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

Junko Nio-Kobayashi[1, 2], Jennifer Trendell[1], Sevasti Giakoumelou[1], Lyndsey Boswell[1], Linda Nicol[1], Masataka Kudo[3], Noriaki Sakuragi[3], Toshihiko Iwanaga[2], William Colin Duncan[1]

[1]MRC Centre for Reproductive Health, The Queen's Medical Research Institute, The University of Edinburgh, 47 Little France Crescent, Edinburgh, EH16 4TJ, UK, [2]Laboratory of Histology and Cytology, [3]Department of Reproductive Endocrinology and Oncology, Hokkaido University Graduate School of Medicine, Kita 15-Nishi 7, Kita-ku, Sapporo 060-8638, JAPAN.

Abbreviated title: BMPs are mediators of luteolysis in the human CL

Key terms: BMP / Activin A / luteinized granulosa cell / hCG

Word count: 4099 words

Corresponding author and person to whom reprint requests:

Junko Nio-Kobayashi, VMD, PhD, Laboratory of Histology and Cytology, Hokkaido University Graduate School of Medicine, Kita 15-Nishi 7, Kita-ku, Sapporo 060-8638, JAPAN

Tel. & Fax: +81-11-706-7151, E-mail: niojun@med.hokudai.ac.jp

Grants*:* This study was supported by the Cunningham Trust to WCD, a Postdoctoral Fellowship for Research Abroad from Japan Society for the Promotion of Science and the Suntory Foundation for Life Sciences to JN-K.

Disclosure Statement: The authors have nothing to disclose.

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

121 re-suspended in culture medium (DMEM/Ham's F-12 containing 2 mM L-glutamine, 10
122 unit/mL penicillin, 0.1 mg/mL streptomycin, 2.5 μg/mL amphotericin B, and 1× concentration
123 of ITS-X). Cell suspension was layered over 45% Percoll in culture medium mixture, and
124 centrifuged at 1,200 rpm for 30 min to pellet blood cells. LGCs, visible in the interface, were
125 collected by a pipette and washed three times with Dulbecco's phosphate buffered saline with
126 $MgCl_2$ and $CaCl_2$.

127 One hundred-thousand viable cells were seeded on Matrigel-coated 24 well plates and
128 cultured with 1 mL of culture medium at 37°C in 5% CO_2 in air. The culture medium was
129 changed every two days, and cells were treated on day six of culture for 8 or 24 hours with
130 either 1) 100 ng/mL hCG, 2) 50 ng/mL BMP2, BMP4, and BMP6 (R&D Systems Inc.,
131 Minneapolis, MN, USA), 3) 50 ng/mL Activin A (R&D Systems Inc.), 4) PI3-kinase inhibitor
132 (LY294002; 20 μM, Cell Signaling Technology Inc., Danvers, MA, USA), 5) cAMP/Protein
133 kinase A (PKA) inhibitor (H89; 10 μM, Merck Millipore, Nottingham, UK), or 6) Protein kinase
134 C (PKC) activator (Phorbol 12-myristate 13-acetate: PMA; 10 ng/mL, Merck Millipore). Each
135 experiment was performed in duplicate and repeated at least three times.
136
137 *Quantitative RT-PCR (qRT-PCR)*
138 The CLs used for a quantitative gene expression analysis were classified as early-luteal (1-5
139 days after the LH surge, n=4), mid-luteal (6-10 days, n=6), late-luteal (11-14 days, n=6),
140 menstrual phase (over 14 days, n=3), and rescued by hCG injection (n=4). Total RNA was
141 extracted from the frozen human CL or cultured LGCs using RNeasy Mini Kit (Qiagen Ltd.,
142 Crawley, UK) following the manufacturer's protocol. RNA (200 ng) was used to prepare cDNA
143 using the TaqMan Reverse Transcription regents (Applied Biosystems, Foster City, CA, USA).

144 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 H_2O, 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 $^{\Delta}$Ct or $^{\Delta\Delta}$Ct 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^{TM} 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; Figure 3A). In LGCs *in vitro*, hCG significantly decreased the mRNAs expression of all three *BMPs* (P<0.001; Figure 3B). This confirms that the expression of BMP transcripts is hormonally regulated in luteal steroidogenic cells.

