Regulatory role of BMP-9 in steroidogenesis by rat ovarian granulosa cells.

Takeshi Hosoya[1], Fumio Otsuka[2], Eri Nakamura[2], Tomohiro Terasaka[1], Kenichi Inagaki[1], Naoko Tsukamoto-Yamauchi[1], Takayuki Hara[1], Kishio Toma[1], Motoshi Komatsubara[1] and Hirofumi Makino[1]

[1]Department of Medicine and Clinical Science and [2]Department of General Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kitaku, Okayama 700-8558, Japan.

Running Title: BMP-9 inhibits ovarian steroidogenesis.

Key words: Bone morphogenetic protein (BMP), Follicle-stimulating hormone (FSH), Granulosa cells, Oocyte and Steroidogenesis.

Corresponding Author: Fumio Otsuka, M.D., Ph.D.
Department of General Medicine, Okayama University of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kitaku, Okayama City, 700-8558, Japan.
Phone: +81-86-235-7342, Fax: +81-86-235-7345
E-mail: fumiotsu@md.okayama-u.ac.jp

Abbreviations:

ALK, activin receptor-like kinase
ActRI, activin type-I receptor
ActRII, activin type-II receptor
BMP, bone morphogenetic protein
BMPRI, BMP type-I receptor
BMPRII, BMP type-II receptor
ECD, extracellular domain
FSH, follicle-stimulating hormone
FSHR, FSH receptor
GDF, growth differentiation factor
3βHSD, 3β-hydroxysteroid dehydrogenase
IBMX, 3-isobutyl-1-methylxanthine
MAPK, mitogen-activated protein kinase
P450scc, P450 steroid side-chain cleavage enzyme
StAR, steroidogenic acute regulatory protein
TGF-β, transforming growth factor

Abstract

BMPs expressed in the ovary differentially regulate steroidogenesis by granulosa cells. BMP-9, a circulating BMP, is associated with cell proliferation, apoptosis and differentiation in various tissues. However, the effects of BMP-9 on ovarian function have yet to be elucidated. Here we investigated BMP-9 actions on steroidogenesis using rat primary granulosa cells. BMP-9 potently suppressed FSH-induced progesterone production, whereas it did not affect FSH-induced estradiol production by granulosa cells. The effects of BMP-9 on FSH-induced steroidogenesis were not influenced by the presence of oocytes. FSH-induced cAMP synthesis and FSH-induced mRNA expression of steroidogenic factors, including StAR, P450scc, 3βHSD2 and FSHR, were suppressed by treatment with BMP-9. BMP-9 mRNA expression was detected in granulosa cells but not in oocytes. BMP-9 readily activated Smad1/5/8 phosphorylation and Id-1 transcription in granulosa cells. Analysis using ALK inhibitors indicated that BMP-9 actions were mediated via type-I receptors other

than ALK-2, -3 and -6. Furthermore, experiments using extracellular domains (ECDs) for BMP type-I and -II receptor constructs revealed that the effects of BMP-9 were reversed by ECDs for ALK-1 and BMPRII. Thus, the functional receptors for BMP-9 in granulosa cells were most likely to be the complex of ALK-1 and BMPRII. Collectively, the results indicate that BMP-9 contributes to the regulation of luteinization as a circulating as well as autocrine/paracrine factor in granulosa cells during folliculogenesis.

Introduction

Bone morphogenetic proteins (BMPs), which belong to the transforming growth factor (TGF)-β superfamily, were originally identified as inducers for ectopic bone growth and cartilage formation. There has been substantial progress in elucidation of the multiple roles of BMPs ranging from roles in bone to other systemic actions [1]. BMPs regulate cell growth, differentiation, and apoptosis of various cell types, and they are crucial for tissue morphogenesis and differentiation.

Ovarian follicle growth and maturation occur as a result of elaborate interactions between pituitary gonadotropins and autocrine/paracrine growth factors produced within the ovary. Recent studies have established the concept that members of the TGF-β superfamily, including BMPs, growth differentiation factors (GDFs), activins and inhibins, play key roles as autocrine/paracrine factors in female fertility in mammals [2-4]. BMP ligands and receptors, including BMP-2, -3, -3b, -4, -6, -7 and -15, GDF-9, and BMP

type-I and type-II receptors, are expressed in cell-specific patterns in ovarian cells that undergo dynamic changes during follicular development and corpora luteal formation [3].

