Bone morphogenetic protein-2 enhances gonadotropin-independent follicular development via sphingosine kinase 1

Running title: BMP-2 and SPHK1 in early follicular development

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ABSTRACT

Problem: Pre-ovulatory mature follicles are not readily induced from gonadotropin (Gn)-independent early follicles in the poor ovarian response (POR) state, characterized by reduced number of retrieved oocytes. Bone morphogenetic protein (BMP), which is expressed in the ovary, contributes to early folliculogenesis, but its precise underlying mechanism remains unknown. The purpose of this study was to examine the effects of BMP-2 on granulosa cells (GCs) of Gn-independent early follicles.

Method of study: Sphingosine kinase 1 (SPHK1) localization, which produces sphingosine 1-phosphate (S1P), was examined in human early follicles by immunohistochemistry. *SPHK1* mRNA levels were examined in Gn-independent bovine GCs (bGCs) and human nonluteinized granulosa cell line (HGrC1) cells. Phosphorylated Yes-associated protein (YAP) expression was evaluated by Western blot, and its localization was evaluated immunocytochemically in bGCs. Verteporfin, a selective YAP inhibitor, was used to explore the influence of YAP on BMP-2–induced bGCs proliferation.

Results: The expression of SPHK1 was observed in human GCs of primary and secondary follicles. BMP-2 significantly induced *SPHK1* mRNA expression in bGCs and HGrC1 cells. Both BMP-2 and S1P decreased phosphorylated YAP protein levels and induced the nuclear translocation of YAP significantly, thereby increasing the number of bGCs by suppressing the Hippo pathway. This BMP-2–induced cell proliferation was completely blocked by verteporfin.

Conclusions: This is a first report showing that BMP-2 up-regulated *SPHK1* mRNA expression in

GCs and promoted GCs proliferation through Hippo pathway suppression. Thus, BMP-2 contributes

to Gn-independent folliculogenesis via SPHK1, suggesting a potential therapeutic strategy for the POR

patients with follicular dysgenesis.

KEYWORDS

bone morphogenetic protein (BMP), gonadotropin-independent early follicular development, Hippo

pathway, poor ovarian response (POR), sphingosine kinase 1 (SPHK1), sphingosine 1-phosphate

(S1P)

1. INTRODUCTION

Poor ovarian response (POR) patients to ovarian stimulation usually indicates a reduction in follicular response, resulting in a reduced number of retrieved oocytes in *in vitro* fertilization (IVF).[1] Although the ovary in patients with POR still has multiple secondary follicles,[2] these early follicles rarely to never proceed to pre-ovulatory mature follicles by routine follicle stimulating hormone (FSH) administration. Since FSH is currently the main factor used for fertility treatment, a new strategy for these people is needed.

Bone morphogenetic protein (BMP) is an extracellular signaling molecule that is a member of the transforming growth factor (TGF)-β superfamily, and plays multiple roles in regulation of the growth, differentiation, and apoptosis of numerous cell types.[3] A recent study revealed that TGF-β superfamily members, including BMP and growth differentiation factor (GDF)-9 are involved in female fertility in mammals.[4-6] Follicles, which are composed of oocytes, granulosa cells (GCs), and theca cells, produce these TGF-β superfamily member proteins. Follicular development is classified into three stages: the gonadotropin (Gn)-independent initial development stage, FSH-dependent progression stage, and luteinizing hormone-responsive maturation stage.[7] TGF-β superfamily member proteins, but not Gn, mainly contribute to development of early follicles in the Gn-independent preantral phase.[4] BMP-15 was shown to promote the primordial-to-primary follicle transition in sheep,[8] and GDF-9–deficient female mice are infertile due to the arrest of follicle development at the

primary stage.[6] Moreover, BMP-2 was shown to promote follicular survival by maintaining the

proliferation of GCs during antral follicle growth.[4] Follicular development depends on the

proliferation, differentiation, and survival of the component cells.[9] As a reason to promote follicular

development, TGF-β superfamily member proteins can promote the proliferation of GCs.[5] In humans,

patients with hypergonadotropic ovarian failure with a *BMP15* gene mutation show reduced

proliferation rates of GCs.[10] In fact, the BMP-2 expression level in GCs is significantly increased

during the primary-to-secondary follicle transition, along with a concomitant increase during early

follicular development in ovaries of adult cycling rats.[5] In this regard, my group previously reported

that BMP-2 and BMP-6 were expressed in GCs, and BMP-7 in theca cells of human ovary.[11-13]

However, the precise underlying mechanism by which BMP influences Gn-independent early

folliculogenesis has not been elucidated to date. BMP-2 is widely used in orthopedic surgery and bone

tissue engineering because of its strong osteogenic activity.[14] Moreover, only BMP-2, produced by

GCs, and BMP-7, producer by theca cells, have received the approval from the United States Food

and Drug Administration (FDA) to be used in combination with collagen type I, in orthopedic

surgery.[14] Looking ahead to future clinical use of BMP in the field of reproductive medicine, I focused

my attention on BMP-2, produced by GCs, in *in vitro* experiments.

Sphingosine 1-phosphate (S1P) is recognized as an essential stimulator of follicular

development in the Gn-independent preantral phase.[9] S1P is a bioactive sphingolipid metabolite and

an important lipid mediator in many biological processes.[15] In particular, S1P is known to suppress the Hippo pathway, which contributes to organ size control and tumorigenesis.[16,17] Activation of the Hippo pathway suppresses cell proliferation, resulting in limited organ sizes or suppressed tumorigenesis in various tissues. Suppression of the Hippo pathway by S1P is mediated by Yes-associated protein (YAP), which is dephosphorylated via the inhibition of large tumor suppressor (LATS)1/2 kinase by S1P, resulting in cell proliferation.[17] In folliculogenesis, S1P promotes ovarian follicle growth by suppressing the Hippo pathway in mice.[18] S1P expression has been detected in human follicular fluid,[19] and two S1P receptors, S1PR1 and S1PR3, were found to be expressed at a higher level than S1PR2 or S1PR5 in human luteinized GCs.[20] The production of S1P, formed by the phosphorylation of sphingosine, is catalyzed by sphingosine kinase 1 (SPHK1) in the cytosol.[21] SPHK1 promotes cell proliferation via increasing the S1P expression level.[22] However, the regulation of SPHK1 in early folliculogenesis remained elusive.

In the present study, I evaluated the role of BMP-2 in early follicular development via its effects on SPHK1 expression. I further explored the underlying mechanism with respect to GCs proliferation and the effects of BMP-2 on the Hippo pathway through S1P mediated-YAP activation. Elucidation of these mechanism might contribute to new insight for developing a treatment for the follicular dysgenesis in patients with POR.

This dissertation is based on an original paper[23] accepted to an academic journal.

2. MATERIALS AND METHODS

2.1 Reagents and materials

Fetal bovine serum (FBS) and an antibiotics mixture of penicillin, streptomycin, and amphotericin B (161-23181) were purchased from Wako (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM)-high glucose (D5796) and verteporfin (VP; 129497-78-5) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human serum albumin/sphingosine 1-phosphate complex (Huzzah S1P; 360492) was obtained from Avanti Polar Lipids, Inc. (Alabama, AL, USA). Recombinant human BMP-2 was a gift from Osteopharma Inc. (Osaka, Japan). Human nonluteinized granulosa cell line (HGrC1)[24] cells were the gift from Dr. Iwase (Gunma University, Gunma, Japan).

2.2 Collection of ovarian tissues

The experimental procedures were approved by the institutional review board of University of Toyama (ORB-No. 22-20). Informed consent was obtained from each patient. Six tissue specimens of human ovaries were obtained from six women who underwent salpingo-oophorectomy for the treatment of uterine cervical cancer. All patients had normal ovarian cycles prior to the surgery without exogenous hormone treatment, and no histological abnormality was detected in the ovarian tissues.