In addition, hCG reduced the mRNA expression of BMP receptors in cultured LGCs (*BMPR1A* P<0.05; *BMPR1B* P<0.01; *BMPR2* P<0.05; Figure 3C). HCG also altered the transcript abundance of other regulators involved in BMP function (Figure 3D). Expression of an antagonist for BMPs, *GREM2*, was up-regulated in LGCs by hCG (P<0.001). In contrast, the other BMP antagonists, *NOG* (P<0.01) and *GREM1* (P<0.001) were decreased by hCG as was an inhibitor of Smad activation, *SMAD6* (P<0.05; Figure 3D). These data suggest that hCG would be consistent with an inhibition of luteal BMP activity by down-regulating the expression of BMPs and their receptors.

BMPs are differentially regulated at a molecular level

To reveal the regulatory mechanism of luteal 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 BMP transcripts in LGCs. Inhibition of PI3-kinase (LY294002) tended to reduce *BMP* mRNA abundances although only *BMP6* was significantly decreased (P<0.001; Figure 4A). Interestingly, manipulation of the PKA and PKC pathways revealed different effects according to BMP subtypes. Inhibition of PKA (H89) increased the expression of both *BMP2* (P<0.05) and *BMP4* (P<0.05) while the expression of *BMP6* was reduced (P<0.01; Figure 4B). Activation of PKC (PMA) inhibited *BMP4* and *BMP6* expression in LGCs (P<0.0001; Figure 4C) while dramatically increasing the expression of *BMP2* (P<0.01; 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 BMP expression after 8 hours of treatment. This resulted in

265 the same changes as seen after 24 hours (Supplementary Figure 1). Although BMPs are all
266 down-regulated by hCG, there are differential molecular pathways involved in their regulation
267 in human LGCs.
268
269 *BMPs have a similar effect to Activin on LGC function*
270 Activin has an opposite effect to hCG in regulating the function of human LGCs *in vitro* (11).
271 Administration of hCG increased the expression of *LHCGR* (P<0.001; Table 2) and
272 steroidogenic acute regulatory protein (*STAR*; P<0.001; Table 2) whereas addition of Activin A
273 decreased the expression of *LHCGR* (P<0.001; Table 2) and *STAR* (P<0.001; Table 2).
274 Treatment of cells with BMP2, BMP4 or BMP6 also reduced the expression of both *LHCGR*
275 (P<0.001; Table 2) and *STAR* (P<0.001; Table 2). Like Activin A, all three BMPs up-regulated
276 the expression of follicular stimulating hormone receptor (*FSHR*; P<0.01-0.001; Table 2) and
277 *CYP19A1* (P<0.05–0.01; Table 2).
278 Activin A promoted its own synthesis by increasing production of Inhibin/Activin β
279 subunits, *INHBA* (P<0.05; Table 2) and *INHBB* (P<0.01; Table 2). BMP4 and BMP6 had
280 similar effects to Activin A and displayed opposite effects to hCG on the expression of
281 Inhibin/Activin β subunits with marked stimulatory effects on *INHBB* (P<0.05–0.01; Table 2).
282 Unlike the effect of Activin A on stimulating the synthesis of its consistent subunits, BMPs
283 suppressed their own synthesis in LGCs (P<0.05-0.01; Table 2), and Activin A also suppressed
284 BMP expression (P<0.01; Table 2). Thus the effects of BMPs during luteolysis are similar to
285 those of Activin A.
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289 **Discussion**

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

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

308 If BMPs have a role in the regulation of the CL, it would be expected that their
309 expression in the CL would change across the luteal phase. We showed that BMP mRNA
310 abundance remarkably changed across the luteal phase: the mRNA expression of *BMP2*, *BMP4*,
311 and *BMP6* all increased in the late-luteal phase. This would be consistent with that BMPs have
312 an important role during luteolysis. Although the mechanism of luteolysis in rodents differs

313 from that in women, the expression of BMP2 and BMP6 in the rodent CL increased at the time
314 of regression (18). This implies that BMP activity during luteolysis may be conserved in
315 disparate species with different paradigms of CL regulation.