Regulation of FSH responsiveness in granulosa cells is critical for the steps of follicular selection, dominant follicle formation and subsequent ovulation in mammals. This process elicited by FSH receptor (FSHR) signaling is further modulated by many autocrine/paracrine factors [2]. BMPs play a key role in female fertility by regulating steroidogenesis and mitosis in granulosa cells. In particular, BMP ligands commonly suppress FSH-induced progesterone synthesis [3-6]. For instance, oocyte-derived BMP-15 attenuates FSH actions by suppressing FSHR expression in granulosa cells. Oocyte- and granulosa-derived BMP-6 reduces FSH-induced progesterone level by suppressing adenylate cyclase activity. BMP-2 from granulosa cells and BMP-4 and -7 from theca cells also suppress FSH-induced progesterone production. The finding that FSH-induced progesterone synthesis is inhibited by ovarian BMPs suggests a key role of BMPs as luteinization inhibitors.

BMP-9, also called GDF-2, is mainly expressed in the liver and shows hematopoietic, hepatogenic and osteo-chondrogenic activities [7]. BMP-9 also acts as a metabolic regulator for glucose, lipid and iron as well as a neuronal differentiation factor [8, 9]. BMP-9 exists in human serum and plasma, in which the circulating concentration was found to be higher at the order of "ng/ml" compared with concentrations of other BMPs [10]. Recently, it was reported that BMP-9 is also associated with cancer cell proliferation, apoptosis and differentiation in the ovary, prostate, breast and thyroid tissues [11, 12]. These findings indicate the possible involvement of BMP-9 in regulation of reproductive functions. However, the effects of BMP-9 on ovarian steroidogenesis and folliculogenesis have yet to be elucidated.

In the present study, we investigated regulatory mechanism of BMP-9 on steroidogenesis by rat primary granulosa cells. It was found that BMP-9 acts as an endocrine regulator of progesterone production as a new luteinizing inhibitor in the process of ovarian folliculogenesis.

Materials and Methods

Reagents and supplies

Female Sprague-Dawley (SD) rats were purchased from Charles-River (Wilmington, MA). Medium 199, McCoy's 5A medium and HEPES buffer were purchased from Invitrogen Corp. (Carlsbad, CA). Diethylstilbestrol (DES), ovine pituitary FSH, 4-androstene-3,17-dione, 3-isobutyl-1-methylxanthine (IBMX), bovine serum albumin, and penicillin-streptomycin solution were from Sigma-Aldrich Co. Ltd. (St. Louis, MO). Recombinant human BMP-4, -9, noggin and extracellular domains (ECDs) that lack trans-membrane and intra-cellular domains of human ALK-1, -2, -3, -6, ActRIIA, ActRIIB and BMPRII [13, 14] were purchased from R&D Systems, Inc. (Minneapolis, MN).

Primary culture of granulosa cells and oocytes

Silastic capsules containing 10 mg of DES were implanted in 22-day-old female SD rats to increase the number of granulosa cells. After 4

days of DES exposure, ovarian follicles were punctured with a double-thickness needle of 27 gauge, and the isolated mixture of granulosa cells and oocytes was cultured in serum-free McCoy's 5A medium supplemented with penicillin-streptomycin at 37°C in an atmosphere of 5% CO_2. Granulosa cell and oocyte numbers were counted in an oocyte/granulosa cell suspension that was filtered by cell strainers (100-μm nylon mesh; BD Falcon, Bedford, MA) to eliminate cell aggregation. For indicated experiments, granulosa cells were separated from oocytes by filtering the oocyte/granulosa cell suspension through an additional 40-μm nylon mesh (BD Falcon) that allowed granulosa cells but not oocytes to pass through as previously reported [15]. The animal protocols were approved by Okayama University Institutional Animal Care and Use Committee.

Measurements of estradiol, progesterone and cAMP

Rat granulosa cells (1×10^5 viable cells in 200 μl) with or without oocytes (100 oocytes/ml) were cultured in 96-well plates with serum-free

McCoy's 5A medium containing 100 nM of androstenedione, a substrate for aromatase. Cells were cultured either alone or in combination with indicated concentrations of FSH and BMP-9 in the presence of ALK inhibitors and BMP-receptor ECDs for 48 h. The levels of estradiol and progesterone in the media were determined by a chemiluminescent immunoassay using Architect estradiol and progesterone kits (Abbott Co., Ltd., Tokyo, Japan). Steroid contents were undetectable (progesterone < 0.1 ng/ml and estradiol < 8 pg/ml) in cell-free medium. To assess cellular cAMP synthesis, rat granulosa cells (1 $\times 10^5$ viable cells in 200 μl) without oocytes were cultured in 96-well plates with serum-free McCoy's 5A medium containing 0.1 mM of IBMX (a phosphodiesterase inhibitor) for 48 h. The extracellular contents of cAMP were determined by an enzyme immunoassay (Assay Designs, Ann Arbor, MI) after acetylation of each sample with assay sensitivity of 0.039 nM.