2.3 Immunohistochemistry

Immunohistochemistry was performed as described previously.[25] In brief, formalin-fixed, paraffin-embedded tissues were sliced to a 5-μm thickness and mounted on slides. The ovarian sections were deparaffinized in xylene, rehydrated through a graded series of ethanol, and washed in water. Antigen retrieval was performed using sodium citrate buffer (10 mmol/l, pH 6.0) by microwaving for 10 min and then cooling to room temperature. The primary antibodies (Abs) were SPHK1 rabbit polyclonal Ab (LS-A3106, LSBio, Seattle, WA, USA) and normal rabbit IgG (sc-2027, Santa Cruz Biotechnology, Dallas, TX, USA). The secondary Ab was from the Envision+ System/ horseradish peroxidase (HRP) rabbit (DAB+) kit (K5027, DAKO, Santa Clara, CA, USA). Slide staining with the primary and secondary Abs was performed according to the manufacturer's instructions. In brief, the specimen was primarily treated with SPHK1 Ab (dilution 1:100) or rabbit IgG as a negative control, and subsequently treated with the Envision+ System/HRP rabbit (DAB+) kit. Images were taken with a Keyence BZ-X801 microscope (Keyence, Osaka, Japan).

2.4 Preparation of bovine GCs (bGCs)

Bovine ovaries with a regressing corpus luteum were collected from adult, cycling heifers at a local abattoir, and immediately transported to the laboratory in an ice cold-buffered salt solution. bGCs at the Gn-independent phase were prepared as described previously.[26] In brief, the GCs were harvested from small-sized follicles (diameter of 4 mm or less) using aseptic needle aspiration. In bovine, these

GCs are assumed to be in the Gn-independent phase just prior to their recruitment into a follicular wave.[27] The bGCs were washed in ammonium-chloride-potassium lysing buffer twice, and resuspended in a culture medium comprising DMEM supplemented with 10% FBS and 1% antibiotics.

2.5 Treatment of cultured cells

To evaluate the effect of BMP-2 on *SPHK1* mRNA induction, bGCs were incubated with different concentrations of BMP-2 (10, 50, 100, 200, or 300 ng/ml) without FBS for 4–72 h. BMP-2 exerted its maximum effect on *SPHK1* expression at 100 ng/ml; hence, 100 ng/ml BMP-2 was selected for the following experiments. HGrC1 cells were incubated with 100 ng/ml BMP-2 without FBS for 8 h for *SPHK1* mRNA induction. For FSH receptor (*FSHR)* mRNA induction, primary bGCs were incubated with 100 ng/ml BMP-2 without FBS for 24 h.

To evaluate the effect of S1P on cell proliferation, bGCs were incubated with S1P (300 nM) in DMEM with 2.5% FBS. Administration of S1P to bGCs was performed for 1 h without FBS after a 12-h period of serum starvation for experiments other than cell proliferation assay, based on a previous study.[28]

For immunofluorescent staining, bGCs were seeded in a chamber slide (30104, SPL Life Sciences, Korea) and maintained in serum-free medium with BMP-2 for 16 h or with S1P for 1h. In Western blot analysis, bGCs were treated with BMP-2 in DMEM with 1% FBS for 48 h or with S1P

in serum-free medium for 1 h. To evaluate the effect of the YAP inhibitor VP on bGCs proliferation,

the cells were pre-treated with VP at different concentrations (10–1000 nM) for 10 min and then

incubated for 24 h as described previously.[28] To evaluate the effect of VP on BMP-2–induced cell

proliferation, bGCs were pre-incubated with 100 nM VP, which did not affect cell proliferation, for 10

min followed by treatment with BMP-2 for 24 h.

2.6 Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNA was extracted from bGCs or HGrC1 cells using ISOGEN (311-07361, Nippon Gene, Tokyo,

Japan) and Ethachinmate (312-01791, Nippon Gene). The amount of total RNA was determined based

on the absorbance measured at 260 nm. RT was performed using ReverTra Ace qPCR Master Mix

with gDNA Remover (FSQ-301, TOYOBO, Tokyo, Japan). Total RNA (500 ng to 1 μg) was reverse-

transcribed in a 20-μl volume to obtain complementary DNA. A 1/20 volume of the resultant solution

(1 μl) was then used as a template in qPCR for quantification of various mRNA levels using the

Mx3000P Real-Time PCR System (401403, Agilent Technologies, Santa Clara, CA, USA), according

to the manufacturer's instructions. The PCR primers used with the SYBR Green method were selected

from different exons of the corresponding genes to discriminate PCR products that might arise from

possible chromosomal DNA contaminants. The relative mRNA levels were calculated using the

standard curve method and were normalized to the mRNA levels of bovine *GAPDH* or human *L19*.

The primer sequences and PCR conditions are shown in Table 1.

2.7 Cell proliferation assay

The cell proliferation assay was performed as described previously.[29] In brief, pre-confluent bGCs

were treated with 100 ng/ml BMP-2 or 300 nM S1P in DMEM with 2.5–5% FBS for at least 24 h, and

the medium was replaced and BMP-2 or S1P was replenished every 24 h. Cell proliferation was

investigated using a Cell Counting Kit-8 (CCK-8; 343-07623, Dojindo, Kumamoto, Japan), based on

the colorimetric assay method. In brief, CCK-8 reagent was added to each well of the plate. The

samples were incubated for 4 h at 37°C in the dark and the absorbance of the supernatant was measured

at a wavelength of 450 nm using a microplate reader (1681135JA, Bio-Rad Laboratories, Hercules,

CA, USA).

2.8 Immunocytochemistry and immunofluorescence

Immunocytochemistry was carried out as described previously.[30] In brief, bGCs were fixed with 4%

formaldehyde diluted in warm phosphate-buffered saline (PBS) for 15 min. The primary Ab was YAP

XP rabbit monoclonal Ab (mAb) (D8H1X; 14074, Cell Signaling Technology, Danvers, MA, USA)

and the secondary Ab was anti-rabbit IgG (H+L), F(ab')$_2$ fragment (Alexa Fluor 488 Conjugate; 4412,

Cell Signaling Technology). The cells were incubated with YAP primary Ab (dilution 1:100) overnight

at 4°C. After washing with PBS, the cells were incubated with the secondary Alexa Fluor 488-conjugated Ab (dilution 1:1000) for 2 h at room temperature in the dark. Coverslipped slides were mounted with VECTASHIELD MOUNTING MEDIUM with DAPI (H-1200, Vector Laboratories, Burlingame, CA, USA), and images were taken with the Keyence BZ-X801 fluorescent microscope. I counted at least 50 YAP-stained cells from five randomly selected fields at 40× magnification, and calculated the proportion of nuclear YAP-positive cells to total counted cells.

2.9 Western blot analysis

Western blot analysis was performed as described previously.[31] In brief, the cells treated as described above were washed with cold PBS, incubated with 10% trichloroacetic acid solution (208-08081, Wako) for 30 min on ice, harvested, and lysed in RIPA buffer (182-02451, Wako) containing a protease inhibitor cocktail (11697498001, Roche, Basel, Switzerland) and 1% phosphatase inhibitor (P5726, Sigma-Aldrich). Protein concentrations were evaluated by the Quick Start Bradford 1× Dye Reagent kit (500-0205, Bio-Rad Laboratories). Samples treated with 2×Laemmli sample buffer (1610730, Bio-Rad Laboratories) and 2-mercaptoethanol (190242, MP Biomedicals, Inc., Solon, OH, USA) were heated at 95°C for 5 min. Electrophoresis was then performed on a SuperSep Ace polyacrylamide gel (191-15031, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). Proteins were blotted onto an Immun-Blot polyvinylidene fluoride membrane (162-0175, Bio-Rad Laboratories). The transferred

membranes were blocked with Blocking Solution in Tris-buffered saline for immunoassays (pH 7.2) (05151-35, Nacalai Tesque, Kyoto, Japan) for 30 min at room temperature. The primary Abs were phospho-YAP (Ser127) rabbit mAb (D9W21; 13008, Cell Signaling Technology), YAP XP rabbit mAb (14074, Cell Signaling Technology), and β-actin mouse mAb (8H10D10; 3700, Cell Signaling Technology). The membranes were incubated with primary Abs overnight at 4°C. The secondary Abs were HRP-conjugated anti-rabbit IgG Ab (7074) and HRP-conjugated anti-mouse IgG Ab (7076), both from Cell Signaling Technology. Images were taken with Multi Imager (Mi-280CB, BioTools Inc., Maebashi, Japan). The band intensities were quantified with ImageJ analysis software (National Institutes of Health, Bethesda, MD, USA; https://imagej.nih.gov/ij/index.html), and the relative ratio of phosphorylated YAP (pYAP) to total YAP (tYAP) was calculated.