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

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

334 In the human CL the mRNA abundance of *BMP2*, *BMP4* and *BMP6* was maximal in the
335 late luteal phase. Interestingly after menstruation had commenced the luteal expression of
336 *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 $PGF_{2\alpha}$, and Priyanka *et al.* reported that BMP2 was increased by $PGF_{2\alpha}$ stimulation in the CL of bonnet monkey (30). Thus locally released $PGF_{2\alpha}$ 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

385 human CL, and are suppressed by luteotrophic hCG both *in vivo* and *in vitro*, suggesting their
386 involvement in the regulation of luteolysis. Like Activin A, BMPs possess opposite effects to
387 hCG, and suppress luteinization and progesterone production in human luteinized steroidogenic
388 cells. The present study provides the evidence that BMPs are one of the predominant mediators
389 of luteolysis in the human CL.
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Acknowledgement

We thank Dr Fiona Connolly and Ms Eva Gay, The University of Edinburgh, for their excellent technical support and kind advice.

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553 **Figure and Table legends**

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

556 Immunoreactivity for p-Smad 2/3, activated by Activin signaling, is found in nuclei of granulosa
557 lutein cells (**A**), theca lutein cells (*insert* in **A**), and endothelial cells (*asterisk* in **A**), as well as
558 non-steroidogenic cells (*arrowheads* in **A**). Intense immunoreaction for p-Smad 1/5/8, which
559 represents an activation of BMP signaling, is found in nuclei of granulosa lutein cells (**B**), theca
560 lutein cells (*insert* in **B**), and endothelial cells (*asterisk* in **B**) but not in non-steroidogenic cells
561 distributed in the CL parenchyma (*arrowheads* in **B**). BMP receptors are immunolocalized to
562 granulosa lutein and theca lutein cells (BMPR1A: **C**, BMPR1B: **D**, BMPR2: **E**) while
563 non-steroidogenic cells in the CL parenchyma are negative in reaction (*arrowheads* in **C**–**E**).
564 Endothelial cells are also immunostained by the antibodies for all BMP receptors (*asterisks* in
565 **C**–**E**). BMP4 (**F** and **G**) and BMP6 (**H** and **I**) immunoreactivities are localized to both
566 granulosa lutein and theca lutein cells (arrows in **F-I**), and absent in negative control (*insert* in
567 **F**). Non-steroidogenic cells in the CL parenchyma are negative in reaction for both BMP4 and
568 BMP6 (*arrowheads* in **F**–**I**). Endothelial cells are also immunostained with both BMP4 and
569 BMP6 (*asterisks* in **F**–**I**). Endothelial cells are immunolabeld with CD31 (*asterisks* in **J**) and
570 steroidogenic cells are positive in 3β-HSD immunoreaction (**K**). Non-steroidogenic cells are
571 distributed between capillary vessels and steroidogenic cells but negative for both
572 immunoreaction (*arrowheads* in **J** and **K**). **L** is a schema of endothelial cells, granulosa lutein
573 cells, and non-steroidogenic cells distinguishable by their morphology, distribution, and
574 immunoreactivity for CD31 or 3β-HSD. The localization of the immunoreactions does not
575 change across luteal phase and the representative tissue sections from the mid-luteal phase are
576 used for illustration. Arrows in **C**–**I** show theca lutein cells.

577

578 **Figure 2. Changes in the expression of BMP transcripts in the human CL throughout the**
579 **luteal phases.**

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

586

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

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

599

600 **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*<0.0001.

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.

