論文の内容の要旨

論文題目　The Role of Bone Morphogenetic Proteins (BMPs) in the Human Ovary

和訳　ヒト卵巣における Bone Morphogenetic Protein (BMP) サイトカインの機能

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As the number of infertility patients is markedly increasing, studies on ovarian physiology, which elucidate the fundamental mechanisms involved in the dysfunction and disease of the reproductive system, will lead to success in infertility treatment.

The physiological roles of the ovary are integrated in the continuous repetitive process of folliculogenesis, ovulation and corpus luteum formation (Figure 1). Folliculogenesis describes the progression of a number of small primordial follicles into large preovulatory follicles. The major steps in folliculogenesis include the primordial/primary transition, the primary/secondary transition, selection and follicle dominance. The ovarian follicle consists of an oocyte, granulosa cells (GCs) and theca

cells. Primordial and primary follicles differ in their diameters, due to differences in the number and size of GCs. When follicles transform to the secondary stage, theca cell layers appear and GCs develop to multiple layers. With the formation of antrum, follicles are defined as tertiary follicles. In the late term of this stage, some healthy follicles are recruited and constitute the population of selectable follicles. Among these, only one follicle becomes dominant and others undergo atresia. Eventually, this dominant follicle grows rapidly to become a preovulatory follicle. During ovulation, this preovulatory follicle develops a stigma and excretes an oocyte with a complement of cumulus cells. The ruptured follicle transforms into the corpus luteum in the process called luteinization. The corpus luteum produces progesterone, making the endometrium receptive to implantation and supportive of the early pregnancy.

Gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are of primary importance in the regulation of folliculogenesis and female fertility. However, FSH receptor (FSHR) and LH receptor (LHR) are not expressed until the secondary follicle stage, which illustrates that the initiation of follicular growth is independent of gonadotropins. Intra-ovarian factors are thought to be important for regulating the early phases of follicular growth. Among these, activins and anti-Müllerian hormone (AMH) are highlighted. Activins induce the proliferation and

differentiation of GCs, whereas AMH inhibits the initiation of primordial follicle growth. However, the precise mechanism of their regulation remains obscure. LH/hCG has a central role in the maintenance of the corpus luteum function. It has been reported that the expression level of LHR of the corpus luteum is 700% compared to that of the preovulatory follicle. On the other hand, progesterone is secreted by the corpus luteum to maintain pregnancy. The process of progesterone synthesis is reported to be primarily mediated by the action of steroidogenic acute regulatory protein (StAR). To date, the precise regulatory mechanism of luteinization still remains uncertain.

Evidence strongly indicates that BMPs play an important role in regulating ovarian follicular development and female fertility in the primate. BMPs, originally isolated from bone tissues as proteins that induce bone and cartilage formation, are structurally classified into the transforming growth factor (TGF)-β superfamily member. To date, more than 20 members of the BMP family have been identified in various species. They can be further classified into several subgroups (Table 1), including BMP-2/4 group, BMP-3 group, BMP-7 group, growth and differentiation factor (GDF)-5/6/7 group, GDF-8/11 group and GDF-9 group. Previous studies have revealed two major types of membrane-bound receptors for the ligands of the TGF-β family, type-I and type-II receptors. BMP ligands bind to type-I receptors first and then recruit type-II receptors or

directly bind to the pre-existing complexes of type-I and type-II receptors, then transphosphorylate the intracellular signaling proteins. Activin receptor–like kinase (ALK)-2, -3 and -6 have been identified as BMP type-I receptors. BMPR-II appears to bind exclusively to BMP ligands as type-II receptor. Moreover, it is reported that BMP and activin membrane-bound inhibitor (BAMBI), structurally related to type-I receptor, competes with the BMP receptors for ligand binding and inhibits the signaling of BMPs.

Among the members of the BMP family, BMP-2, BMP-6, BMP-7 and BMP-15 show specific effects in the ovary. BMP-2 amplifies FSH action on sheep GCs. BMP-6 promotes normal fertility in female mice. BMP-7 increases numbers of primary, secondary, and tertiary follicles in rats. Mutation of BMP-15 in sheep causes infertility due to a block in the early stage of folliculogenesis. However, most studies on BMPs in the ovary have been conducted on non-human animals and there is little data on the human. Whereas BMP-7 and BMP-15 are reported to be expressed in theca cells and oocytes of human ovarian follicles, respectively, localization of BMP-2 and BMP-6 remains to be investigated.