2.10 Statistical analysis

Nonparametric tests (Mann-Whitney U test) were performed using JMP Pro 14 software (SAS Institute Inc., Cary, NC, USA). A p-value of less than 0.05 was considered statistically significant.

3. RESULTS

3.1 SPHK1 localization in human GCs

I first confirmed the localization of SPHK1 during early follicular development in human ovarian specimens by immunohistochemistry (Figure 1). SPHK1 expression was clearly detected in oocytes and GCs of primary, early- secondary, and late-secondary follicles (Figure 1B–D), but not evident in pre-GCs of primordial follicles (Figure 1A). The positive rate of SPHK1 staining in GCs tended to be higher in primary or secondary follicles compared with that in the primordial follicles (Figure 1G). By contrast, the level of SPHK1 expression was stable in the oocytes at each follicular stage. These results indicated that SPHK1 expression in GCs likely increases from the primary follicle stage.

3.2 BMP-2 promotes *SPHK1* and *FSHR* mRNA expression and cell proliferation in GCs.

To clarify the induction of SPHK1 expression in human GCs, I focused on BMP-2, a member of the TGF-β superfamily, which is known to be expressed in human GCs and its level was found to increase significantly during the primary-to-secondary follicle transition in rats.[5] I first examined *SPHK1* mRNA levels in bGCs treated with BMP-2. BMP-2 treatment significantly increased *SPHK1* mRNA levels in bGCs (Figure 2A). The maximum increase of *SPHK1* mRNA was 2.1-fold with 100 ng/ml BMP-2 compared with the control ($p < 0.001$) (Figure 2A); therefore, GCs were treated with 100 ng/ml BMP-2 in subsequent experiments. With respect to the time-dependent effects, BMP-2 induced

SPHK1 mRNA in bGCs at 4 h, and the effect was continuously observed until 72 h (Figure 2B).

Consistent with the increase of *SPHK1* mRNA levels, which is known to enhance cell proliferation in

various cell types,[22] the number of bGCs significantly increased in response to BMP-2 stimulation

(1.08-fold compared with day0 of BMP-2, 1.20-fold compared with the control, $p < 0{,}05$) (Figure 2C).

BMP-2 also enhanced *SPHK1* mRNA expression (Figure 2D) in the HGrC1 cells, human

nonluteinized granulosa cell line, similar to the results found in bGCs. In addition, the mRNA

expression level of *FSHR*, which is required for GCs differentiation, was increased significantly by

BMP-2 treatment in primary bGCs ($p < 0.05$) (Figure 2E). These results suggested that BMP-2

enhanced proliferation via *SPHK1* expression in both bGCs and human GCs.

3.3 S1P stimulated the proliferation of bGCs

SPHK1, which catalyzes the conversion of sphingosine to S1P, promotes cell proliferation of NIH

3T3 fibroblasts.[21,22] S1P is also known to promote ovarian follicular growth in mice.[18] Therefore, I

further investigated the effect of S1P on the proliferation of bGCs by administering S1P to the cells

exogenously. The number of bGCs did not increase in the control culture condition, whereas 300 nM

of S1P significantly stimulated the proliferation of bGCs in a time-dependent manner (1.14-fold

compared with day0 of S1P, 1.26-fold compared with the control, $p < 0{,}05$) (Figure 3). These results

suggested that the S1P mediated-pathway to increase cell numbers, Hippo pathway, might be

responsible for BMP-2–induced cell proliferation in bGCs.

3.4 S1P suppresses the Hippo pathway in bGCs

To further clarify the mechanism of S1P-mediated bGCs proliferation, I investigated the phosphorylation and localization of YAP, which is activated by S1P via suppression of LATS1/2 kinases in the Hippo pathway.[17] Since YAP is dephosphorylated when the Hippo pathway is suppressed, I evaluated the amount of pYAP by Western blot analysis. DMEM with 10% FBS treatment was used as a positive control for pYAP reduction, and norepinephrine was used as a negative control.[17] As shown in Figure 4A, S1P decreased the expression level of pYAP, but did not affect the total amount of YAP in bGCs. The ratio of pYAP to tYAP was significantly lower ($p < 0.01$) in bGCs treated with S1P compared with that of the control (Figure 4B). S1P reduced the pYAP protein level by 48% compared with that of the control, and the mean ratio was similar to that of the cells treated with DMEM with 10% FBS as a positive control (Figure 4B). The dephosphorylated form of YAP, which is the active form, is localized in the nucleus to stimulate cell proliferation.[17] Immunofluorescent microscopic analysis showed that YAP was localized in the cytoplasm under the non-stimulated condition (Figure 4C, D), but rapidly translocated to the nucleus in response to S1P treatment after 1 h (Figure 4E, F). Consistent with the results of the pYAP/tYAP ratio, the rate of nuclear-localized YAP was significantly increased in the S1P-treated group than that in the control group ($41.6 \pm 13.7\%$ vs.

$2.4 \pm 2.7\%$, $p < 0.05$) (Figure 4G). I further analyzed whether S1P increased the mRNA expression

level of connective tissue growth factor (*CTGF*), a proliferation related-gene, which is the direct target

of the YAP-TEA domain transcription factor (TEAD) transcriptional active complex, in bGCs, because

S1P–induced cell proliferation was significant but also relatively "slight" as an effect. I confirmed that

S1P significantly increased the expression level of *CTGF* in bGCs ($p < 0.05$) (Figure 4H). These results

demonstrated that S1P promoted cell proliferation via pYAP reduction and YAP nuclear translocation,

reflecting Hippo pathway suppression, in bGCs.

3.5 YAP is involved in BMP-2–induced bGC proliferation

As shown in Figure 2, BMP-2 increased *SPHK1* mRNA levels and enhanced cell proliferation in bGCs.

As SPHK1 catalytically increases S1P production, which was found to suppress the Hippo pathway in

bGCs as described above, I next evaluated the effects of BMP-2 on the dephosphorylation and nuclear

translocation of YAP. Western blot analysis showed that BMP-2 treatment significantly reduced the

pYAP protein level by 25% compared with that of the control ($p < 0.05$) (Figure 5A, B).

Immunofluorescent microscopic analysis further showed that the nuclear translocation of YAP protein

was induced by BMP-2 treatment after 16 h (Figure 5E, F). Similar to the results shown in Figure 4G,

the level of nuclear YAP was significantly increased in response to BMP-2 treatment compared with

that of the control group ($25.4 \pm 16.1\%$ vs. $2.4 \pm 2.5\%$, $p < 0.05$) (Figure 5G).

To specifically explore the role of YAP, bGCs were incubated with VP, a benzoporphyrin derivative that inhibits cell proliferation through disruption of the YAP-TEAD complex,[32] at various concentrations (10–1000 nM). Prior to the inhibition experiment, I confirmed that 10–100 nM VP did not affect cell proliferation (Figure 5H). I then examined whether VP suppressed the BMP-2– induced cell proliferation in bGCs. Indeed, the BMP-2–induced cell proliferation was significantly suppressed by 100 nM VP treatment ($p < 0.05$) (Figure 5I). These results indicated that YAP activation, Hippo pathway suppression, plays a role in BMP-2–induced cell proliferation in bGCs, suggesting that BMP-2 promoted GCs proliferation through Hippo pathway suppression mediated by the increase of S1P catalyzed by SPHK1.