625 **Tables**

Table 1. Primers used for qPCR analysis

Gene name	Protein name	Accession no.	Forward primer	Reverse primer	Product size
G6PDH	G6PDH	NM_000402	CGGAAACGGTCGTACACTTC	CCGACTGATGGAAGGCATC	155 bp
BMP2	BMP2	NM_001200	AGAAGGAGGAGGCAAAGAAA	AAGCAGCAACGCTAGAAGAC	181 bp
BMP4	BMP4	NM_001202	TACATGCGGGATCTTTACCG	GGGATGCTGCTGAGGTTAAA	200 bp
BMP6	BMP6	NM_001718	ACAGCATAACATGGGGCTTC	GTCCTGGGACTGGGTAGAGC	217 bp
BMPR1A	BMPR1A	NM_004329	CTCATCAAGAAAAATGGGAGTTG	CCACGATCCCTCCTGTGATA	247 bp
BMPR1B	BMPR1B	NM_001203	AAATGTGGGCACCAAGAAAG	ACAGGCAACCCAGAGTCATC	171 bp
BMPR2	BMPR2	NM_001204	GCAGGTTCTCGTGTCTAGGG	GGATTGACTGTTGGGCTCAC	213 bp
NOG	Noggin	NM_005450	ACCTCATCGAACACCCAGAC	AGCCCTCGGAGAACTCTAGC	252 bp
CHRD	Chordin	NM_003741	CAGGAGTGGGGGACTAACC	CAGCACCTCAGCAAAGCCT	118 bp
GREM1	Gremlin 1	NM_013372	GCAAATACCTGAAGCGAGAC	CGATGGATATGCAACGACAC	288 bp
GREM2	Gremlin 2	NM_022469	CACGTCATTGCAGGATGTTC	AGCACCTCCTTGATCTGGTG	172 bp
SMAD6	Smad6	NM_005585	CTGAAACGGAGGCTACCAAC	CTGGTCGTACACCGCATAGA	155 bp
SMAD7	Smad7	NM_005904	AGGGGGAACGAATTATCTGG	GCACCAGCTGACTCTTGTTG	227 bp

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

	Treatments				
	hCG	Activin A	BMP2	BMP4	BMP6
LHCGR	↑ 3.58 ± 0.43***	↓ 0.19 ± 0.03***	↓ 0.59 ± 0.03***	↓ 0.25 ± 0.04***	↓ 0.26 ± 0.05***
STAR	↑ 18.04 ± 1.56***	↓ 0.31 ± 0.06***	↓ 0.57 ± 0.02***	↓ 0.32 ± 0.04***	↓ 0.23 ± 0.02***
FSHR	↓ 0.16 ± 0.09***	↑ 4.10 ± 0.81**	↑ 2.08 ± 0.31**	↑ 3.35 ± 0.58***	↑ 2.91 ± 0.42***
CYP19A1	→ 1.23 ± 0.14	↑ 1.93 ± 0.29**	↑ 1.75 ± 0.22*	↑ 1.98 ± 0.37**	↑ 2.03 ± 0.17**
INHA	↑ 5.03 ± 0.67***	→ 1.79 ± 0.24	→ 1.63 ± 0.26	↑ 3.12 ± 0.48*	↑ 3.17 ± 0.44**
INHBA	↓ 0.41 ± 0.06**	↑ 1.67 ± 0.19*	→ 1.65 ± 0.15	↑ 3.04 ± 0.49**	↑ 3.00 ± 0.41**
INHBB	↓ 0.02 ± 0.01**	↑ 33.25 ± 11.48**	→ 2.24 ± 0.20	↑ 17.58 ± 4.64*	↑ 23.09 ± 7.05**
BMP2	↓ 0.20 ± 0.02***	↓ 0.13 ± 0.01**	↓ 0.28 ± 0.06**	↓ 0.13 ± 0.02**	↓ 0.14 ± 0.02**
BMP4	↓ 0.28 ± 0.03***	↓ 0.12 ± 0.03**	↓ 0.42 ± 0.08**	↓ 0.11 ± 0.02**	↓ 0.10 ± 0.02**
BMP6	↓ 0.15 ± 0.03***	↓ 0.65 ± 0.09*	↓ 0.69 ± 0.06**	↓ 0.56 ± 0.11**	↓ 0.58 ± 0.10**