Thus, this study focused on the function of several BMPs in the human ovary, by investigating their effects on folliculogenesis and luteinization, to expand our

understanding of human ovarian physiology and pathology.

The localization of BMP-2 and BMP-6 was examined using normal human ovaries. Tissue specimens of human ovaries were obtained under signed informed consent from fifteen women (age range, 28–40 years old) who underwent salpingo-oophorectomy for the treatment of uterine cervical cancer. All patients had normal ovarian cycles prior to surgery and no histological abnormalities and malignant lesions were observed in ovarian tissues. The experimental procedure was approved by the institutional review board of University of Tokyo. Ovarian tissues were fixed in neutral-buffered formalin and embedded in paraffin blocks, and 6-μm sections were prepared. Sections were stained with 2 μg/ml anti-BMP-6 antibodies or mouse IgGs as negative control using an Envision+ System/HRP Mouse (DAB+) kit. In situ hybridization was performed using an ISHR Starting kit according to the manufacturer's instructions. To prepare the digoxigenin (DIG)-labeled RNA anti-sense probes for BMP-2, a primer set (NM_001200: 1022-1041 and 1298-1279) was used. Sense probe hybridization was used as a control for a background level.

The study of the function of BMPs was performed using human GCs. The methods to obtain and culture GCs were described previously. Briefly, follicular fluids with GCs were aspirated from the patients undergoing oocyte retrieval for in vitro fertilization

(IVF). The clinical indications for IVF in these patients were primarily male factor or tubal factor infertility. Patients with ovarian dysfunctions were excluded from the study. The experimental procedures were approved by the institutional review board (Case No. 325), and signed informed consent for use of GCs was obtained from each patient. All of the follicular aspirates from each patient were mixed and centrifuged at 200 x g for 5 minutes, resuspended in PBS with 0.2% hyaluronidase and incubated at 37º C for 30 minutes. The suspension was layered onto Ficoll-Paque and centrifuged at 150 x g for 20 minutes. GCs were collected from the interphase, washed with PBS and cultured in DMEM/F12 media supplemented with 5% FBS and antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin, and 250 ng/ml amphotericin B) for 15 minutes at 37º C, in order to remove contaminating macrophage cells from GCs. Using this method, GCs remained in the supernatant while macrophages were attached to the culture dish. The contamination of monocytes/macrophages and endothelial cells was less than 1% judged by immunohistochemistry for CD68 and von Willebrand factor, respectively.

Collected GCs were cultured in DMEM/F12 containing 5% FBS and antibiotics in 12-well plates at a density of 2 x 10^5 cells/ml and kept at 37º C in a humidified 5% CO_2/95% air environment for 5 days. All GCs used for the experiments were pre-cultured for 5 days prior to treatments to allow GCs to regain sensitivity to FSH

stimulation. In the dose-response study, GCs were cultured with increasing concentrations of BMP-2, BMP-6, BMP-7 or BMP-15 (0–300 ng/ml) for 24 hours. Since the BMPs at 100 ng/ml maximally induced FSHR mRNA expression (data not shown), thus, 100 ng/ml of BMPs was utilized in the subsequent experiments. In the time course experiments, GCs were incubated with or without BMP-2, BMP-6, BMP-7 or BMP-15 (100 ng/ml) for 3, 8, 24 and 48 hours. To evaluate the effects of BMPs, GCs were cultured with or without BMP-2, BMP-6, BMP-7 or BMP-15 (100 ng/ml) for 24 hours. To investigate which receptor was used for induction of FSHR by BMP-7, SB-431542 was used 30 minutes before the stimulation by BMP-7 or activin-A. To investigate the regulation of BMP-6, GCs were cultured with BMP-2 (100 ng/ml), BMP-6 (100 ng/ml), BMP-7 (100 ng/ml), BMP-15 (100 ng/ml), estradiol (E2) (10 ng/ml), activin-A (100 ng/ml), FSH (0.5 IU/ml), or 8-bromo-cyclic adenosine 3′:5′ monophosphate (cAMP) (1 mM) for 24 hours. To investigate the effect of hCG, GCs were cultured with hCG (10 IU/ml) for 3, 8, 24 and 48 hours. Total RNA was extracted from GCs using the RNAeasy minikit. Reverse transcription (RT) was performed using Rever Tra Dash. One microgram of total RNA was reverse transcribed in a 20-μL volume. For the quantification of various mRNA levels, real-time polymerase chain reaction (PCR) was performed using LightCycler according to the manufacturer's

