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Bone morphogenetic proteins are mediators of luteolysis in the human corpus luteum

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Abstract

Bone morphogenetic proteins (BMPs), members of the tumor growth factor β superfamily, play important roles in folliculogenesis in various species, however, little is known about their role in luteal function. In this study, we investigated the expression, regulation, and effects of BMP2, BMP4, and BMP6 in carefully-dated human corpora lutea and cultured human luteinized granulosa cells. The mRNA abundance of BMPs was increased in the regressing corpus luteum in vivo ($P<0.01–0.001$). Human chorionic gonadotropin (hCG) down-regulated BMP2, BMP4, and BMP6 transcripts both in vivo ($P=0.05–0.001$) and in vitro ($P<0.001$), and decreased the mRNA abundance of BMP receptors (BMPR1A, BMPR1B, BMPR2; $P<0.05–0.01$) in vitro. Three BMPs were regulated by differential signaling pathways. H89, a protein kinase A inhibitor, increased the expression of both BMP2 ($P<0.05$) and BMP4 ($P<0.05$) while decreasing BMP6 ($P<0.01$). PMA, a protein kinase C activator, decreased both BMP4 and BMP6 expression ($P<0.0001$) while enhancing the mRNA abundance of BMP2 ($P<0.01$). BMPs significantly down-regulated transcripts for LH/choriogonadotropin receptor (LHCGR; $P<0.001$) and steroidogenic acute regulatory protein (STAR; $P<0.001$), while up-regulating those of follicular stimulating hormone receptor (FSHR; $P<0.01$) and aromatase (CYP19A1; $P<0.05–0.01$) in vitro, possessing an effect opposite to hCG but similar to Activin A. Like Activin A, BMP4 and BMP6 stimulated the expression of Inhibin/Activin subunits with a marked effect on INHBB expression ($P<0.05–0.01$). These data confirm that BMPs are increased during luteal regression and negatively regulated by hCG via differential mechanisms, suggesting that BMPs are one of the mediators of luteolysis in women.
Introduction

The human corpus luteum (CL) is a transient endocrine gland that develops from the dominant follicle after ovulation. It produces large amounts of progesterone, which is essential for blastocyst implantation, maintenance of pregnancy, and preservation of the structure and function of the CL itself (1). The production of progesterone by the CL is dependent on luteinizing hormone (LH) secretion from the pituitary gland, which binds to LH/choriogonadotropin receptor (LHCGR) on the surface of granulosa lutein and theca lutein cells (2). Human chorionic gonadotropin (hCG) secreted from the developing conceptus also binds to LHCGR and activates intracellular signaling pathways to maintain progesterone secretion, suppress luteolytic molecules, and rescue the CL from degradation during early pregnancy (3, 4).

In a non-conception cycle, the CL starts to regress via a degradation process known as luteolysis. This involves a loss of both the functional and structural integrity of the CL where progesterone production declines, and the CL undergoes a marked reduction in size and weight to form an increasingly fibrous avascular structure termed the corpus albicans. Structural regression includes apoptosis of luteal cells, degradation of capillary vessels, and extensive tissue remodeling (4, 5). Tissue remodeling during luteolysis is accompanied by an invasion of the CL by macrophages (6, 7) and proteolytic degradation of the extracellular matrix by matrix metalloproteinases (MMPs) (8).

Although luteal function is fundamentally important in human reproduction, we still do not understand how luteolysis is initiated in the absence of pregnancy. This is because there are no good non-primate animal models that regulate the CL in same way as women. It is believed that a functional withdrawal of LH enhances the expression of paracrine molecules that promote luteolysis and that are suppressed by hCG (9). We previously demonstrated that a member of
transforming growth factor β (TGFβ) superfamily, Activin A, is an important paracrine regulator of luteolysis. It promotes MMP activity and reduces the expression of genes involved in progesterone synthesis (10, 11). Bone morphogenetic proteins (BMPs), other members of the TGFβ superfamily, have now been shown to play a critical role during steroidogenesis in granulosa cells of preovulatory follicles and in granulosa-like tumor cell lines (12-16). The expression of some members of the BMP family and their receptors have been reported in the human ovary (17) and BMPs are expressed in the CL of rats (18) and cows (19). However, limited information is available about the role of BMPs in the human CL.