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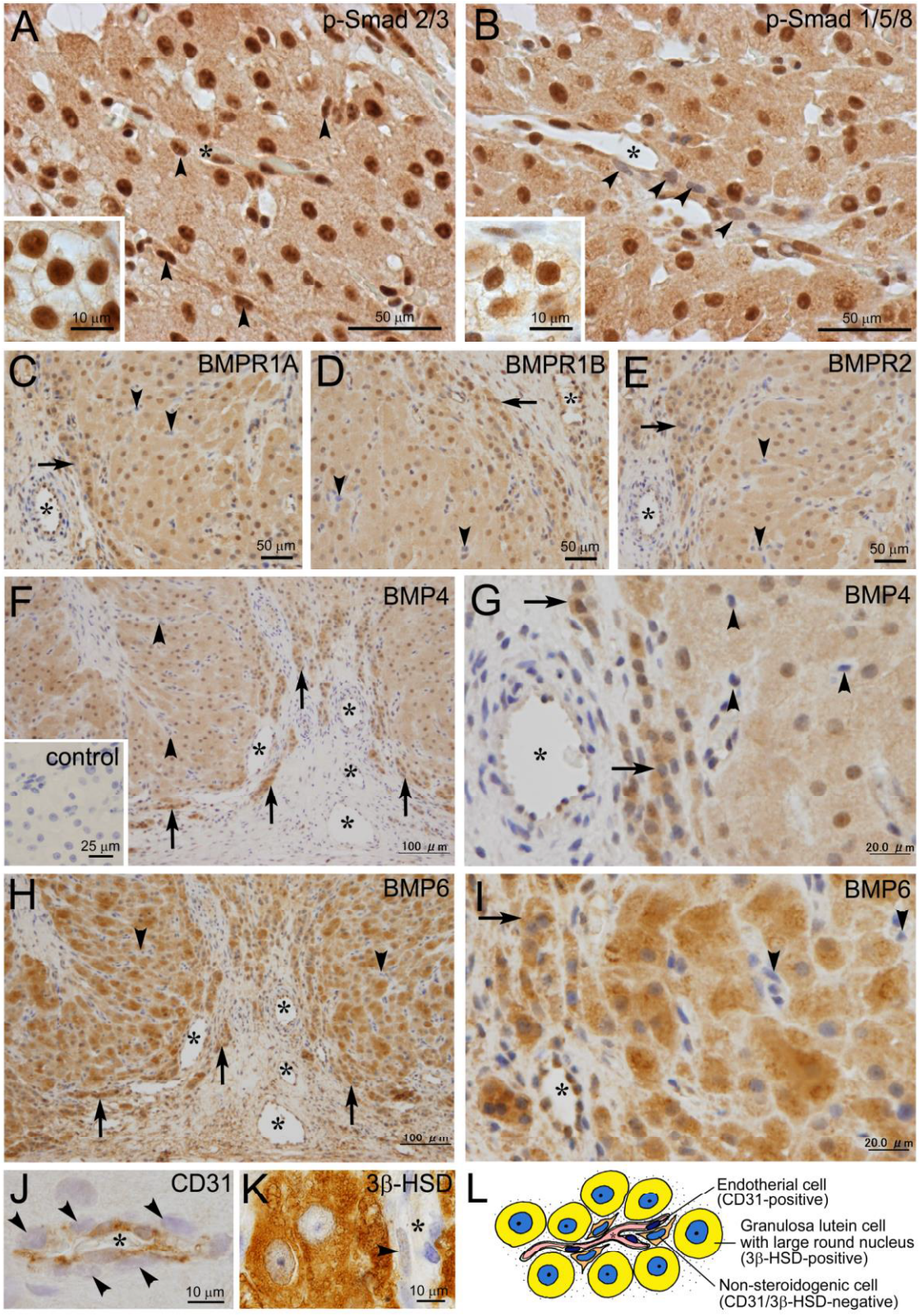


Figure 1 Nio-Kobayashi et al.