4. DISCUSSION

In this study, I revealed a new role of BMP-2 in Gn-independent early follicle development. My hypothesized mechanism is illustrated in Figure 6. Similar to the increase of BMP-2 levels observed during the primary-to-secondary follicle transition in rats,[5] the SPHK1-positive rate in GCs rapidly increased from the primary follicles in humans. BMP-2 enhanced *SPHK1* mRNA expression in bGCs and HGrC1 cells. BMP-2 enhanced cell proliferation ~~not only~~ in bGCs ~~but also in HGrC1 cells.~~ Since SPHK1 promotes S1P production, which suppresses Hippo pathway, to induce cell proliferation,[33] my data suggested that BMP-2 promoted Gn-independent GCs proliferation through Hippo pathway suppression by increasing the level of S1P catalyzed by SPHK1.

As human luteinized GCs obtained from IVF are already differentiated, it is difficult to evaluate the effect of BMP-2 on GCs at the Gn-independent phase in *in vitro* study. In bovine, GCs from small-sized follicles (diameter of 4 mm or less) are assumed to be in the Gn-independent phase just prior to their recruitment into a follicular wave.[27] BMP type I and II receptors (BMPR1A and BMPR2) are expressed in bovine follicles.[34] Although it is not yet clear which S1PR exists in bGCs, five S1PRs have been confirmed in GCs of other species.[21] Therefore, *in vitro* experiments were conducted using Gn-independent bGCs as primary cells or HGrC1 cells as cell line instead of human luteinized GCs from IVF. The concentration of BMP-2 was reported to be approximately 60–400 pg/ml in human plasma and 1–110 ng/ml in the follicular fluid of IVF patients.[35-37] The S1P

concentration was reported to be about 1.5 μM in human plasma and 60–170 nM in the human

follicular fluid from IVF patients.[38-40] The concentrations of BMP-2 and S1P in this study were in

accordance with the physiological status during follicular development.

I also found a time lag of the inhibitory effect of the Hippo pathway between BMP-2 and

S1P; that is, the inhibition was observed within only 1 h of S1P treatment, but at 16–48 h after BMP-

2 treatment in bGCs. I speculated that this time lag was derived from the translational activation of

SPHK1 followed by the production of S1P. In other words, S1P is assumed to be the main player in

the execution phase in the BMP-2–SPHK1-S1P axis in GCs. In addition, the strength of Hippo

pathway inhibition was weaker with BMP-2 treatment than with S1P treatment. BMP-2 decreased the

pYAP protein level by 25% and increased the rate of YAP nuclear localization by $25.4 \pm 16.1\%$

compared with that of the control, whereas S1P decreased the pYAP level by 48% and increased the

nuclear YAP rate by $41.6 \pm 13.7\%$. In fact, the cell proliferation effect of BMP-2 was also weaker than

that of S1P (1.08-fold by BMP-2 vs. 1.14-fold by S1P compared with day0). Thus, BMP-2 and S1P

showed similar effects on the proliferation of bGCs, but the difference in the magnitude and timing of

their effects might be caused by the indirect influence of BMP-2 on the Hippo pathway. The preantral-

to-early antral follicle transition takes approximately 65 days in humans, and the doubling time of GCs

is considered to be 100–250 h during the growth phase.[41] Because preantral follicle development

proceeds much more slowly than that of large antral follicles,[42] the weaker and time-consuming effects

of BMP-2 may be necessary for early folliculogenesis. Although the effect of BMP-2 on GCs takes more time and is weaker than that of S1P, BMP-2 might induce GCs proliferation "slowly" during early follicular development, and this effect would be more consistent with the physiological folliculogenesis process.

Hernandez-Coronado and colleagues reported that FSH, which stimulates GCs proliferation and differentiation, induced S1P through the phosphorylation of SPHK1 in bGCs of medium-sized (4 –7 mm) follicles.[7,43] Moreover, the presence of both BMP-2 and FSH significantly increased the follicular diameter in bovine secondary follicles.[44] My group previously reported that *FSHR* mRNA expression of human luteinized GCs was enhanced by BMP-2, -6, or -7.[11-13] Based on these previous findings, I examined the effect of BMP-2 on *FSHR* mRNA expression in Gn-independent bGCs, which were derived from small-sized (4 mm or less) follicles. In the present study, I also observed an increase of *FSHR* mRNA levels by BMP-2 in Gn-independent primary bGCs. As GCs in early follicles are known to be unaffected by FSH,[45] BMP-2 may up-regulate FSHR expression of Gn-independent GCs in early follicles to enhance their reactivity to FSH. In other words, BMP-2 promotes the transfer of Gn-independent early follicles to the FSH-dependent advanced stages, in which the FSHR level is gradually increased in GCs. This suggests that BMP-2 is not only involved in GCs proliferation but also in GCs differentiation, which is induced from the increase of FSHR levels, and both are required for early follicle development.

In a clinical setting, administration of FSH is currently the main factor used for fertility treatment; however, FSH does not have a notable effect on inducing Gn-independent early follicles to the Gn-dependent mature stage. My data suggest that BMP-2 might be useful to promote Gn-independent early folliculogenesis by enhancement of SPHK1 and FSHR expression in human GCs. For POR, who do not respond to FSH, BMP might be firstly used to induce GCs proliferation and FSHR expression in Gn-independent follicles, and shift them to the Gn-dependent phase. Subsequently, administration of FSH might be able to increase the number of retrieved oocytes. Drug administration to ovary is relatively easy, because the human ovary is transvaginally accessible. Topical administration to ovary has the advantage that the drug concentration in the ovary can be increased. There are also few side effects in the topical administration compared with systemic administration. In 2002, recombinant human BMP-2/absorbable collagen sponge (ACS) at a 1.5-mg/cc concentration (INFUSE Bone Graft, Medtronic Spinal and Biologics, Memphis, TN) was FDA-approved as an autograft replacement for certain interbody spinal fusion procedures.[46] Although my experiments were performed on cells at concentrations in "ng" units, concentrations in "mg" units may be required to act on ovarian tissue as practical use in orthopedics. However, it is very difficult to deliver BMP-2 at appropriate concentrations in human tissues because of its short half-life[47,48] and side effects, such as postoperative inflammation, ectopic bone formation, osteoclast-mediated bone resorption or inappropriate adipogenesis.[49] To solve these problems, various BMP-2 delivery systems

such as a biodegradable sponge and porous poly lactic-co-glycolic acid beads have been developed

and tested in *in vivo* experiments.[48,50]

One of the main strengths of this study was that I used Gn-independent bGCs rather than

human luteinized GCs from IVF, which is in Gn-dependent phase. To clarify the role of BMP-2 in Gn-

independent GCs, the use of bGCs was essential to confirm my hypothesis. This was also a meaningful

result given the ethical difficulties of obtaining human Gn-independent GCs for experiments. In this

study, I used bGCs at the Gn-independent phase to validate my hypothesis and explore the

mechanisms; however, my final aim is to reveal the effects of BMP on Gn-independent early follicular

development in humans (Figure 7). Although further study is needed to determine the more precise

mechanism of BMP in early follicular development, my present results suggest that BMP-2 can be a

candidate therapeutic strategy for women with follicular dysgenesis that is characteristic of POR.

