (FSH) receptor in human granulosa cells. Fertil Steril 93:1273-1279
227. **Ge W** 2005 Intrafollicular paracrine communication in the zebrafish ovary: the state of the art of an emerging model for the study of vertebrate folliculogenesis. Mol Cell Endocrinol 237:1-10
228. **Wang Y, Ge W** 2004 Cloning of epidermal growth factor (EGF) and EGF receptor from the zebrafish ovary: evidence for EGF as a potential paracrine factor from the oocyte to regulate activin/follistatin system in the follicle cells. Biol Reprod 71:749-760
229. **Kohli G, Hu S, Clelland E, Di Muccio T, Rothenstein J, Peng C** 2003 Cloning of transforming growth factor-β1 (TGF-β1) and its type II receptor from zebrafish ovary and role of TGF-β1 in oocyte maturation. Endocrinology 144:1931-1941
230. **Cheng GF, Yuen CW, Ge W** 2007 Evidence for the existence of a local activin follistatin negative feedback loop in the goldfish pituitary and its regulation by activin and gonadal steroids. J Endocrinol 195:373-384
231. **Pang Y, Ge W** 1999 Activin stimulation of zebrafish oocyte maturation *in vitro* and its potential role in mediating gonadotropin-induced oocyte maturation. Biol Reprod 61:987-992
232. **Miyazono K, Maeda S, Imamura T** 2005 BMP receptor signaling: transcriptional targets, regulation of signals, and signaling cross-talk. Cytokine Growth Factor Rev 16:251-263