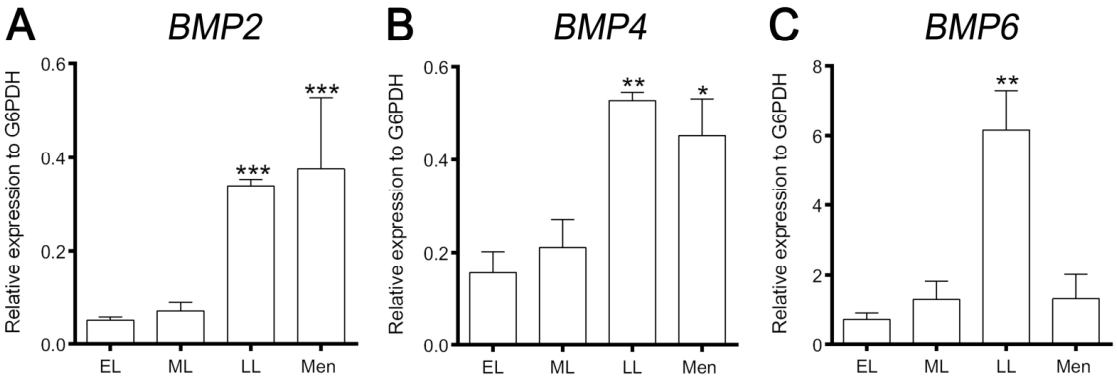


Figure 2 Nio-Kobayashi et al.