Herein we investigate 1) The localization of BMPs and their site of reception and action in the human CL; 2) If BMP transcript expression in the human CL changes across the luteal phase; 3) Whether hCG regulates BMP expression in the human CL *in vivo* and in human luteinized granulosa cells (LGCs) *in vitro*; 4) Whether hCG regulates the expression of transcripts for BMP receptors and other BMP regulatory molecules in human LGCs *in vitro*; 5) Which molecular pathways are involved in the regulation of BMP transcripts in LGCs; and finally 6) What effects BMPs have on the transcription of genes associated with LGC function. We aimed to determine whether, like Activin A, BMPs are locally produced and are active molecules that have a role in the induction and regulation of luteolysis in the human CL.
Materials and Methods

Ethics

All experiments using human tissues and cells were approved by the Medical Research Ethics Committee, and all women gave informed consent.

Reagents

Reagents were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA) unless otherwise stated. Insulin-Transferrin-Selenium (ITS)-X was obtained from Life Technologies Inc. (Carlsbad, CA, USA). Matrigel is a product of BD Biosciences (San Jose, CA, USA).

Collection of the human CL

Human CLs were collected at the time of surgery from women undergoing hysterectomy for benign conditions. The stages of the CLs were determined by endometrial morphology and by the concentration of LH in daily urine samples, obtained prior to surgery as reported previously (20). The enucleated CLs were halved upon collection; one half was snap-frozen and stored at −80°C, and the other half was fixed with formalin and processed into paraffin wax. Some women were given daily doubling injections of hCG (Profasi®, Serono Laboratories, Welwyn Garden City, UK), starting at 125 IU from 7 days after the LH surge for 5–8 days until surgery. This regimen has been shown to rescue the CL and mimic the hormonal changes of early pregnancy (20). Only frozen tissues of rescued CL were available from these women.

Human LGC culture

Follicular aspirates were obtained from patients undergoing assisted conception. Oocytes were removed and the remaining fluid was centrifuged at 1,500 rpm for 10 min, and cells were
re-suspended in culture medium (DMEM/Ham’s F-12 containing 2 mM L-glutamine, 10 unit/mL penicillin, 0.1 mg/mL streptomycin, 2.5 μg/mL amphotericin B, and 1× concentration of ITS-X). Cell suspension was layered over 45% Percoll in culture medium mixture, and centrifuged at 1,200 rpm for 30 min to pellet blood cells. LGCs, visible in the interface, were collected by a pipette and washed three times with Dulbecco’s phosphate buffered saline with MgCl₂ and CaCl₂.

One hundred-thousand viable cells were seeded on Matrigel-coated 24 well plates and cultured with 1 mL of culture medium at 37°C in 5% CO₂ in air. The culture medium was changed every two days, and cells were treated on day six of culture for 8 or 24 hours with either 1) 100 ng/mL hCG, 2) 50 ng/mL BMP2, BMP4, and BMP6 (R&D Systems Inc., Minneapolis, MN, USA), 3) 50 ng/mL Activin A (R&D Systems Inc.), 4) PI3-kinase inhibitor (LY294002; 20 μM, Cell Signaling Technology Inc., Danvers, MA, USA), 5) cAMP/Protein kinase A (PKA) inhibitor (H89; 10 μM, Merck Millipore, Nottingham, UK), or 6) Protein kinase C (PKC) activator (Phorbol 12-myristate 13-acetate: PMA; 10 ng/mL, Merck Millipore). Each experiment was performed in duplicate and repeated at least three times.

**Quantitative RT-PCR (qRT-PCR)**

The CLs used for a quantitative gene expression analysis were classified as early-luteal (1-5 days after the LH surge, n=4), mid-luteal (6-10 days, n=6), late-luteal (11-14 days, n=6), menstrual phase (over 14 days, n=3), and rescued by hCG injection (n=4). Total RNA was extracted from the frozen human CL or cultured LGCs using RNeasy Mini Kit (Qiagen Ltd., Crawley, UK) following the manufacturer’s protocol. RNA (200 ng) was used to prepare cDNA using the TaqMan Reverse Transcription regents (Applied Biosystems, Foster City, CA, USA).

The sequences of the primer sets used for this study are listed in Table 1. Primers were
pre-validated by standard PCR and by generating standard curves using qRT-PCR. Each reaction buffer contained 5.0 µL 2×PowerSYBR® Green PCR Master Mix (Applied Biosystems), 0.5 µL primer pair (5 µM), 3.5 µL of nuclease free H₂O, and 1.0 µL cDNA, and each reaction was conducted in duplicate. The qRT-PCR cycling program consisted of a denaturing step (95°C for 10 min), annealing and extension step (95°C for 15 sec and 60°C for 1 min repeated for 40 cycles), and a dissociation step (95°C, 60°C, and 95°C for 15 sec each) using a 7900 Sequence Detection System (Applied Biosystems). The relative expression levels of each target to the housekeeping gene (glucose-6-phosphate dehydrogenase: G6PDH), previously validated using GeNorm analysis (Primerdesign Ltd, Southampton, UK), were quantified using the ΔCt or ΔΔCt methods. After testing for normality, all statistical analyses were performed using unpaired t-tests or one-way ANOVA, with pairwise comparison, using GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA), and P<0.05 was regarded as significant. Values in the graphs represent the mean ± SEM of relative expression to G6PDH.