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Table 1. Primer sequences used for RT-qPCR

species	Gene symbol	Primer sequence (5' - 3')		PCR cycle	denaturation	annealing	extension
bovine	SPHK1	F :	GAGATGGGCTGATCCACGAG	40	95℃ for 10seconds	64℃ for 10 seconds	64℃ for 20 seconds
		R :	CTCGTACCCGGCGTAATAGT				
	CTGF	F :	AGCTGAGCGAGTTGTGTACC	40	95℃ for 10seconds	62℃ for 10 seconds	62℃ for 30 seconds
		R :	GGGCACCATCTTTAGCGGT				
	FSHR	F :	AACCTGCTATACATCGACCCTGAT	40	95℃ for 10seconds	64℃ for 10 seconds	64℃ for 20 seconds
		R :	GCTTAATACCTGTGTTGGATATTAACAGA				
	GAPDH	F :	CCGCATCCCTGAGACAAGATG	40	95℃ for 10seconds	62℃ for 10 seconds	62℃ for 20 seconds
		R :	CATTGATGGCGACGATGTCC				
human	SPHK1	F :	CTTGCAGCTCTTCCGGAGTC	40	95℃ for 10seconds	58℃ for 10 seconds	72℃ for 10 deconds
		R :	GCTCAGTGAGCATCAGCGTG				
	L19	F :	GGGATTTGCATTCAGAGATCAG	40	95℃ for 10seconds	54℃ for 10 seconds	72℃ for 10 seconds
		R :	GGAAGGGCATCTCGTAAG				

RT-qPCR indicates reverse transcription and real-time quantitative polymerase chain reaction

FIGURE LEGENDS

FIGURE 1   SPHK1 localization in human GCs.

SPHK1 expression was investigated by immunohistochemistry. The sections were stained with specific antibodies to SPHK1 (A–D) or rabbit IgG (E, F) as negative control. (A and E) Primordial follicles. (B) Primary follicle. (C, D, and F) Secondary follicles. (G) Rate of SPHK1-positive staining in human GCs; primordial, primary, early secondary or, late secondary follicles. The SPHK1-positive rate in human GCs increased from primary follicles to subsequent stages. Scale bar = 50 μm.

FIGURE 2   BMP-2 promotes *SPHK1* and *FSHR* mRNA expression and cell proliferation in GCs.

(A and B) Bovine GCs were treated with BMP-2 at various concentrations and for various times. Bovine GCs were incubated with 10–300 ng/ml BMP-2 for 8 h (A) and with 100 ng/ml BMP-2 for 4–72 h (B) without FBS. Total RNA was extracted from the cells and subjected to RT-qPCR to determine the *SPHK1* mRNA levels. (C) Effect of BMP-2 on cell proliferation. bGCs were treated with 100 ng/ml BMP-2 in DMEM with 2.5–5% FBS and the numbers of bGCs were measured using the CCK-8 assay. The medium was exchanged and BMP-2 was replenished every 24 h. (D) HGrC1 cells were treated with 100 ng/ml BMP-2 for 8 h without FBS. The Y-axis indicates the relative *SPHK1* mRNA levels. (E) Primary bGCs were treated with 100 ng/ml BMP-2 for 24 h without FBS. The Y-axis

indicates the *FSHR* mRNA levels. Data were normalized by bovine *GAPDH* mRNA levels in bGC (A, B and E) or human L19 in HGrC1 cells (D) to show the relative abundance. (A, B, D and E) The primer sequences and PCR conditions are shown in Table 1. (A) n = 8 per group; (B) n = 6 per group; (C) n = 10 per group. (D) n = 6 per group. (E) n = 12 per group. Data from at least three different experiments were combined, and are shown as the mean ± SEM relative to an adjusted value of 1.0 for the mean value of each control or day 0. n.s.= not significant. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

FIGURE 3   Sphingosine 1-phosphate (S1P) stimulated the proliferation of bovine granulosa cells (bGCs).

Effect of S1P on bGCs proliferation. Bovine GCs were treated with 300 nM S1P for the indicated times in DMEM with 2.5–5% FBS. The medium was exchanged and S1P was replenished every 24 h. The number of bGCs were measured using the CCK-8 assay. n = 10 per group. Data from at least three different experiments were combined, and are shown as the mean ± SEM relative to an adjusted value of 1.0 for the mean value of the control or day 0. n.s.= not significant. *$p < 0.05$.

FIGURE 4   Effect of sphingosine 1-phosphate (S1P) on the Hippo pathway in bovine granulosa cells (bGCs).

S1P suppressed the Hippo pathway in bGCs. Bovine GCs were cultured in serum-free medium for 12

h and then stimulated with 300 nM S1P for 1 h. (A) The lysates were subjected to Western blotting for

β-actin, bovine total YAP (tYAP), and phosphorylated YAP (pYAP) (Ser127). DMEM with 10% FBS

treatment is shown as a positive control of pYAP reduction, and norepinephrine (NE) was used as a

negative control. Bovine GCs of non-stimulated control group were incubated with vehicle of S1P. (B)

The Y-axis indicates pYAP/tYAP ratio obtained from at least three different experiments. The results

are expressed relative to an adjusted value of 1.0 for the mean value of the control. Bars in the graph

indicate the average values in each group. (C–G) S1P induced the nuclear translocation of YAP in

bGCs. Localization of endogenous YAP (green) was determined by immunofluorescent staining and

counterstaining of DAPI for DNA (blue) in control (C and D) and S1P-treated (E and F) cells.

Magnified pictures of E and F were also shown; scale bar = 50 μm. (G) Quantification of YAP nuclear

localization. Data are expressed as the mean ± SEM. (H) *CTGF*, the proliferation-related gene, is the

direct target of the YAP-TEAD transcriptional active complex. Bovine GCs were treated with 300 nM

S1P for 1 h after 12 h of serum starvation. The Y-axis indicates relative *CTGF* mRNA levels. Data

were normalized by the bovine *GAPDH* mRNA level to show the relative abundance. The primer

sequences and PCR conditions are shown in Table 1. (A, C–G) Representative data from at least three

different experiments; (H) Data from at least three different experiments were combined, and are

shown as the mean ± SEM relative to an adjusted value of 1.0 for the mean value of the control. (B) n

= 7 per group. (H) n = 9 per group. $**p < 0.01$, $*p < 0.05$.

FIGURE 5 Bone morphogenic protein (BMP)-2–induced bovine granulosa cells (bGCs) proliferation is mediated by Hippo pathway suppression.

(A) Bovine GCs were stimulated with 100 ng/ml BMP-2 in DMEM with 1% FBS for 48 h. The lysates were subjected to Western blotting for β-actin, bovine total YAP (tYAP), and phosphorylated YAP (pYAP) (Ser127). (B) The Y-axis indicates the pYAP/tYAP ratio obtained from at least three different experiments. The results are expressed relative to an adjusted value of 1.0 for the mean value of the control. Bars in the graph indicate the average values in each group. (C–G) Bovine GCs were cultured with 100 ng/ml BMP-2 in serum-free medium for 16 h. BMP-2 induced the nuclear translocation of YAP in bGCs. Localization of endogenous YAP (green) was determined by immunofluorescent staining with counterstaining of DAPI for DNA (blue) in control (C and D) and BMP-2-treated (E and F) cells. Magnified pictures of E and F were also shown; scale bar = 50 μm. (G) Quantification of YAP nuclear localization. (H and I) Effect of verteporfin (VP), a YAP inhibitor, on BMP-2–induced proliferation of bGCs. (H) The Y-axis indicate the number of bGCs pre-treated with various concentrations of VP (10–1000 nM) for 10 min and then incubated for 24 h in DMEM with 2.5% FBS. In the control, cells were pre-treated with dimethyl sulfoxide (DMSO) for 10 min. (I) The Y-axis indicates the number of bGCs, which were pre-treated with 100 nM VP for 10 min, followed by incubation with 100 ng/ml BMP-2 for 24 h in DMEM with 2.5% FBS. In the vehicle control, cells

were pre-treated with dimethyl sulfoxide (DMSO) for 10 min, followed by treatment with 0.5 mM

HCl for 24 h. (A, C–G) Representative data from at least three different experiments; (B) n = 12 per

group; (H) n = 6 per group. (I) n = 8 per group. (B and H-I) Results are expressed relative to an adjusted

value of 1.0 for the mean value of the control or DMSO control. (G and H-I) Data are expressed as the

mean ± SEM. *$p < 0.05$.

FIGURE 6   Schema of our hypothesis from this study.