instructions. To assess the levels of cAMP synthesis, GCs were cultured in 48-well plates with DMEM/F12 containing 1% FBS and antibiotics with or without BMP-7 (100 ng/ml) for 24 hours. Then, cells were cultured with 0.1 mmol/L IBMX, a phosphodiesterase inhibitor, in the presence or absence of FSH (0.5 IU/ml) for 2 hours. Conditioned medium was collected and the extracellular content of cAMP was determined using a cAMP enzyme immunoassay kit. Data were analyzed by Student's t-test for paired comparison and one-way ANOVA with post hoc test for multiple comparisons using Statview software. A p-value of less than 0.05 was considered statistically significant.

In the present study, BMP-6 was localized in the oocyte of human ovary, and human GCs expressed BMP-2 and BMP-6. Furthermore, gene expression of the receptors for BMPs such as ALK-3, ALK-6 and BMPR-II was confirmed in human GCs by PCR (Table 2). The BMPs (BMP-2, BMP-6, BMP-7 and BMP-15) significantly increased FSHR, inhibin/activin β-subunits, and AMH mRNA levels. On the other hand, the BMPs (BMP-2, BMP-6, BMP-7 and BMP-15) inhibited mRNA levels of LHR and StAR. In addition, hCG decreased gene expression of BMP-2 and increased that of BAMBI (Table 3).

Folliculogenesis involves a series of sequential steps in which a growing follicle either develops to the preovulatory stage or dies by apoptosis. The major steps in folliculogenesis include the primordial/primary transition, the primary/secondary transition, selection and follicle dominance. The emerging concept is that folliculogenesis is accompanied by a precise spatial and temporal pattern of expression of the BMP family.

Primordial/primary transition

In primordial and primary follicles, which do not express FSHR, the mechanism of follicular growth is poorly understood, but there is evidence that BMPs are involved; BMP-15 is expressed in human oocytes from the very early stage and BMP-6 is strongly expressed in the oocytes of primordial and primary follicles. These patterns of expression suggest that BMPs might be essential for the initiation of follicular growth in the human.

In the early stage of folliculogenesis, inhibin α-subunit is barely detected and activins are preferentially synthesized rather than inhibins. Activins are known to induce the proliferation of GCs. Therefore, the present findings that the BMPs increased mRNA levels of inhibin/activin β-subunits suggest that oocyte-derived BMP-6 and

BMP-15 might induce activins, regulating folliculogenesis in the primordial and primary stages.

Primary/secondary transition

Progressively, follicles grow to the secondary stage, theca cell layers appear, and follicles are served by one or two arterioles, terminating in an anastomotic network just outside the basal lamina. The physiological importance of this event is emphasized by the fact that the follicle becomes directly exposed to the factors circulating in the blood, such as FSH. As the secondary follicle is being formed, GCs develop FSHR, by which all effects of FSH can be mediated. Therefore, the mechanism responsible for the regulation of FSHR is important. Several factors, such as activins, FSH, cAMP stimulants and cAMP analogs, are known to modulate the synthesis of FSHR mRNA in GCs. The present study showed that the BMPs (BMP-2, BMP-6, BMP-7 and BMP-15) induced FSHR mRNA expression in human GCs, which suggests that BMPs enhance folliculogenesis by promoting the expression of FSHR. The observation that the BMPs increased mRNA levels of not only FSHR but also inhibin/activin β-subunits led me to examine the possibility that the increase in FSHR mRNA might be mediated by an increase in activin protein synthesis. However, SB-431542, an inhibitor of activins but not BMP-7 signaling, failed to suppress BMP-7–induced FSHR mRNA expression.

Thus, it is speculated that BMPs and activin-A act on different receptors to increase FSHR mRNA expression.

What's more, in view of the present finding that BMP-2, BMP-7, BMP-15 and activin-A increased mRNA levels of BMP-6, these factors might induce FSHR partially by up-regulation of BMP-6 in GCs.