Immunohistochemistry

Fixed human CL at the early-luteal (n=6), mid-luteal (n=3), late-luteal (n=3), and menstrual phases (n=3) were used for immunohistochemical analysis. The tissues were dehydrated through ethanol and embedded into paraffin according to the conventional method. Tissue sections (5 µm thick) were de-waxed and washed twice in distilled water, then antigen retrieval was performed for 5 minutes in 0.01 M citrate buffer (pH 6.0) using a pressure cooker. After washing twice in phosphate-buffered saline (PBS), the sections were incubated with 3% hydrogen peroxide for 20 min followed by Avidin/Biotin blocking solution (Vector Laboratories Inc., Burlingame, CA, USA) for 15 min each. Then the sections were incubated with normal
goat serum for 60 min at room temperature. Thereafter they were incubated with mouse
anti-human BMP4 or BMP6 (1:200; MAB1049 or MAB1048, Merck Millipore), rabbit
anti-human BMP receptors (1:500 or 1:1,000; kind gifts from Dr. Heldin, Uppsala University),
rabbit anti-human phospho-Smad 1/5/8 (p-Smad 1/5/8; 1:500; 9511, Cell Signaling Technology
Inc.), or rabbit anti-human phospho-Smad 2/3 (p-Smad 2/3; 1:500; sc-11769R, Santa Cruz
Biotechnology Inc., Dallas, TX, USA) in goat serum at 4°C overnight. Control sections were
incubated with non-immune serum and the disappearance of immunoreaction was confirmed.

After washing twice in PBS, the sections were incubated with biotinylated anti-mouse or
anti-rabbit IgG (1:500; Vector laboratories Inc.) for 60 min at room temperature. The reaction
sites were visualized using a Vectastain ABC Elite kit (Vector Laboratories Inc.) for 60 min
followed by ImmPACT™ DAB Peroxidase Substrate Kit (Vector Laboratories Inc.) for 1 min.
The sections were counterstained with haematoxylin and observed under a light microscope
(BX51; Olympus corporation, Tokyo, Japan). Identification of granulosa lutein, theca lutein,
endothelial and other non-steroidogenic cell types was done using cell localization and
morphology with reference to our previous detailed studies using immunohistochemical cell
identification in the human CL (4-6, 21, 22). Some sections were immunostained with mouse
anti-CD31 antibody (1:20; Dako Japan ltd., Tokyo, Japan) or rabbit anti-3β-HSD (1:1,000; kind
gift from Prof. Ian Mason, The University of Edinburgh) to identify the endothelial cells and
steroidogenic cells, and the reaction site were visualized as described above using appropriate
peroxidase or biotin-labeled secondary antibodies.
Results

**BMP signaling is active in the human CL**

TGFβ ligand-receptor binding leads to phosphorylation of receptor mediated Smad proteins. Activin activates Smad 2 and 3 through type I and type II Activin receptors, whereas BMPs activate Smad 1, 5, and 8 through type I and type II BMP receptors (23). Activin signaling in luteal cells is associated with luteolysis (10, 11), and Smad 2/3 phosphorylation can be detected in the nuclei of granulosa lutein cells, theca lutein cells, endothelial cells, and non-steroidogenic cells in the CL parenchyma (Figure 1A). Interestingly, Smad 1/5/8 phosphorylation can also be detected in nuclei of granulosa lutein and theca lutein cells (Figure 1B), suggesting that BMP signaling is also involved in the function of these cells. Endothelial cells also demonstrated a positive immunoreactivity for p-Smad 1/5/8 (asterisk in Figure 1B), however, non-steroidogenic cells in the CL parenchyma did not express p-Smad 1/5/8 (arrowheads in Figure 1B).

BMPs initially bind to type II BMP receptor (BMPR2) that in turn leads to phosphorylation and activation of type I receptors (BMPR1A and BMPR1B). These BMP receptors showed the same cellular distribution as p-Smad 1/5/8; they were mainly localized to steroidogenic granulosa lutein and theca lutein cells (Figures 1C–E). Endothelial cells were also immunostained for all three receptors but other non-steroidogenic cells were negative in reaction (Figure 1C–E). Thus the predominant targets for BMPs in the human CL are luteal steroidogenic cells and endothelial cells.