The numbers in the scheme correspond to the figure numbers for the associated results. SPHK1 is

expressed in gonadotropin-independent granulosa cells (Fig. 1). BMP-2 up-regulates SPHK1

expression (Fig. 2) and SPHK1 catalyzes the conversion of sphingosine (SPH) to S1P. BMP-2 and

S1P suppress the Hippo pathway, as evidenced by the dephosphorylation and nuclear translocation of

YAP (Figs. 4 and 5). YAP acts mainly through TEA domain transcription factor (TEAD) family

transcription factors to stimulate expression of its target genes (Fig. 4) that promote cell proliferation

(Figs. 2 and 3). BMP-2–induced cell proliferation required YAP-TEAD complex formation (Fig. 5).

Black lines, data from previous reports; red lines, data from the present study.

FIGURE 7   Schema of my final aim for determining the mechanism of gonadotropin (Gn)-

independent early folliculogenesis by bone morphogenic protein (BMP)-2 in humans.

BMP-2 is expressed in the early follicles at each stage of Gn-independent initial development. BMP-2 expression levels increase during early follicular development. BMP-2 induces granulosa cells (GCs) proliferation by suppressing the Hippo pathway and induces GCs differentiation by increasing follicle stimulating hormone receptor (FSHR) expression, which might sensitize the follicles to Gn. Pre-antral follicles, which exhibit an increase in cell number and FSHR levels under BMP-2 stimulation, proceed to Gn-dependent early-antral follicles.

Figure 1

SPHK1

Primordial

Early-secondary

Primary

Late-secondary

negative control

Primordial

Late secondary

G

SPHK1-positive rate (%)