Cellular RNA extraction, RT and quantitative real-time PCR

Isolated granulosa cells and oocytes were cultured in 12-well plates in

serum-free conditions. Rat granulosa cells (5×10^5 viable cells in 1 ml) without oocytes were cultured either alone or in combination with indicated concentrations of FSH and BMP-9 in serum-free McCoy's 5A medium. After 48-h culture, the medium was removed and total cellular RNA was extracted using TRIzol® (Invitrogen Corp.) and quantified by measuring the absorbance of the sample at 260 nm. Primer pairs for steroidogenic acute regulatory protein (StAR), P450 steroid side-chain cleavage enzyme (P450scc), 3β-hydroxysteroid dehydrogenase type 2 (3βHSD2), FSHR, BMPRII and ribosomal protein L19 (RPL19) were selected as we reported previously [16]. PCR primer pairs for other target genes were selected from different exons of the corresponding genes to discriminate PCR products that might arise from possible chromosome DNA contaminants as follows: BMP-9, 310-330 and 581-602 (from GenBank accession #NM_001106096), and ALK-1, 1499-1519 and 1949-1972 (NM_022441). The extracted RNA (1 µg) was subjected to an RT reaction using a First-Strand cDNA Synthesis System (Invitrogen Corp.) with random hexamer (2 ng/µl), reverse transcriptase (200 U) and deoxynucleotide

triphosphate (dNTP; 0.5 mM) at 42°C for 50 min and at 70°C for 10 min. For the quantification of mRNA levels of each target gene, real-time PCR was performed using the LightCycler® Nano real-time PCR system (Roche Diagnostic Co., Tokyo, Japan) under optimized annealing conditions at 60-62°C. The relative expression of each mRNA was calculated by the Δ threshold cycle (Ct) method, in which ΔCt is the value obtained by subtracting the Ct value of RPL19 mRNA from the Ct value of the target mRNA, and the amount of target mRNA relative to RPL19 mRNA was expressed as $2^{-(\Delta Ct)}$. The data are expressed as the ratio of target mRNA to RPL19 mRNA.

Western immunoblot analysis

Rat granulosa cells (5×10^5 viable cells in 1 ml) without oocytes were cultured in 12-well plates in serum-free McCoy's 5A medium. After preculture with noggin (30 ng/ml) for 24 h to exclude endogenous effects of BMPs, cells were treated with BMP-4 and BMP-9 for 60 min. Cells were solubilized by a sonicator in 100 µl RIPA lysis buffer (Upstate Biotechnology, Lake Placid, NY)

containing 1 mM Na_3VO_4, 1 mM NaF, 2% SDS, and 4% β-mercaptoethanol. The cell lysates were then subjected to SDS-PAGE/immunoblotting analysis using anti-phospho-Smad1/5/8 (pSmad1/5/8) antibody (Cell Signaling Technology, Inc., Beverly, MA) and anti-actin antibody (Sigma-Aldrich Co. Ltd.). The integrated signal density of each protein band was analyzed by the C-DiGit® Blot Scanner System (LI-COR Biosciences, NE). For evaluating the target protein levels, ratios of the signal intensities of pSmad/actin were calculated.

Statistical analysis

All results are shown as means ± SEM of data from at least three separate experiments, each performed with triplicate samples. Differences between groups were analyzed for statistical significance using ANOVA with Tukey-Kramer's post hoc test or unpaired *t*-test, when appropriate, to determine differences (StatView 5.0 software, Abacus Concepts, Inc., Berkeley, CA). *P* values < 0.05 were accepted as statistically significant.

Results

We first investigated the effects of BMP-9 on steroidogenesis by rat primary granulosa cells. Treatment with BMP-9 (10 to 100 ng/ml) had no significant effect on basal estradiol or progesterone level in granulosa cells. As shown in **Fig. 1A**, FSH (30 ng/ml)-induced estradiol production was not significantly altered by treatment with BMP-9 (3 to 300 ng/ml). However, BMP-9 potently suppressed FSH-induced progesterone production in a concentration-dependent manner (3 to 300 ng/ml) (**Fig. 1B**). The effects of BMP-9 on FSH-induced steroidogenesis were not influenced by the presence of oocytes (**Fig. 1**).

To determine the mechanism by which BMP-9 suppresses FSH-induced progesterone production in granulosa cells, the effects of BMP-9 on cAMP synthesis were examined. As shown in **Fig. 2**, FSH (30 ng/ml)-induced cAMP synthesis was significantly inhibited by treatment with BMP-9 (3 to 300 ng/ml). In addition, the effects of BMP-9 on FSH-induced

cAMP production were not changed by the actions of the oocytes.