**BMPs are autocrine and/or paracrine mediators in luteal cells**

We next investigated the cellular sources of luteal BMPs. Both BMP4 and BMP6 were expressed in the CL, and primarily localized to the cytoplasm of granulosa lutein (Figures 1F–I) and theca lutein cells (arrows in Figures 1F–I). Endothelial cells were also immunostained for
BMP4, while other non-steroidogenic cells and stromal cells outside the CL lacked BMP4 immunoreactivity (arrowheads in Figure 1G). Immunoreactivity for BMP6 was also detected in endothelial cells and stromal cells outside the CL (Figure 1H), while non-steroidogenic cells in the CL parenchyma were not immunostained (arrowheads in Figure 1I). Thus BMPs are mainly produced by luteal steroidogenic cells.

BMP mRNA abundance in the human CL peaks during luteolysis

As luteal steroidogenic cells were the main source of BMPs in the CL, we next investigated their mRNA expression across the luteal phase by qRT-PCR. The mRNAs for BMP2, BMP4, and BMP6 were all expressed in the human CL, with BMP6 mRNA expression being particularly abundant (Figure 2). While there were no significant changes in their expression in the CL between the early- and mid-luteal phases, the mRNA expression of all three genes was significantly increased in the CL during the late-luteal phase (BMP2 $P<0.001$; BMP4 $P<0.01$; BMP6 $P<0.001$; Figure 2). During menstruation, the expression of BMP2 ($P<0.001$) and BMP4 ($P<0.01$) in the CL remained high while only the expression of BMP6 was significantly reduced (Figure 2). This suggests that BMPs are up-regulated during luteolysis and possibly have differential roles in luteal cells.

HCG reduces luteal BMP and BMP receptor mRNA abundance

We next analyzed the effect of hCG, which rescues the human CL from luteolysis, on the mRNA expression of BMPs and the related genes for BMP function. Both BMP2 and BMP4 mRNAs were down-regulated by in vivo administration of hCG when compared to the late-luteal CL in the absence of hCG ($P<0.001$; Figure 3A). BMP6 mRNA expression also decreased in the rescued CL, however, the suppressive effect of hCG did not reach a significant
level \( (P=0.0548; \text{Figure 3A}) \). In LGCs \textit{in vitro}, hCG significantly decreased the mRNAs expression of all three \textit{BMPs} \( (P<0.001; \text{Figure 3B}) \). This confirms that the expression of \textit{BMP} transcripts is hormonally regulated in luteal steroidogenic cells.

In addition, hCG reduced the mRNA expression of \textit{BMP} receptors in cultured LGCs \( (\text{BMPRI}A P<0.05; \text{BMPRI}B P<0.01; \text{BMPRI}2 P<0.05; \text{Figure 3C}) \). HCG also altered the transcript abundance of other regulators involved in \textit{BMP} function (Figure 3D). Expression of an antagonist for \textit{BMPs}, \textit{GREM2}, was up-regulated in LGCs by hCG \( (P<0.001) \). In contrast, the other \textit{BMP} antagonists, \textit{NOG} \( (P<0.01) \) and \textit{GREM1} \( (P<0.001) \) were decreased by hCG as was an inhibitor of Smad activation, \textit{SMAD6} \( (P<0.05; \text{Figure 3D}) \). These data suggest that hCG would be consistent with an inhibition of luteal \textit{BMP} activity by down-regulating the expression of \textit{BMPs} and their receptors.

\textit{BMPs are differentially regulated at a molecular level}

To reveal the regulatory mechanism of luteal \textit{BMPs} at a molecular level, we examined the effect of a PI3-kinase inhibitor (LY294002), a cAMP/PKA inhibitor (H89), and a PKC activator (PMA) on the expression of \textit{BMP} transcripts in LGCs. Inhibition of PI3-kinase (LY294002) tended to reduce \textit{BMP} mRNA abundances although only \textit{BMP6} was significantly decreased \( (P<0.001; \text{Figure 4A}) \). Interestingly, manipulation of the PKA and PKC pathways revealed different effects according to \textit{BMP} subtypes. Inhibition of PKA (H89) increased the expression of both \textit{BMP2} \( (P<0.05) \) and \textit{BMP4} \( (P<0.05) \) while the expression of \textit{BMP6} was reduced \( (P<0.01; \text{Figure 4B}) \). Activation of PKC (PMA) inhibited \textit{BMP4} and \textit{BMP6} expression in LGCs \( (P<0.0001; \text{Figure 4C}) \) while dramatically increasing the expression of \textit{BMP2} \( (P<0.01; \text{Figure 4C}) \). It is known the PMA can have time dependent effects on PKC activation (24) so we analysed the early effect of PMA on \textit{BMP} expression after 8 hours of treatment. This resulted in
the same changes as seen after 24 hours (Supplementary Figure 1). Although BMPs are all
down-regulated by hCG, there are differential molecular pathways involved in their regulation
in human LGCs.