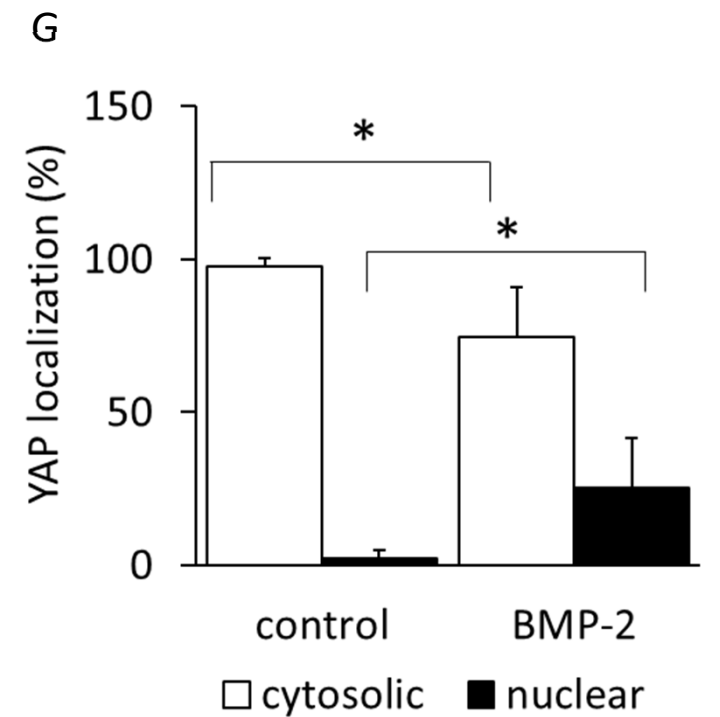primordial primary early secondary late secondary
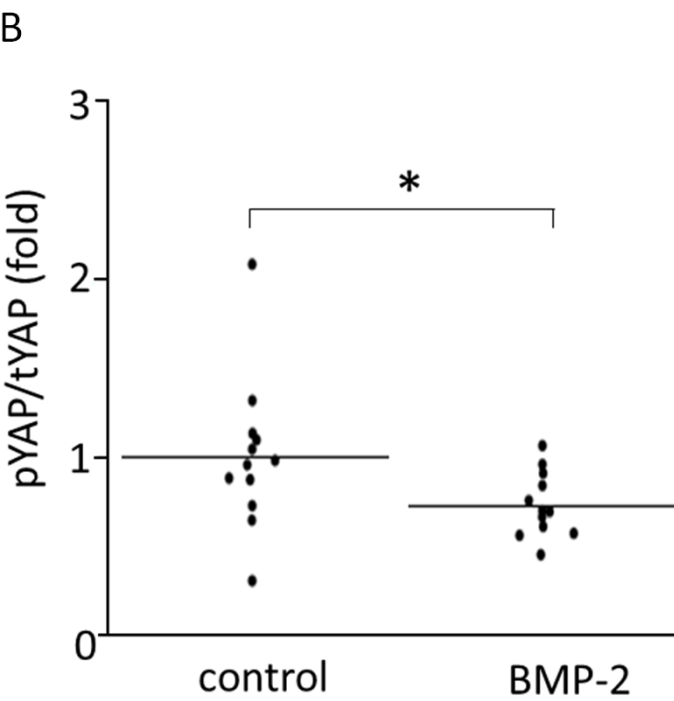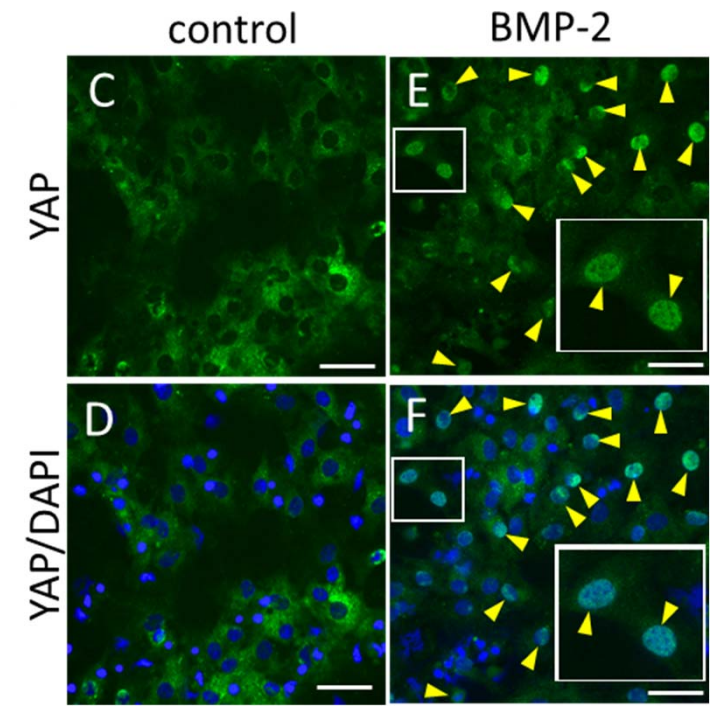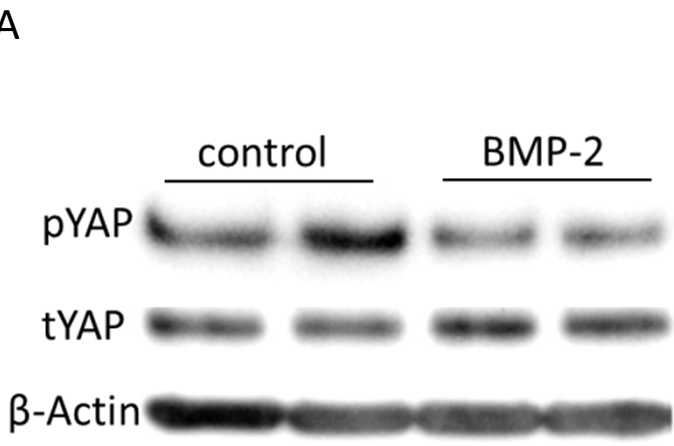
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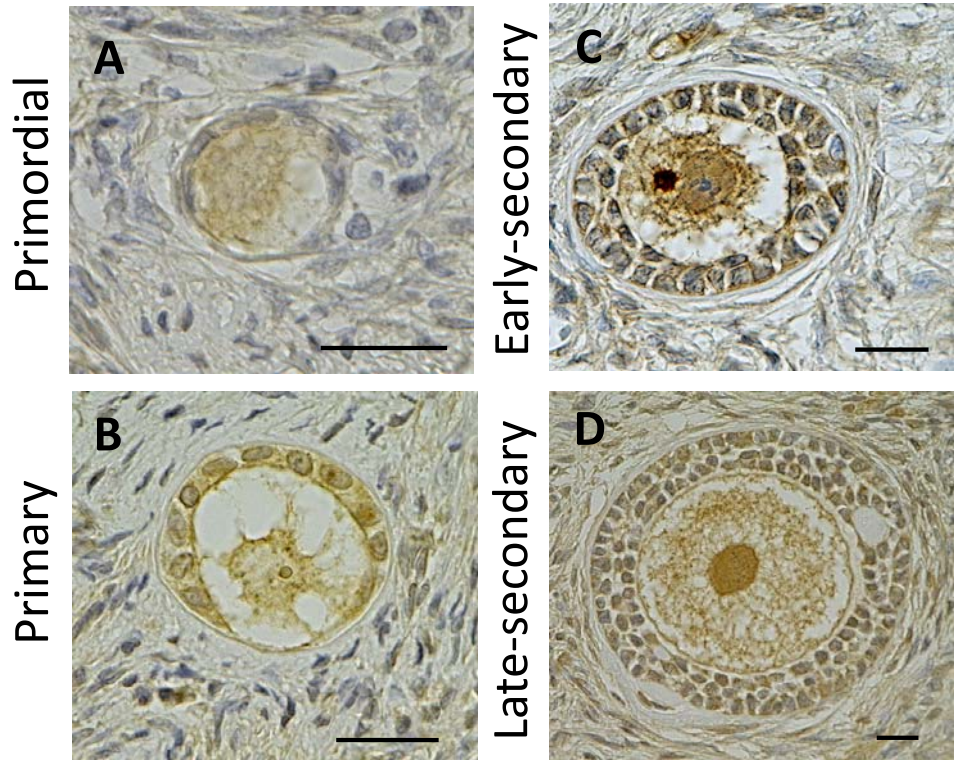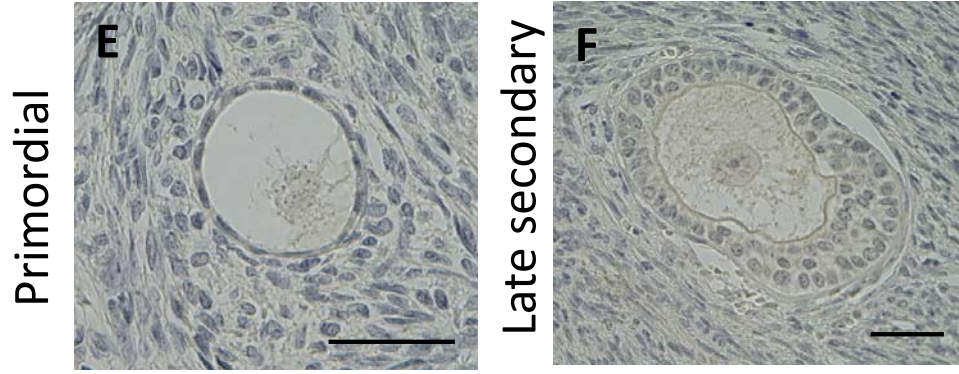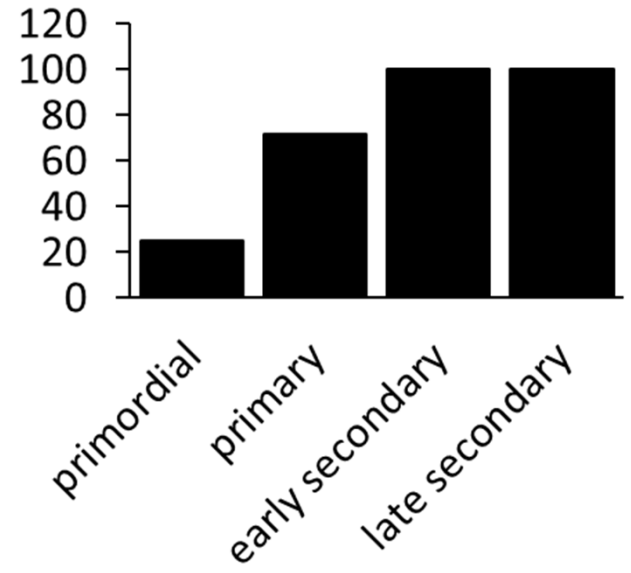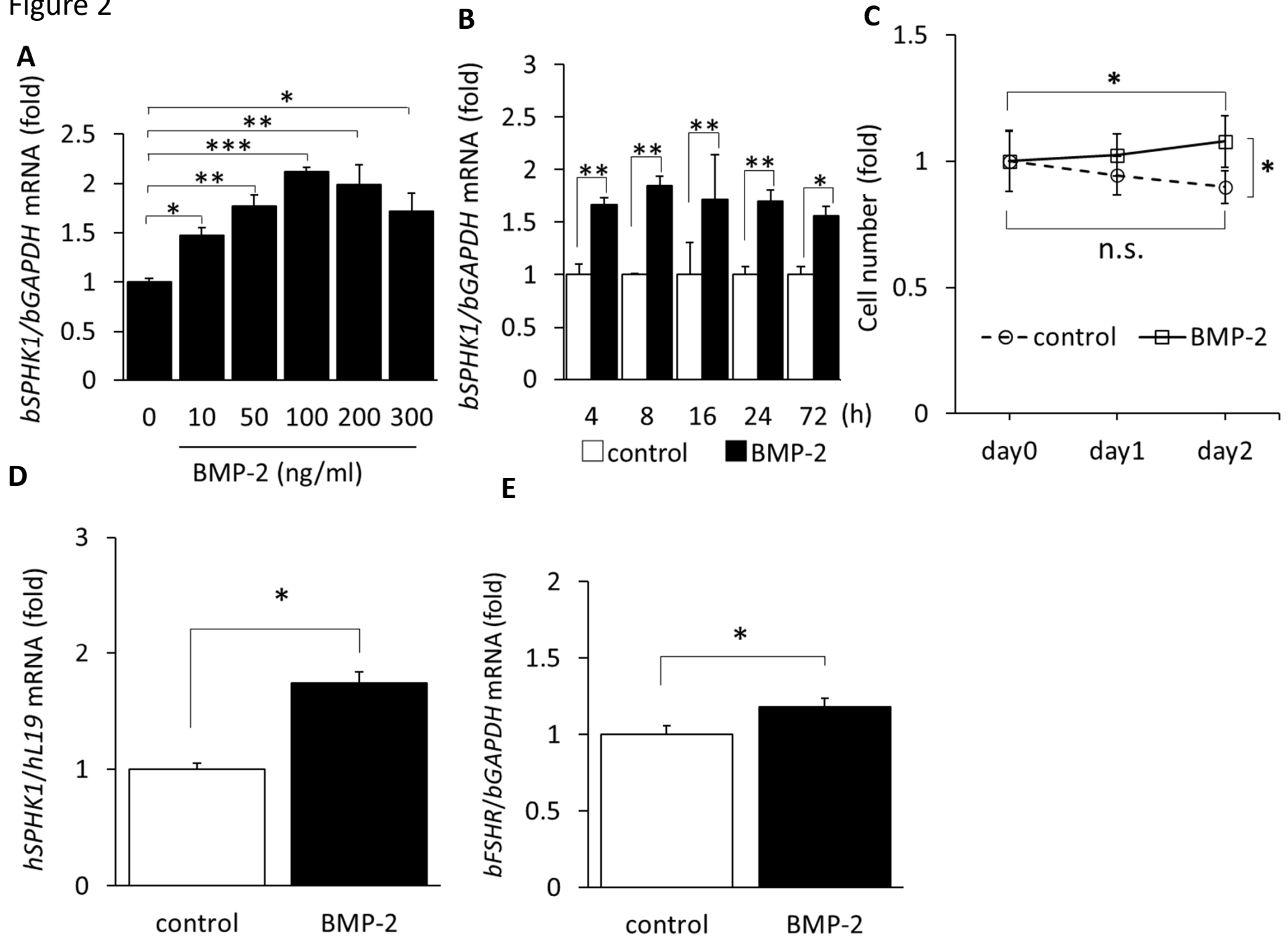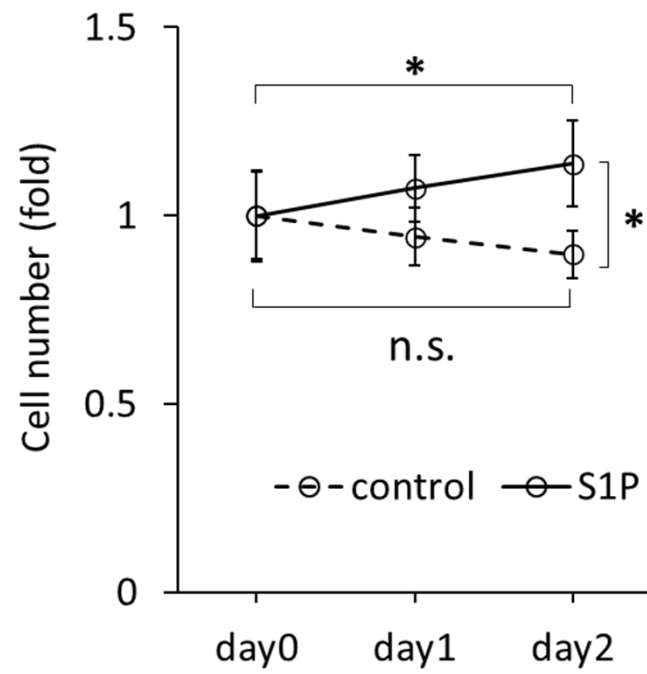
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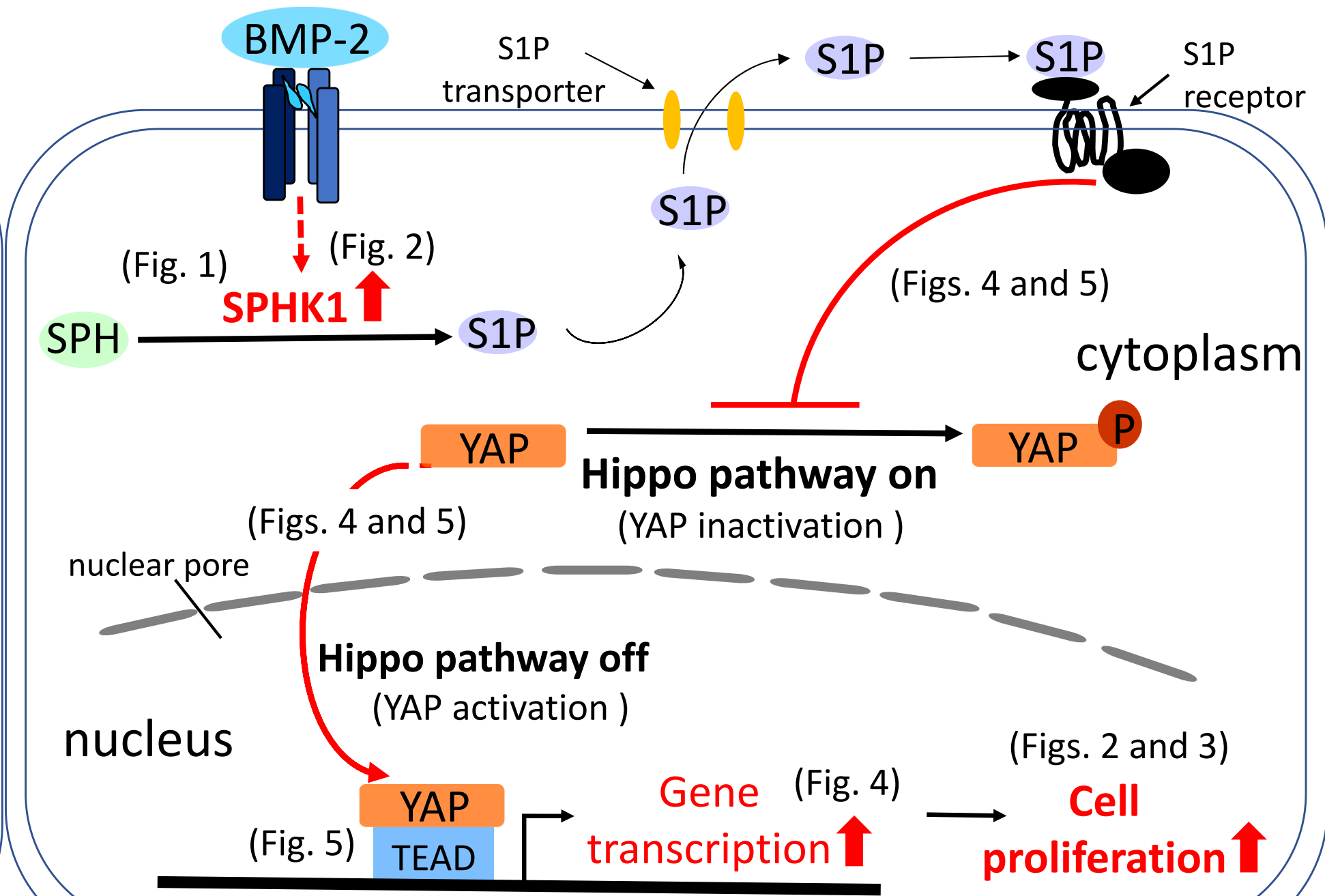
Figure 3