Selection and follicle dominance

Not all follicles are guaranteed to ovulate because growing follicles are vulnerable to atresia. Healthy follicles will be selected from growing follicles to be a preovulatory follicle. The immunohistochemistry study of BMP-6 uncovered the significance of BMP in follicular selection. In the human ovary, BMP-6 was found to be strongly expressed in GCs of healthy tertiary follicles. In contrast, BMP-6 was only weakly expressed in GCs of atretic follicles. These data indicated that BMP-6 might be an important factor in the selection of follicle. The in vitro experiment showed that BMP-6 increased mRNA levels of inhibin/activin β-subunits in GCs. In addition, the observed expression pattern of BMP-6 in GCs is consistent with the finding that inhibin/activin β-subunits are expressed in healthy follicles, but not in the similarly sized atretic follicles.

Serum FSH concentration decreases in the latter half of the follicular phase. Therefore, the sensitivity of follicles to FSH during this period is critical and determines whether follicles become atretic or dominant. In view of the present finding that BMPs increased the expression of FSHR, follicles with high BMP expression might be more likely to survive the decrease in serum FSH, thus increasing the chances of surviving to the dominant follicle stage.

Another intra-ovarian factor, AMH is produced by GCs of growing follicles. Female mice lacking AMH show accelerated depletion of follicles, suggesting that AMH inhibits recruitment of primordial follicles into the growing pool. Although it is reported that FSH and estradiol down-regulate AMH expression in GCs, no AMH up-regulators have been identified to date. The present study showed that the BMPs (BMP-2, BMP-6, BMP-7 and BMP-15) increased the expression level of AMH. Thus, I hypothesize that BMPs in the healthy growing follicles up-regulate AMH, which, in turn, suppresses the growth of the surrounding primordial follicles, thereby preserving the ovarian reserve.

LHR is a key factor for GCs in the tertiary follicle to undergo luteinization. During folliculogenesis, it is important to prevent GCs from precocious luteinization which might lead to premature ovulation. The present data showed that the BMPs (BMP-2,

BMP-6, BMP-7 and BMP-15) suppressed LHR mRNA expression in human GCs, suggesting a possible role of BMPs in the inhibition of premature luteinization and eventual promotion of follicle development to the stage of ovulation. Pangas et al. reported that in Smad4 conditional knockout mouse, GCs underwent premature luteinization and expressed higher levels of LHR and lower levels of FSHR compared with control. Smad4 is a common Smad for TGF-β superfamily signaling. Given that BMPs also use the Smad signaling pathway, the present data appear to be consistent with the phenotype of Smad4 knockout mouse.

Luteinization

After ovulation, residual GCs undergo luteinization and the follicle transforms to the corpus luteum. Progesterone, produced by luteinized GCs, sustains the initiation and maintenance of pregnancy. If embryo implantation does not occur, the corpus luteum stops synthesizing progesterone and degenerates. LH/hCG has a central role in the maintenance of the corpus luteum function. StAR, which mediates translocation of cholesterol from the outer to the inner mitochondrial membrane, is one of the rate-limiting factors in progesterone production. Any perturbation of these events, such as luteinization failure, can impair reproduction. The finding that the BMPs (BMP-2, BMP-6, BMP-7 and BMP-15) suppressed gene expression of LHR and StAR indicates

that BMPs might play a role as anti-luteinization factors in the ovary. Because BMP-6 and BMP-15 are derived from oocyte, it is reasonable to conclude that the release of the oocyte at ovulation results in the promotion of luteinization in GCs. The finding that hCG suppressed BMP-2 mRNA levels in cultured GCs suggests that the expression of BMP-2 might be attenuated in response to LH surge in vivo. In situ hybridization in the present study further confirmed this hypothesis. BMP-2 mRNA was expressed in GCs of tertiary follicles whereas its expression was almost undetectable in the corpus luteum. The vanishment of BMP-2 expression in the corpus luteum would facilitate luteinization. In this context, it should also be noted that hCG induced the expression of BAMBI in GCs. BAMBI inhibits dimerization of type-I receptors, thereby inhibiting BMP signaling. It is speculated that LH surge not only inhibits BMP-2 expression but increases BAMBI expression in GCs to extinguish the effects of BMPs that impede the establishment of the corpus luteum.