Changes in expression levels of steroidogenic factors and enzymes mRNA were also examined to elucidate the mechanism by which BMP-9 suppresses FSH-induced progesterone production in granulosa cells. As shown in **Fig. 3**, FSH (30 ng/ml) potently stimulated mRNA expression involved in ovarian steroidogenesis in graunulosa cells. Of note, the induced mRNA expression of StAR, P450scc, 3βHSD2 and FSHR was significantly suppressed by treatment with BMP-9 (100 ng/ml).

To examine the expression of endogenous BMP-9 in granulosa cells and/or oocytes, RT-PCR was performed using mRNAs extracted from granulosa cells and oocytes. As shown in **Fig. 4A**, BMP-9 expression was detected in granulosa cells but not in oocytes. We previously reported the expression of BMP type-I receptors including ALK-2, -3 and -6, type-II receptors including ActRII and BMPRII, and Smads1, 4, 6, 7 and 8 in our granulosa cell culture [6]. Crystal structure analysis of BMP-9 has suggested that BMP-9 can bind to ALK-1 and BMPRII [17], although the functional receptors for BMP-9

have not been well characterized. As shown in **Fig. 4A**, RT-PCR analysis also revealed the expression of both ALK-1 and BMPRII in granulosa cells but not in oocytes. It was also found that BMP-9 (100 ng/ml) readily activated Smad1/5/8 phosphorylation as shown in cells stimulated by the same concentration of BMP-4 by rat granulosa cells (**Fig. 4B**). This experiment was performed following preculture with noggin (30 ng/ml) to eliminate the effects of endogenous BMPs other than BMP-9 [10]. Treatment with BMP-9 (100 ng/ml) also induced mRNA expression of Id-1, a target gene of BMP signaling (**Fig. 4C**).

To determine the signal-transducing receptors for BMP-9 in granulosa cells, we examined the changes in BMP-9 effects on progesterone production in the presence of ALK inhibitors. Dorsomorphin, which was originally isolated as an inhibitor of AMP-activated protein kinase (AMPK), selectively inhibits BMP signaling transmitted by ALK-2, -3 and -6 [18]. LDN-193189 also inhibits ALK-2 and -3 actions with higher activity and specificity [19]. Both compounds thereby specifically prevent Smad1/5/8 phosphorylation mediated via BMP

type-I receptors. As shown in **Fig. 4D**, neither dorsomorphin (100 to 1000 nM) nor LDN-193189 (100 to 1000 nM) affected the inhibitory effects of BMP-9 (100 ng/ml) on FSH (30 ng/ml)-induced progesterone production, indicating that BMP-9 acts on granulosa cells through BMP receptors other than ALK-2, -3 and -6.

To determine the functional receptors for BMP-9, we further examined the changes in BMP-9 activity by using the ECDs of BMP/activin type-I and -II receptors, which inhibit ligands from binding to target receptors (**Fig. 5**). The changes in FSH-induced progesterone production inhibited by BMP-9 (10 ng/ml) were examined in the presence of ECD proteins (0.3 μg/ml) of type-I and -II receptors. As shown in **Fig. 5A**, the effect of BMP-9 (10 ng/ml) on FSH-induced progesterone synthesis was not altered by treatments with ALK-2, -3 and -6-ECDs or ActRIIA- and ActRIIB-ECDs (0.3 μg/ml), suggesting that BMP-9 has insignificant affinity to these receptor subtypes. However, as shown in **Fig. 5B**, BMP-9-induced reduction of FSH-induced progesterone level was significantly reversed by BMPRII-ECD (0.3 μg/ml) and further restored by

ALK-1-ECD (0.3 µg/ml) or by the pair of BMPRII/ALK-1-ECDs. The results suggested that the functional receptors for BMP-9 in granulosa cells are ALK-1 and BMPRII, with the role of ALK-1 likely being predominant. Thus, BMP-9 suppressed FSH-induced progesterone production as a new luteinizing inhibitor in the process of ovarian folliculogenesis through a receptor complex of ALK-1/BMPRII and subsequent Smad1/5/8 activation (**Fig. 6**).

Discussion

In the present study, we uncovered the expression and functional roles of the BMP-9 system in rat primary granulosa cells. It was revealed that BMP-9 suppressed FSH-induced progesterone, but not estradiol, production. FSH-induced cAMP synthesis and mRNA levels of StAR, P450scc, 3βHSD and FSHR were downregulated by BMP-9. The results suggested that BMP-9 functionally contributes to progesterone reduction as a new luteinizing inhibitor in the process of ovarian folliculogenesis (**Fig. 6**).