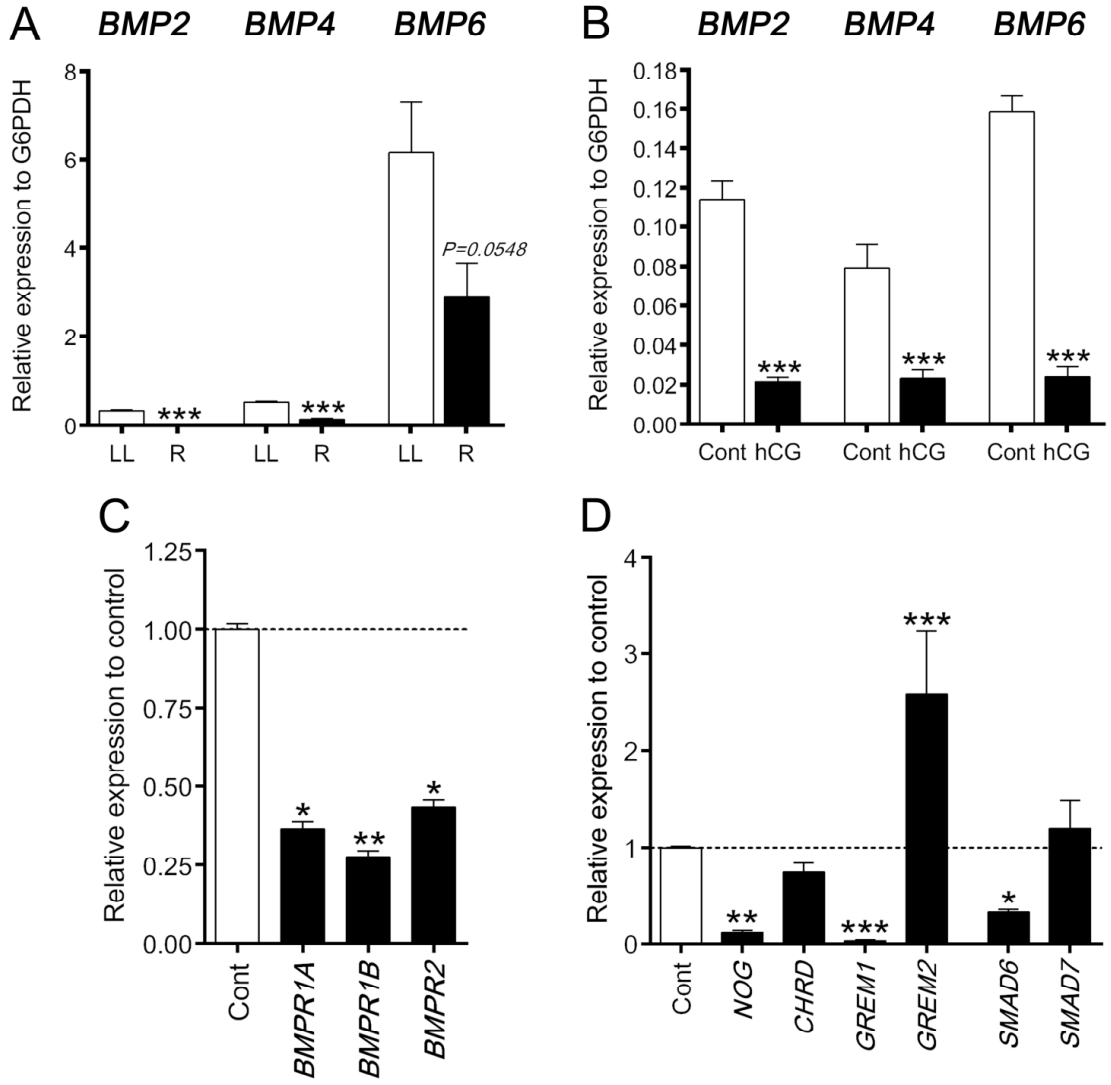


Figure 3 Nio-Kobayashi et al.

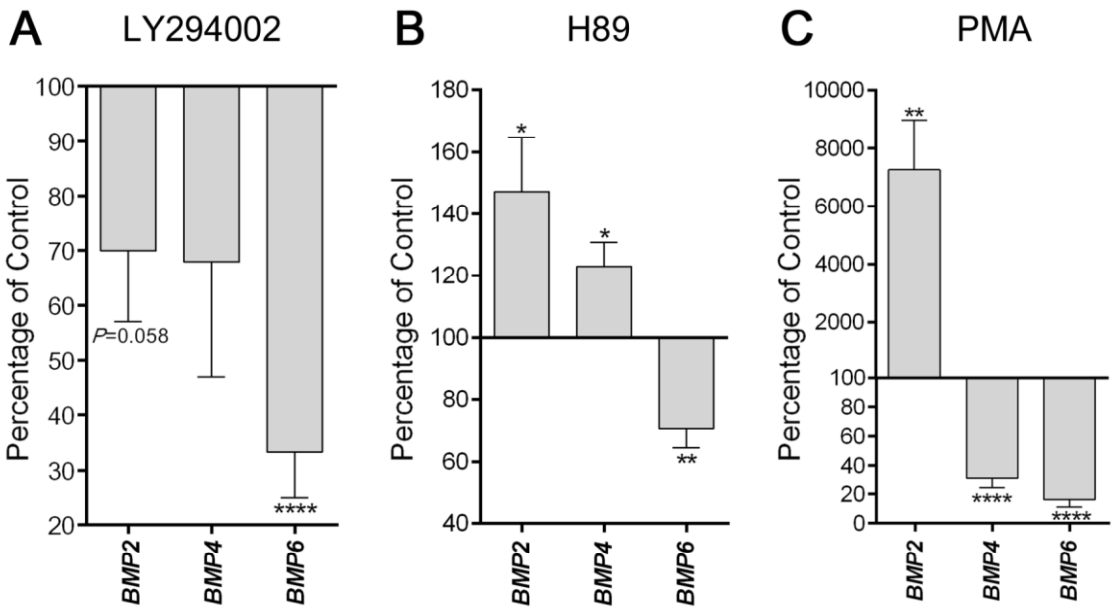
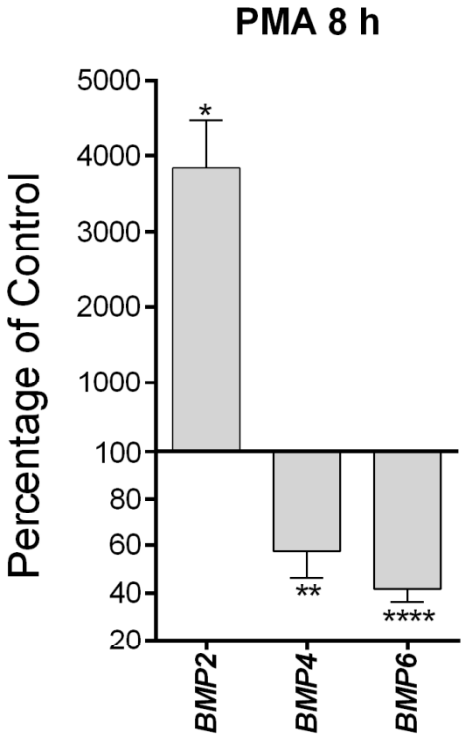


Figure 4 Nio-Kobayashi et al.



Supplementary Figure 1
Nio-Kobayashi et al.