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# Differential regulation of gonadotropin receptors by bone morphogenetic proteins in the zebrafish ovary

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ABSTRACT

Follicle-stimulating hormone receptor (*fshr*) and luteinizing hormone/choriogonadotropin receptor (*lhcgr*) exhibit differential temporal expression patterns during zebrafish folliculogenesis with *fshr* being dominant during vitellogenic growth and *lhcgr* increasing its expression dramatically before maturation. The dynamic and distinct expression patterns of *fshr* and *lhcgr* suggest that they are under tight regulatory control. However, the underlying mechanisms for the differential expression of the two receptors remain unknown. We have recently demonstrated that members of bone morphogenetic protein (BMP) family are largely expressed in the oocyte, while their receptors are exclusively localized on the follicle cells, suggesting a potential paracrine signaling from the oocyte to the follicle cells by BMPs. In this study, we investigated the effects of zebrafish BMP2b (zfBmp2b) and BMP4 (zfBmp4) on the expression of *fshr* and *lhcgr* using a novel co-culture approach. The recombinant zfBmp2b or zfBmp4-producing CHO cells were co-cultured with the zebrafish follicle cells followed by real-time qPCR analysis of *fshr* and *lhcgr* expression. Our results showed that zfBmp2b and zfBmp4 both down-regulated *fshr*, while up-regulated *lhcgr* expression at 24 h of co-culturing. This finding, together with the high expression level of BMP receptors in the follicle cells prior to oocyte maturation, strongly suggests a potential role for BMPs in the differential expression of *fshr* and *lhcgr,* especially in the full-grown follicles before maturation. As BMPs are largely expressed in the oocyte, this also implies an important role for the oocyte in orchestrating the differentiation and function of the follicle cells.



## 1. Introduction

Follicle development is a dynamic process subjected to control by a regulatory network both outside and within the follicle [5,12]. Among the vast number of regulatory factors, the two pituitary gonadotropins, namely follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are indispensable in ovarian development. FSH is responsible for follicle growth, while LH is responsible for oocyte maturation and ovulation [14,36]. FSH and LH signal through their cognate receptors, namely follicle-stimulating hormone receptor (FSHR) and luteinizing hormone/choriogonadotropin receptor (LHCGR), in the ovary. Both FSHR and LHCGR are G-protein-coupled receptors with a large extracellular domain, seven transmembrane helices and a carboxy-terminal intracellular tail [24]. Similar to the situation in mammals, fish also have two gonadotropin receptors that are differentially expressed during folliculogenesis [2,11,16,19–21]. In the Atlantic halibut, *fshr* is highly expressed during primary growth and vitellogenesis, yet it decreases during final oocyte maturation. On the contrast, *lhcgr* mRNA is undetectable at early stages, yet it drastically increases at final oocyte maturation [20]. In the European sea bass, the expression of both gonadotropin receptors is low at primary growth stage. When the follicle enters the secondary growth phase, its expression of *fshr* increases and reaches the peak at early vitellogenic stage, but this is followed by a drop at mid-vitellogenic and late-vitellogenic stage. In contrast, *lhcgr* expression shows a steady increase in expression as the follicle develops, but surges in expression at the late-vitellogenic stage [11]. In the Japanese eel, *fshr* mRNA level is more abundant than that of *lhcgr* in follicles of early stages, indicating that *fshr* is the dominant receptor mediating actions of pituitary gonadotropins at early stages [16]. In the bamboo leaf wrasse, the mRNA level of *fshr* is high in early yolk-stage but decreases at the end of vitellogenesis. On the other hand, *lhcgr* expression is low during vitellogenesis, yet it increases at the end of vitellogenesis and early migratory nucleus stage, followed by a drop towards late mature stage [19]. In the Atlantic salmon, *fshr* expression is detectable in small and large vitellogenic follicles, whereas *lhcgr* expression can only be detected in large vitellogenic follicles [2].

In the zebrafish, the two gonadotropin receptors and their expression profiles during folliculogenesis have also been characterized [21,22]. During primary growth (PG) stage, the expression of both *fshr* and *lhcgr* is low. The expression of *fshr* increases significantly at the transition from PG to pre-vitellogenic (PV) stage and

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continues to rise until reaching the peak at mid-vitellogenic (MV) stage. Its expression decreases significantly at the late vitellogenic (LV) and full-grown (FG) stage prior to oocyte maturation and ovulation. Interestingly, the expression of *fshr* is not accompanied by *lhcgr*, whose expression lags behind that of *fshr*. The expression of *lhcgr* increases slowly at early stages of folliculogenesis; however it shows a significant increase at MV stage, followed by a surge at FG stage [21,45]. The differential expression of the gonadotropin receptors during folliculogenesis in the zebrafish and other teleosts provides strong support to the notion that FSH and LH from the pituitary play important roles in follicle growth and maturation, respectively [41]; however, the mechanisms that control the expression of *fshr* and *lhcgr* in the ovary of teleosts are largely unknown.