**BMPs have a similar effect to Activin on LGC function**

Activin has an opposite effect to hCG in regulating the function of human LGCs *in vitro* (11).
Administration of hCG increased the expression of *LHCGR* (*P*<0.001; Table 2) and
steroidogenic acute regulatory protein (*STAR*; *P*<0.001; Table 2) whereas addition of Activin A
decreased the expression of *LHCGR* (*P*<0.001; Table 2) and *STAR* (*P*<0.001; Table 2).
Treatment of cells with BMP2, BMP4 or BMP6 also reduced the expression of both *LHCGR*
(*P*<0.001; Table 2) and *STAR* (*P*<0.001; Table 2). Like Activin A, all three BMPs up-regulated
the expression of follicular stimulating hormone receptor (*FSHR*; *P*<0.01-0.001; Table 2) and
*CYPI9A1* (*P*<0.05–0.01; Table 2).

Activin A promoted its own synthesis by increasing production of Inhibin/Activin β
subunits, *INHBA* (*P*<0.05; Table 2) and *INHBB* (*P*<0.01; Table 2). BMP4 and BMP6 had
similar effects to Activin A and displayed opposite effects to hCG on the expression of
Inhibin/Activin β subunits with marked stimulatory effects on *INHBB* (*P*<0.05–0.01; Table 2).
Unlike the effect of Activin A on stimulating the synthesis of its consistent subunits, BMPs
suppressed their own synthesis in LGCs (*P*<0.05-0.01; Table 2), and Activin A also suppressed
BMP expression (*P*<0.01; Table 2). Thus the effects of BMPs during luteolysis are similar to
those of Activin A.
Discussion

Members of the TGFβ superfamily regulate cell proliferation and differentiation, and are involved in various biological and pathological events including tissue remodeling and cancer. There are more than 40 molecules in this superfamily, which are classified into TGFβ, Inhibin/Activin, BMP, anti-müllerian hormone (AMH), and growth and differentiation factor (GDF) subfamilies. In the ovary, it is well established that most of the TGFβ superfamily proteins, especially Activins, BMPs, and GDF are involved in the regulation of steroidogenic cells during follicle growth (25-27). However, limited information was available about their role in the CL. We previously demonstrated that Activin A plays an important role in the regulation of luteolysis in women (10, 11). Here we provide the evidence that supports a role for BMPs in the regulation of luteolysis in women.

We showed in this study that luteal steroidogenic cells were sites of synthesis and reception of BMPs and that BMP-stimulated cell signaling (p-Smad 1/5/8) was present in these cells. Previously BMP expression in granulosa cells has been shown to be maintained after luteinization in women (15, 16, 28). The present study further demonstrated that BMP4 and BMP6 are localized to both granulosa lutein and theca lutein cells in the CL of women, and that BMPs are able to act on luteal cells in a paracrine and/or autocrine manner. This suggests that locally produced BMPs may have a role in the regulation of the CL as they do during folliculogenesis (25-27).

If BMPs have a role in the regulation of the CL, it would be expected that their expression in the CL would change across the luteal phase. We showed that BMP mRNA abundance remarkably changed across the luteal phase: the mRNA expression of BMP2, BMP4, and BMP6 all increased in the late-luteal phase. This would be consistent with that BMPs have an important role during luteolysis. Although the mechanism of luteolysis in rodents differs...
from that in women, the expression of BMP2 and BMP6 in the rodent CL increased at the time
of regression (18). This implies that BMP activity during luteolysis may be conserved in
disparate species with different paradigms of CL regulation.

If BMPs are the molecules with a role in luteolysis in women, it would be expected that
hCG, which protects the human CL from luteolysis (9), would negatively regulate BMP
eexpression. Here we were able to show that luteotropic hCG down-regulated BMP2, BMP4, and
BMP6 mRNAs not only in luteal steroidogenic cells *in vitro* but also in rescued CL *in vivo*. Our
*in vitro* data agrees with previous studies showing the suppressive effect of hCG on the
expression of BMP2 and BMP3 in human LGCs (27, 28). We also revealed here that hCG
down-regulates BMP receptors in cultured LGCs. This would serve to reduce BMP action and
would further support a potential luteolytic role for BMPs.