BMP-9 is predominantly expressed in non-parenchymal cells in the liver such as endothelial, stellate and Kupffer cells [7] and in the septum and spinal cord of embryos [20]. In addition, BMP-9 is associated with cell proliferation, apoptosis and differentiation in the ovary, prostate, breast and thyroid [11, 12]. Regarding BMP-9 actions in the ovary, Herrera *et al.* showed that BMP-9 functions as a proliferative factor for immortalized human ovarian surface epithelial and epithelial ovarian cancer cells [11]. Different from results of previous studies showing that BMP-9 inhibits vascular endothelial cell

proliferation signaling through ALK-1 [21, 22], Herrera et al. showed that BMP-9 promoted ovarian epithelial cell proliferation through an ALK-2-to-Smad1/4 pathway [11]. In their study, dorsomorphin abrogated BMP-9-induced proliferation in immortalized ovarian cells [11], suggesting the involvement of non-ALK-1 pathways in BMP-9 actions in ovarian cancer. In contrast, both dorsomorphin and LDN-193189, inhibitors of ALK-2, -3 and -6 pathways, failed to inhibit the effects of BMP-9 on FSH-induced progesterone synthesis in our study, implying that BMP-9 signaling is likely to be mediated via ALK-1 on normal granulosa cells.

In the physiological aspect of ovarian folliculogenesis and steroidogenesis, follicle-derived growth factors play key roles in the communication network between oocytes and somatic follicular cells, which is important for the maturation of oocytes and growth and differentiation of surrounding granulosa and theca cells [23, 24]. In the present study, BMP-9 expression was clearly detected in granulosa cells but not in oocytes, and the suppressive effect of BMP-9 on FSH-induced progesterone production were not

influenced by the presence of oocytes.

Of note, BMP-9 is known to be a circulating factor in serum and plasma at a concentration of ~5 ng/ml as a biologically active form [10, 25]. In endothelial cells, BMP-9 has been recognized as a major endothelial-stimulating factor via Smad1/5/8 activation. Because of the inhibitory effect of BMP-9 on angiogenesis, BMP-9 has also been proposed to be a circulating vascular quiescence factor [10]. Considering our results in granulosa cells, it is possible that BMP-9 also plays a quiescent role in ovarian steroidogenesis by specifically regulating FSH-induced progesterone production.

In the present study, BMP-9 activated Smad1/5/8 phosphorylation and Id-1 transcription in granulosa cells as do other BMP ligands. The functional receptors for BMP-9 in granulosa cells were revealed to be ALK-1 and BMPRII, based on results using specific ECDs and ALK inhibitors. It has been reported that BMP-9 binds to ALK-1 and ActRIIB with a high affinity, while it also binds to ALK-2 and other type-II receptors, BMPRII and ActRIIA, with lower affinities [26].

In the cellular situation with a low expression level of ALK-1, the signaling could be initiated via complex formation with some lower-affinity type-I receptors such as ALK-2 [11, 21].

In the clinical aspect, ALK-1 is implicated in the pathogenesis of hereditary hemorrhagic telangiectasia type 2, also known as Rendu-Osler disease type 2 [27], being characterized by recurrent nosebleeds, cutaneous telangiectases and arteriovenous malformations. ALK-1 inactivation results in embryonic lethality in mice, supporting critical roles of BMP-9 and BMP-10 in major angiogenesis [28]. On the other hand, BMP-9 null mice were born and developed normally into fertile adults [29], possibly due to the replaced functions by BMP-10 during embryogenesis. However, given that BMP-9 knockout mice showed decreased lymphatic draining efficiency [30], BMP-9 might play a role in development of the lymphatic vascular network surrounding ovarian follicles.

BMP-9 binding to its receptors elicits phosphorylation of Smad1/5/8 in various cells, regardless of the receptors bound to BMP-9 [31, 32]. In addition,

BMP-9 may induce Smad2/3 phosphorylation [21, 33] and activate non-Smad pathways including mitogen-activated protein kinases (MAPKs), PI3K/AKT, NF-κB, Wnt, Rho-GTPase and modulation of microRNAs in some cells [34]. We have focused on oocyte-granulosa cell communication through BMP signaling and MAPKs in growing follicles [5, 6]. FSH-induced ERK1/2 signaling is suppressed by BMP-7, followed by the enhancement of FSH-induced estradiol production [6]. The FSH-induced p38 pathway is enhanced by BMP-2 and -4, leading to efficient estradiol synthesis by granulosa cells [5]. Oocytes modulate these interactions between the BMP system and MAPK signaling in granulosa cells, which may be crucial for the optimal regulation of FSH-induced steroidogenesis in developing follicles. Although the BMP-9-induced non-Smad pathway has yet to be elucidated, BMP-9 may also be involved in non-Smad activity as a circulating factor for oocyte-granulosa cell communication.

Collectively, the results indicate that BMP-9 functionally contributes to progesterone reduction in the process of ovarian folliculogenesis. This is the

first report regarding the effects of BMP-9 on ovarian steroidogenesis. Not only the autocrine/paracrine roles of BMPs expressed in the ovary but also circulating BMP-9 may be involved in fine-tuning of ovarian steroidogenesis. Hence, BMP-9 functionally contributes to progesterone reduction as a luteinizing inhibitor in accordance with other BMP effects in the ovary. Future *in vivo* study is needed to clarify the endocrine role of BMP-9 in ovarian physiology.