In mammals, the gonadotropin receptors are known to be controlled by various endocrine or paracrine factors including pituitary hormones, steroids and local growth factors [15,17,18,27–29,35,37,44]. In the zebrafish ovary, the expression levels of *fshr* and *lhcgr* in the follicle cells are clearly dependent on the stage of the oocyte, suggesting a potential role for the oocyte in regulating their expression and follicle cell function in general. Our earlier studies have identified that the bone morphogenetic protein (BMP) family members are largely expressed in the oocyte and their receptors are exclusively expressed in the follicle cells, indicating a potential BMP-mediated paracrine signaling from the oocyte to the follicle cells [25]. What is particularly of interest is that the expression of BMP receptors reaches the highest level at the FG stage [25], which happens to be the period during which the differential expression of the two gonadotropin receptors occurs with *fshr* decreasing its expression whereas *lhcgr* increasing the expression. This led us to hypothesize that the increased BMP signaling at the FG stage could be a potential mechanism for the differential regulation of the gonadotropin receptors. In mammals, there have been reports that the oocyte-derived factors such as growth differentiation factor 9 (GDF9), BMP15 and other forms of BMPs could target the granulosa cells to regulate the expression of gonadotropin receptors [8,30,31,42].

In this study, we investigated the effects of zfBmp2b and zfBmp4 on the expression of *fshr* and *lhcgr* in cultured zebrafish follicle cells using a novel co-culture approach. Two recombinant CHO cell lines stably expressing zfBmp2b and zfBmp4 were established as described in our previous report [25]. These BMP-producing CHO cells were co-incubated with the zebrafish follicle cells at 28 °C, which happens to be the optimal temperature for both follicle cell growth and production of recombinant proteins by the CHO cells [40]. The zfBMPs produced by the CHO cells were expected to act directly on the neighboring follicle cells in culture, which would mimic the paracrine signaling of BMPs from the oocyte to the follicle cells in the follicle.

## 2. Materials and methods

### 2.1. Chemicals

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

### 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.

### 2.3. 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 medium (Gibco Invitrogen, Carlsbad, CA) several times in a 15-ml Falcon tube (BD, Franklin Lakes, NJ) and filtered through a sieve to remove large FG follicles. The filtered follicles were then washed several times with medium M199 (Gibco Invitrogen) and plated into 10-cm culture dishes. The follicles were grown for 6 days at 28 °C in M199 with 10% FBS (Hyclone, Logan, UT) with the medium changed once on day 3. After the 6-day incubation, the follicle cells were suspended by trypsinization, washed, and added to the 12-well plates already plated with the recombinant CHO cells one day earlier (see details below).

### 2.4. Culture of CHO cells

The CHO cell lines expressing recombinant zfBmp2b and zfBmp4 were established as described in the previous report [25]. A control cell line carrying the empty vector (pcDNA5/FRT) was also established. The CHO cell lines were maintained in F-12 medium (Gibco Invitrogen) supplied with 6 g/L HEPES (USB, Cleveland, OH), 10% FBS (Hyclone) and 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% confluence.

### 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 at 200,000 cells per well in 0.5 ml F-12 medium with 10% FBS. The CHO cells were allowed to attach in 24 h at 37 °C. On the next day, the follicle cells from the incubated follicles were added to each well at 200,000 cells per well in 0.5 ml F-12 medium with 10% FBS (incubation medium was changed from M199 to F-12 during sub-culture at the last step 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 per well (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 proteins by the CHO cells. After 24 h of co-incubation, the follicle cells became attached to the well surface. The cells were washed once with 1 ml of F-12 medium and the medium was changed to serum-free F-12 for another 24 h incubation. At the end of incubation, total RNA was extracted with Tri-Reagent (Molecular Research Center, Cincinnati, OH) and reverse transcribed. For the control wells, the control CHO cells carrying the expression vector only were seeded at 200,000 cells per well, followed by adding 200,000 follicle cells. In the dose response experiments, the lower dose group included 100,000 zfBMP-producing CHO cells plus 100,000 control CHO cells, followed by adding 200,000 follicle cells, resulting in the total of 400,000 cells per well. The higher dose group included 200,000 zfBMP-producing CHO cells and 200,000 follicle cells, reaching the fixed total of 400,000 cells.

### 2.6. RNA isolation and reverse transcription

Total RNA was isolated from the co-cultured cells using Tri-Reagent according to manufacturer's instruction (Molecular Research Center). Total RNA from the co-cultured cells was used for reverse