We have previously shown that Activin A is a paracrine effector of luteolysis (10, 11) and
hCG is known to reduce Activin A activity at multiple levels. HCG increased the expression of
Follistatin which functions as an antagonist by binding to and inactivating Activins (10). We
therefore analyzed the effect of hCG on mRNAs for BMP-specific antagonists expecting that it
would up-regulate these genes. Interestingly, the effect of hCG was not consistent. Among
BMP-specific antagonists, *NOG* and *GREM1*, which were predominantly expressed in human
LGCs, were suppressed by hCG. In contrast, *GREM2*, another BMP-specific antagonist, was
significantly up-regulated by hCG although its expression was relatively low. However, overall
these results would suggest that BMP signaling is suppressed by hCG in luteal cells via
down-regulation of the expression of both BMPs and their receptors.

In the human CL the mRNA abundance of *BMP2*, *BMP4* and *BMP6* was maximal in the
late luteal phase. Interestingly after menstruation had commenced the luteal expression of
*BMP6* was not maintained in contrast to that of *BMP2* and *BMP4*. While this may be due to the
different cellular localization of BMP6, which is expressed in non-steroidogenic cells outside the CL as well as luteal steroidogenic cells, we also wondered if the molecular regulation of BMP6 differed to that of BMP2 and BMP4. As hCG can stimulate different intracellular signalling pathways (29), we therefore investigated which pathways regulated the mRNA abundance of the different BMPs. The present study provided the evidence for the first time that the expression of BMP2, BMP4, and BMP6 transcripts in human LGCs are regulated by different intracellular signaling pathways. HCG is classically considered to work through PKA activation (29). Accordingly, blocking of the PKA pathway in vitro led to the expected increase the expression of both BMP2 and BMP4. However BMP6 mRNA abundance was reduced by PKA inhibition suggesting that the PKA pathway may not be involved in the hCG-mediated inhibition of BMP6 in LGCs.

HCG is also known to activate PKC (29), and the activation of this pathway reduced BMP6 expression. This suggests that BMP6 mRNA in LGCs may be primarily regulated by PKC activation rather than PKA. Thus, although the effect of hCG is same, it is likely that the reduction of BMP2 by hCG treatment is mainly through PKA, BMP6 is mainly through PKC, and BMP4 is through both PKA and PKC. It is well known that PKC pathway is activated by luteolytic PGF₂α, and Priyanka et al. reported that BMP2 was increased by PGF₂α stimulation in the CL of bonnet monkey (30). Thus locally released PGF₂α might be also involved in an increased expression of BMP2 in the regressing CL. We also showed a possibility that another signaling pathway, PI3-kinase, regulates the production of BMPs, especially that of BMP2 and BMP6, in human luteal cells. Since all three BMPs are increased during luteolysis, our findings suggest that a reduction of PKA and an activation of PKC and PI3-kinase are likely to be important during luteolysis. The differential signaling of the LHCGR across the luteal phase and how it is regulated may be of major interest in the understanding of the molecular regulation of
human luteolysis.

If BMPs are luteolytic it is expected that they would reduce the steroidogenic function of luteal steroidogenic cells. BMPs in granulosa cells stimulate estradiol synthesis and suppress progesterone synthesis as reported in various animals (14, 31-35), while little is known about the function of BMPs in luteal cells. Shi et al. (15) recently reported that BMP2 increased the expression of FSHR and CYP19A1, but down-regulated the expression of LHCGR and STAR in cultured human LGCs. We confirmed the same effects of both BMP4 and BMP6 on the expression of these genes in human LGCs, indicating that BMPs seem to suppress luteinization of human granulosa cells like Activin A (11). Importantly, the factors that regulate follicle growth and suppress luteinization of granulosa cells are also likely to be involved in the suppression of progesterone synthesis in luteal cells during functional luteolysis.

We previously showed that Activin A promoted its own synthesis in human luteal cells (11). This leads to a cycle of positive reinforcement of Activin action during luteolysis. We reported here that BMPs enhance the expression of Inhibin/Activin β subunits in human LGCs, possibly stimulating Activin synthesis in luteal cells and contributing to the increased action of Activin.

This is consistent with the previous report showing that BMP up-regulates the production of Inhibin/Activin βB subunit in human LGCs (28). It is likely that local Activin synthesis is stimulated by BMPs, however, further work on the resulting Inhibin/Activin balance is required because BMP4 and BMP6 also slightly increased the mRNA expression of Inhibin α subunit (Table 2). On the other hand, unlike Activin A that enhanced Activin/Inhibin β subunits synthesis, we showed that the mRNA abundance of BMPs was reduced in response to BMPs. The net effect of BMPs is uncertain but there may be a self-limitation in the BMP effect during luteolysis.