Acknowledgements

This work was supported in part by Grants-in-Aid for Scientific Research (No. 24591364), Foundation for Growth Science and Astellas Foundation for Research on Metabolic Disorders (Japan).

Disclosure Statement:
FO receives speaker honoraria from Novo Nordisk. HM is a consultant for AbbVie, Astellas and Teijin, receives speaker honoraria from Astellas, Boehringer-ingelheim, Chugai, Daiichi Sankyo, Dainippon Sumitomo, Kyowa

Hakko Kirin, MSD, Novartis, Pfizer, Takeda, and Tanabe Mitsubishi, and receives grant support from Astellas, Boehringer-ingelheim, Daiichi Sankyo, Dainippon Sumitomo, Kyowa Hakko Kirin, Mochida, MSD, Novartis, Novo Nordisk, Pfizer, Takeda, and Tanabe Mitsubishi.

References

[1] Wagner DO, Sieber C, Bhushan R, Borgermann JH, Graf D, Knaus P: BMPs: from bone to body morphogenetic proteins. Science signaling 3 (2010): mr1.

[2] Findlay JK, Drummond AE, Dyson ML, Baillie AJ, Robertson DM, Ethier JF: Recruitment and development of the follicle: the roles of the transforming growth factor-beta superfamily. Mol Cell Endocrinol 191 (2002): 35-43.

[3] Shimasaki S, Moore RK, Otsuka F, Erickson GF: The bone morphogenetic protein system in mammalian reproduction. Endocr Rev 25 (2004): 72-101.

[4] Otsuka F, McTavish KJ, Shimasaki S: Integral role of GDF-9 and BMP-15 in ovarian function. Mol Reprod Dev 78 (2011): 9-21.

[5] Inagaki K, Otsuka F, Miyoshi T, Yamashita M, Takahashi M, Goto J, Suzuki J, Makino H: p38-Mitogen-activated protein kinase stimulated steroidogenesis in granulosa cell-oocyte cocultures: role of bone morphogenetic proteins 2 and 4. Endocrinology 150 (2009): 1921-1930.

[6] Miyoshi T, Otsuka F, Inagaki K, Otani H, Takeda M, Suzuki J, Goto J, Ogura T, Makino H: Differential regulation of steroidogenesis by bone morphogenetic proteins in granulosa cells: involvement of extracellularly regulated kinase signaling and oocyte actions in follicle-stimulating hormone-induced estrogen production. Endocrinology 148 (2007): 337-345.

[7] Miller AF, Harvey SA, Thies RS, Olson MS: Bone morphogenetic protein-9. An autocrine/paracrine cytokine in the liver. J Biol Chem 275 (2000): 17937-17945.

[8] Lamplot JD, Qin J, Nan G, Wang J, Liu X, Yin L, Tomal J, Li R, Shui W, Zhang H et al: BMP9 signaling in stem cell differentiation and osteogenesis. American journal of stem cells 2 (2013): 1-21.

[9] Herrera B, Dooley S, Breitkopf-Heinlein K: Potential roles of bone morphogenetic protein (BMP)-9 in human liver diseases. International journal of molecular sciences 15 (2014): 5199-5220.

[10] David L, Mallet C, Keramidas M, Lamande N, Gasc JM, Dupuis-Girod S, Plauchu H, Feige JJ, Bailly S: Bone morphogenetic protein-9 is a circulating vascular quiescence factor. Circulation research 102 (2008): 914-922.

[11] Herrera B, van Dinther M, Ten Dijke P, Inman GJ: Autocrine bone morphogenetic protein-9 signals through activin receptor-like kinase-2/Smad1/Smad4 to promote ovarian cancer cell proliferation. Cancer Res 69 (2009): 9254-9262.

[12] Na KY, Kim HS, Lee SK, Jung WW, Sung JY, Kim YW, Park YK: Papillary thyroid carcinoma with bone formation. Pathology, research and practice 209 (2013): 14-18.

[13] Moore RK, Otsuka F, Shimasaki S: Molecular basis of bone morphogenetic protein-15 signaling in granulosa cells. J Biol Chem 278 (2003): 304-310.

[14] Matsumoto Y, Otsuka F, Hino J, Miyoshi T, Takano M, Miyazato M, Makino H, Kangawa K: Bone morphogenetic protein-3b (BMP-3b) inhibits osteoblast differentiation via Smad2/3 pathway by counteracting Smad1/5/8 signaling. Mol Cell Endocrinol 350 (2012): 78-86.