Taken together, BMPs play crucial roles in controlling folliculogenesis and luteinization in the human ovary. During gonadotropin-independent phase, BMPs are expressed in the oocytes of the early follicular stage and contribute to the initiation of follicular growth by inducing activin expression in GCs. With the growth of follicle, the suppression of LHR by BMPs prevents GCs from precocious luteinization, which is

essential for follicle maturation. In turn, BMPs increase AMH expression to suppress the growth of the surrounding primordial follicles, thereby preserving the ovarian reserve. Because BMPs induce FSHR mRNA expression in GCs, sufficient FSHR allows follicles to fully respond to serum FSH to support follicular growth. With continuous stimulation of FSH, LHR is dramatically induced through FSHR signaling to respond to LH surge, and eventually induces ovulation. After ovulation, LH/hCG suppresses BMPs directly or indirectly to maintain the corpus luteum function. Here we can see human reproduction is controlled by the harmonious cooperation of BMPs and gonadotropins. BMPs regulate gonadotropins in the early stage of folliculogenesis, and then gonadotropins become predominant to promote follicular growth. Ultimately, BMPs are inhibited by gonadotropin to maintain the corpus luteum function (Figure 2).

The present study provides a comprehensive insight into the biological activities of the BMPs (BMP-2, BMP-6, BMP-7 and BMP-15) in the human ovary. The physical ovarian function is dependent on the action of BMP family within the follicle, including: 1) initiation of follicular growth from the very early stage, 2) modulation of folliculogenesis, and 3) inhibition of luteinization. Better understanding of BMP system may expand our concept of the diverse mechanisms of ovarian physiology, providing the potential for a novel approach to the infertility treatment.

Figure 1

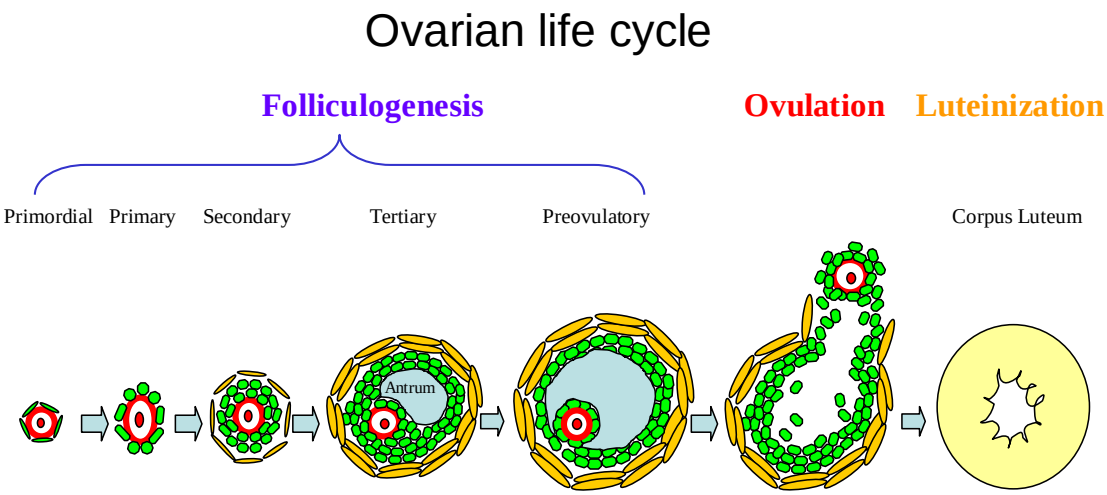

Figure 1

The physiological roles of the ovary are integrated in the continuous repetitive process of folliculogenesis, ovulation and corpus luteum formation.

Folliculogenesis describes the progression of a number of small primordial follicles into large preovulatory follicles.

The ovarian follicle consists of an oocyte, granulosa cells (GCs), and theca cells (TCs).