In conclusion, the expression of BMP2, BMP4, and BMP6 is increased in the regressing...
human CL, and are suppressed by luteotrophic hCG both *in vivo* and *in vitro*, suggesting their involvement in the regulation of luteolysis. Like Activin A, BMPs possess opposite effects to hCG, and suppress luteinization and progesterone production in human luteinized steroidogenic cells. The present study provides the evidence that BMPs are one of the predominant mediators of luteolysis in the human CL.
Acknowledgement

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16. Shi J, Yoshino O, Osuga Y, Nishii O, Yano T, Taketani Y. Bone morphogenetic protein 7 (BMP-7) increases the expression of follicle-stimulating hormone (FSH) receptor in human


Figure 1. The expression and localization of p-Smad, BMP receptors, and BMPs in the human CL at mid-luteal phase.

Immunoreactivity for p-Smad 2/3, activated by Activin signaling, is found in nuclei of granulosa lutein cells (A), theca lutein cells (insert in A), and endothelial cells (asterisk in A), as well as non-steroidogenic cells (arrowheads in A). Intense immunoreaction for p-Smad 1/5/8, which represents an activation of BMP signaling, is found in nuclei of granulosa lutein cells (B), theca lutein cells (insert in B), and endothelial cells (asterisk in B) but not in non-steroidogenic cells distributed in the CL parenchyma (arrowheads in B). BMP receptors are immunolocalized to granulosa lutein and theca lutein cells (BMPR1A: C, BMPR1B: D, BMPR2: E) while non-steroidogenic cells in the CL parenchyma are negative in reaction (arrowheads in C–E).

Endothelial cells are also immunostained by the antibodies for all BMP receptors (asterisks in C–E). BMP4 (F and G) and BMP6 (H and I) immunoreactivities are localized to both granulosa lutein and theca lutein cells (arrows in F–I), and absent in negative control (insert in F). Non-steroidogenic cells in the CL parenchyma are negative in reaction for both BMP4 and BMP6 (arrowheads in F–I). Endothelial cells are also immunostained with both BMP4 and BMP6 (asterisks in F–I). Endothelial cells are immunolabeled with CD31 (asterisks in J) and steroidogenic cells are positive in 3β-HSD immunoreaction (K). Non-steroidogenic cells are distributed between capillary vessels and steroidogenic cells but negative for both immunoreaction (arrowheads in J and K). L is a schema of endothelial cells, granulosa lutein cells, and non-steroidogenic cells distinguishable by their morphology, distribution, and immunoreactivity for CD31 or 3β-HSD. The localization of the immunoreactions does not change across luteal phase and the representative tissue sections from the mid-luteal phase are used for illustration. Arrows in C–I show theca lutein cells.
Figure 2. Changes in the expression of BMP transcripts in the human CL throughout the luteal phases.

Quantitative RT-PCR showed that BMP6 mRNA is more intensely expressed in the human CL than BMP2 and BMP4 mRNAs. The mRNA expression of BMP2, BMP4, and BMP6 is significantly increased in the CL at late-luteal (LL) phase. The elevated mRNA expression of BMP2 and BMP4 is maintained during menstruation (Men), while the expression of BMP6 mRNA falls in the CL at menstruation. EL: early-luteal phase, ML: mid-luteal phase. Asterisks show significant differences versus EL and ML. *P<0.05, **P<0.01, ***P<0.001.

Figure 3. Regulation of transcripts for BMPs, BMP receptors, and modulators for BMP signaling by hCG.

BMP2 and BMP4 mRNAs are significantly down-regulated and BMP6 mRNA tends to decrease by in vivo administration of hCG (A). Treatment with hCG significantly decreased all three BMP mRNA expression in cultured human LGCs (B). The mRNA abundances for BMP receptors (BMPR1A, BMPR1B, and BMPR2) are decreased by hCG treatment in vitro (C). The mRNAs of BMP-specific antagonists, NOG and GREM1, are significantly down-regulated, while GREM2 is significantly increased by hCG in vitro (D). The mRNA of an inhibitory Smad, SMAD6, is significantly decreased by hCG treatment in vitro (D). All black bars represent the hCG-treated samples. Dotted lines in C and D represent the expression level in control which have been normalized to 1.0. Cont: control, LL: late-luteal phase, n.s.: not significant, R: rescued CL by in vivo administration of hCG. *P<0.05, **P<0.01, ***P<0.001.

Figure 4. Regulation of the mRNA expression of BMPs via differential signaling pathway
in LGCs.