[15] Otsuka F, Shimasaki S: A negative feedback system between oocyte bone morphogenetic protein 15 and granulosa cell kit ligand: its role in regulating granulosa cell mitosis. Proc Natl Acad Sci USA 99 (2002): 8060-8065.

[16] Nakamura E, Otsuka F, Inagaki K, Miyoshi T, Matsumoto Y, Ogura K, Tsukamoto N, Takeda M, Makino H: Mutual regulation of growth hormone and bone morphogenetic protein system in steroidogenesis by rat granulosa cells. Endocrinology 153 (2012): 469-480.

[17] Brown MA, Zhao Q, Baker KA, Naik C, Chen C, Pukac L, Singh M, Tsareva T, Parice Y, Mahoney A et al: Crystal structure of BMP-9 and functional interactions with pro-region and receptors. J Biol Chem 280 (2005): 25111-25118.

[18] Yu PB, Hong CC, Sachidanandan C, Babitt JL, Deng DY, Hoyng SA, Lin HY, Bloch KD, Peterson RT: Dorsomorphin inhibits BMP signals required for embryogenesis and iron metabolism. Nat Chem Biol 4 (2008): 33-41.

[19] Cuny GD, Yu PB, Laha JK, Xing X, Liu JF, Lai CS, Deng DY, Sachidanandan C, Bloch KD, Peterson RT: Structure-activity relationship study of bone morphogenetic protein (BMP) signaling inhibitors. Bioorg Med Chem Lett 18 (2008): 4388-4392.

[20] Lopez-Coviella I, Berse B, Thies RS, Blusztajn JK: Upregulation of acetylcholine synthesis by bone morphogenetic protein 9 in a murine septal cell line. Journal of physiology, Paris 96 (2002): 53-59.

[21] Scharpfenecker M, van Dinther M, Liu Z, van Bezooijen RL, Zhao Q, Pukac L, Lowik CW, ten Dijke P: BMP-9 signals via ALK1 and inhibits bFGF-induced endothelial cell proliferation and VEGF-stimulated angiogenesis. J Cell Sci 120 (2007): 964-972.

[22] David L, Mallet C, Mazerbourg S, Feige JJ, Bailly S: Identification of BMP9 and BMP10 as functional activators of the orphan activin receptor-like kinase 1 (ALK1) in endothelial cells. Blood 109 (2007): 1953-1961.

[23] Kidder GM, Vanderhyden BC: Bidirectional communication between oocytes and follicle cells: ensuring oocyte developmental competence. Canadian journal of physiology and pharmacology 88 (2010): 399-413.

[24] Gilchrist RB: Recent insights into oocyte-follicle cell interactions provide opportunities for the development of new approaches to in vitro maturation. Reproduction, fertility, and development 23 (2011): 23-31.

[25] Herrera B, Inman GJ: A rapid and sensitive bioassay for the simultaneous measurement of multiple bone morphogenetic proteins. Identification and quantification of BMP4, BMP6 and BMP9 in bovine and human serum. BMC cell biology 10 (2009): 20.

[26] Townson SA, Martinez-Hackert E, Greppi C, Lowden P, Sako D, Liu J, Ucran JA, Liharska K, Underwood KW, Seehra J et al: Specificity and structure of a high affinity activin receptor-like kinase 1 (ALK1) signaling complex. J Biol Chem 287 (2012): 27313-27325.

[27] Johnson DW, Berg JN, Baldwin MA, Gallione CJ, Marondel I, Yoon SJ, Stenzel TT, Speer M, Pericak-Vance MA, Diamond A et al: Mutations in the activin receptor-like kinase 1 gene in hereditary haemorrhagic telangiectasia type 2. Nat Genet 13 (1996): 189-195.

[28] Urness LD, Sorensen LK, Li DY: Arteriovenous malformations in mice lacking activin receptor-like kinase-1. Nat Genet 26 (2000): 328-331.

[29] Chen H, Brady Ridgway J, Sai T, Lai J, Warming S, Chen H, Roose-Girma M, Zhang G, Shou W, Yan M: Context-dependent signaling defines roles of BMP9 and BMP10 in embryonic and postnatal development. Proc Natl Acad Sci U S A 110 (2013): 11887-11892.

[30] Levet S, Ciais D, Merdzhanova G, Mallet C, Zimmers TA, Lee SJ, Navarro FP, Texier I, Feige JJ, Bailly S et al: Bone morphogenetic protein 9 (BMP9) controls lymphatic vessel maturation and valve formation. Blood 122 (2013): 598-607.

[31] Li Q, Gu X, Weng H, Ghafoory S, Liu Y, Feng T, Dzieran J, Li L, Ilkavets I, Kruithof-de Julio M et al: Bone morphogenetic protein-9 induces epithelial to mesenchymal transition in hepatocellular carcinoma cells. Cancer science 104 (2013): 398-408.