Folliculognesis	**Primordial**	**oocyte; one layer of flat GCs**
	Primary	**oocyte; one layer of cuboidal GCs**
	Secondary	**oocyte; multiple layers of GCs; appearance of theca cell layer**
	Tertiary	**oocyte; GCs; TCs; formation of antrum**
	Preovulatory	**only one tertiary follicle can become dominant and grow rapidly to the preovulatory stage**
Ovulation	**preovulatory follicle excretes the oocyte with a complement of cumulus cells**	
Luteinization	**ruptured follicle transforms into the corpus leteum, which produces progesterone to support the early pregnancy**	

Figure 2

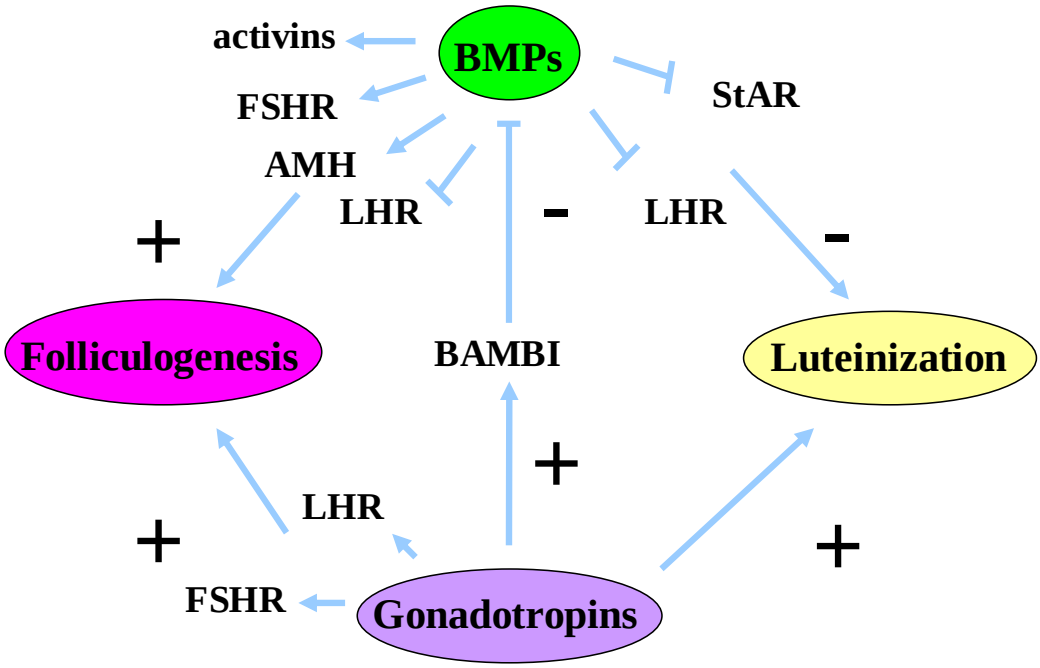

Figure 2

BMPs and gonadotropins cooperate with mutual communication to regulate the human ovarian function.

BMPs regulate gonadotropins in the early stage of folliculogenesis, and then gonadotropins become predominant to promote follicular growth. Ultimately, BMPs are inhibited by gonadotropin to maintain the corpus luteum function.

Table 1

List of Bone Morphogenetic Proteins (BMPs)

Group	BMP	Known Functions
BMP-2/4	BMP-2	Amplifies FSH action on sheep GCs
	BMP-4	Mutation of BMP-4 in mice results in defects in mesoderm formation
BMP-3	BMP-3	Controls bone mass
BMP-7	BMP-5	Performs function in cartilage development
	BMP-6	Promotes normal fertility in female mice
	BMP-7	Increases numbers of primary, secondary, and tertiary follicles in rats
GDF-5/6/7	GDF-5	Induces cartilage and tendon-like tissues
	GDF-6	Induces cartilage and tendon-like tissues
	GDF-7	Induces cartilage and tendon-like tissues
GDF-8/11	GDF-8	Controls the growth of muscle tissue
	GDF-11	Controls anterior-posterior patterning
GDF-9	GDF-9	Deficiency of GDF-9 in mice causes infertility
	BMP-15	Mutation of BMP-15 in sheep causes infertility

Table 2

Expression of BMPs and their receptors in the human ovary

Oocyte	Granulosa cell
BMP-6	BMP-2 BMP-6 ALK-3 ALK-6 BMPR-II

Table 3

Biological activities of BMPs regulating the human ovarian function

	FSHR	inhibin/activin β A-subunit	inhibin/activin β B-subunit	AMH	LHR	StAR
BMP-2	↑	↑	↑	↑	↓	↓
BMP-6	↑	↑	↑	↑	↓	↓
BMP-7	↑	↑	↑	↑	↓	↓
BMP-15	↑	↑	↑	↑	↓	↓

	BMP-2	BAMBI
hCG	↓	↑