PI3-kinase inhibitor, LY294002, tended to suppress the mRNA expression of BMP2 ($P=0.0582$) and significantly suppressed BMP6 mRNA abundance (A). The treatment of H89, a protein kinase A inhibitor, significantly increased the expression of both BMP2 and BMP4, while it decreased BMP6 expression (B). PMA, an activator of protein kinase C, dramatically increased the expression of BMP2, whereas it suppressed the expression of both BMP4 and BMP6 (C). No change is indicated as 100% of control while a doubling is 200% and a halving is 50%.

* $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Table 2. The effects of hCG, Activin A, and BMPs on gene transcript expression in LGCs.

Relative expression (mean ± SEM) of candidate genes compared to control samples, which have been normalized to 1.0 are listed. The effects of treatments were analyzed after 24 hours and values with asterisks are significantly different when compared to the controls. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Supplementary Figure 1. Early effects of PMA on the regulation of the mRNA expression of BMPs.

Like cells treated for 24 hours with PMA, an activator of protein kinase C, the BMP2 mRNA expression is up-regulated whereas the expression of both BMP4 and BMP6 are suppressed by 8 hours PMA treatment. * $P<0.05$, ** $P<0.01$, **** $P<0.0001$. 
### Table 1. Primers used for qPCR analysis

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<tr>
<th>Gene name</th>
<th>Protein name</th>
<th>Accession no.</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Product size</th>
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<td>BMP4</td>
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Table 2. The effects of hCG, Activin A, and BMPs on gene transcript expression in LGCs.

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<tr>
<th>Treatments</th>
<th>hCG</th>
<th>Activin A</th>
<th>BMP2</th>
<th>BMP4</th>
<th>BMP6</th>
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<tr>
<td>LHCGR</td>
<td>↑ 3.58 ± 0.43***</td>
<td>↓ 0.19 ± 0.03***</td>
<td>↓ 0.59 ± 0.03***</td>
<td>↓ 0.25 ± 0.04***</td>
<td>↓ 0.26 ± 0.05***</td>
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<tr>
<td>STAR</td>
<td>↑ 18.04 ± 1.56***</td>
<td>↓ 0.31 ± 0.06***</td>
<td>↓ 0.57 ± 0.02***</td>
<td>↓ 0.32 ± 0.04***</td>
<td>↓ 0.23 ± 0.02***</td>
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<tr>
<td>FSHR</td>
<td>↓ 0.16 ± 0.09***</td>
<td>↑ 4.10 ± 0.81**</td>
<td>↑ 2.08 ± 0.31**</td>
<td>↑ 3.35 ± 0.58***</td>
<td>↑ 2.91 ± 0.42***</td>
</tr>
<tr>
<td>CYP19A1</td>
<td>→ 1.23 ± 0.14</td>
<td>↑ 1.93 ± 0.29**</td>
<td>↑ 1.75 ± 0.22**</td>
<td>↑ 1.98 ± 0.37**</td>
<td>↑ 2.03 ± 0.17**</td>
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<tr>
<td>INHA</td>
<td>↑ 5.03 ± 0.67***</td>
<td>→ 1.79 ± 0.24</td>
<td>→ 1.63 ± 0.26</td>
<td>↑ 3.12 ± 0.48*</td>
<td>↑ 3.17 ± 0.44**</td>
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<td>INHBA</td>
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<td>↑ 1.67 ± 0.19*</td>
<td>→ 1.65 ± 0.15</td>
<td>↑ 3.04 ± 0.49**</td>
<td>↑ 3.00 ± 0.41**</td>
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<tr>
<td>INHBB</td>
<td>↓ 0.02 ± 0.01**</td>
<td>↑ 33.25 ± 11.48**</td>
<td>→ 2.24 ± 0.20</td>
<td>↑ 17.58 ± 4.64*</td>
<td>↑ 23.09 ± 7.05**</td>
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<td>↓ 0.14 ± 0.02**</td>
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<td>↓ 0.12 ± 0.03**</td>
<td>↓ 0.42 ± 0.08**</td>
<td>↓ 0.11 ± 0.02**</td>
<td>↓ 0.10 ± 0.02**</td>
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<tr>
<td>BMP6</td>
<td>↓ 0.15 ± 0.03***</td>
<td>↓ 0.65 ± 0.09*</td>
<td>↓ 0.69 ± 0.06**</td>
<td>↓ 0.56 ± 0.11**</td>
<td>↓ 0.58 ± 0.10**</td>
</tr>
</tbody>
</table>
Figure 1 Nio-Kobayashi et al.
Figure 2 Nio-Kobayashi et al.
Figure 3 Nio-Kobayashi et al.
Figure 4 Nio-Kobayashi et al.
Supplementary Figure 1
Nio-Kobayashi et al.