[32] Herrera B, Garcia-Alvaro M, Cruz S, Walsh P, Fernandez M, Roncero C, Fabregat I, Sanchez A, Inman GJ: BMP9 is a proliferative and survival factor for human hepatocellular carcinoma cells. PLoS One 8 (2013): e69535.

[33] Upton PD, Davies RJ, Trembath RC, Morrell NW: Bone morphogenetic protein (BMP) and activin type II receptors balance BMP9 signals mediated by activin receptor-like kinase-1 in human pulmonary artery endothelial cells. J Biol Chem 284 (2009): 15794-15804.

[34] Herrera B, Sanchez A, Fabregat I: BMPS and liver: more questions than answers. Current pharmaceutical design 18 (2012): 4114-4125.

Figure Legends:

Fig. 1. Effects of BMP-9 on FSH-induced (A) estradiol and (B) progesterone production by rat granulosa cells. Rat granulosa cells were cultured in the absence or presence of oocytes. FSH was added to the culture medium either alone or in combination with BMP-9. After 48-h culture, the levels of estradiol and progesterone in the medium were determined. Results are shown as means ± SEM. The results were analyzed by ANOVA. For each result within a panel, the values with different superscript letters are significantly different at $P < 0.05$.

Fig. 2. Effects of BMP-9 on FSH-induced cAMP synthesis by rat granulosa cells. Granulosa cells were cultured in serum-free medium containing IBMX. FSH was added to the culture medium either alone or in combination with BMP-9. After 48-h culture, the levels of cAMP in the medium were determined. Results are shown as means ± SEM. The results were analyzed by ANOVA.

For each result within a panel, the values with different superscript letters are significantly different at $P < 0.05$.

Fig. 3. Effects of BMP-6 on FSH-induced expression of steroidogenesis-related factors by rat granulosa cells. Total cellular RNA was extracted from granulosa cells treated with FSH and BMP-9 for 48 h, and StAR, P450scc, 3βHSD and FSHR mRNA levels were determined by quantitative PCR. The expression levels of target gene mRNA were standardized by RPL19 level in each sample, and then levels of mRNA of target genes were expressed as fold changes. The results were analyzed by ANOVA. *, $P < 0.05$ vs. control group.

Fig. 4. Expression of BMP-9 system and its receptor signaling in rat granulosa cells. A) Total cellular RNAs extracted from granulosa cells and oocytes were subjected to RT-PCR for detecting the expression of BMP-9 (293 bp), ALK-1 (474 bp), BMPRII (520 bp) and RPL19 (192 bp). MM indicates

molecular weight marker. **B)** After preculture with noggin (30 ng/ml) for 24 h, granulosa cells were stimulated with BMP-9 and BMP-4 for 60 min. Cell lysates were subjected to SDS-PAGE/immunoblotting for pSmad1/5/8 analysis. **C)** BMP-9 effects on Id-1 transcription. Total cellular RNA was extracted from granulosa cells treated with BMP-9 for 48 h, and then Id-1 mRNA levels were determined by quantitative PCR. Data were standardized by RPL19, and levels of Id-1 mRNA were expressed as fold changes. **D)** Granulosa cells were treated with FSH (30 ng/ml) and BMP-9 (10 ng/ml) in combination with dorsomorphin or LDN-193189 (100-1000 nM). After 48-h culture, the levels of progesterone in the medium were determined. Results are shown as means ± SEM. The results were analyzed by the unpaired *t*-test (C) or ANOVA (D). *, $P < 0.05$ vs. control group (C). The values with different superscript letters are significantly different at $P < 0.05$ (D).

Fig. 5. Effects of BMP-receptor inhibition by ECDs on BMP-9 activity in granulosa cells. Granulosa cells were treated with FSH (30 ng/ml) and

BMP-9 (10 ng/ml) **(A)** in the presence of ALK-2-, -3-, -6-, ActRIIA- and ActRIIB-ECDs (0.3 μg/ml) **(B)** and either alone or in combination with ALK-1- and BMPRII-ECDs (300 μg/ml). After 48-h culture, the levels of progesterone in the medium were determined. Results are shown as means ± SEM. The results were analyzed by ANOVA. For each result within a panel, the values with different superscript letters are significantly different at $P < 0.05$.

Fig. 6. Effects of BMP-9 on steroidogenesis and its receptor signaling in rat granulosa cells. In granulosa cells, BMP-9 inhibits FSH-induced progesterone production via the ALK-1/BMPRII receptor complex and subsequent Smad1/5/8 phosphorylation as a luteinizing inhibitor in the process of ovarian folliculogenesis. AC, adenylate cyclase.