Figure 4
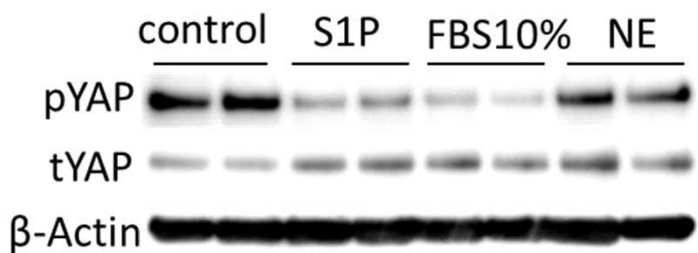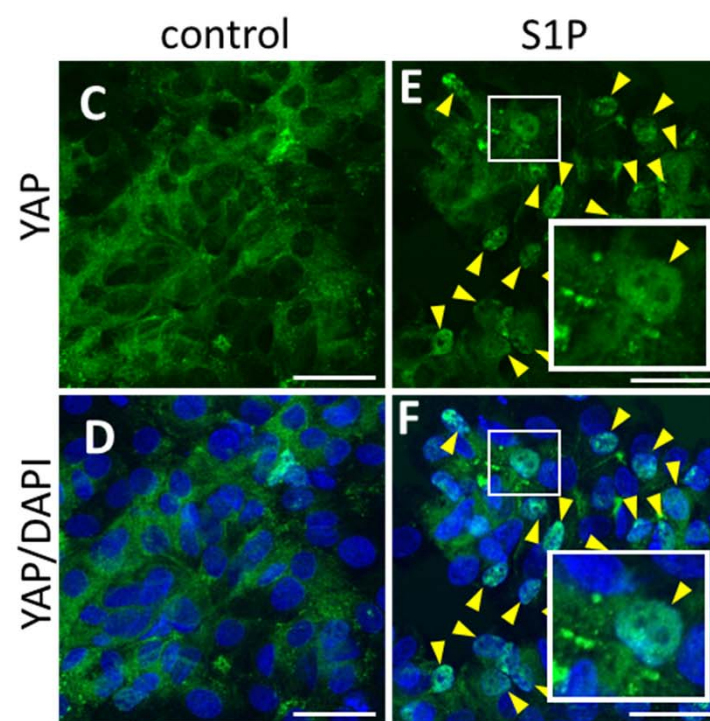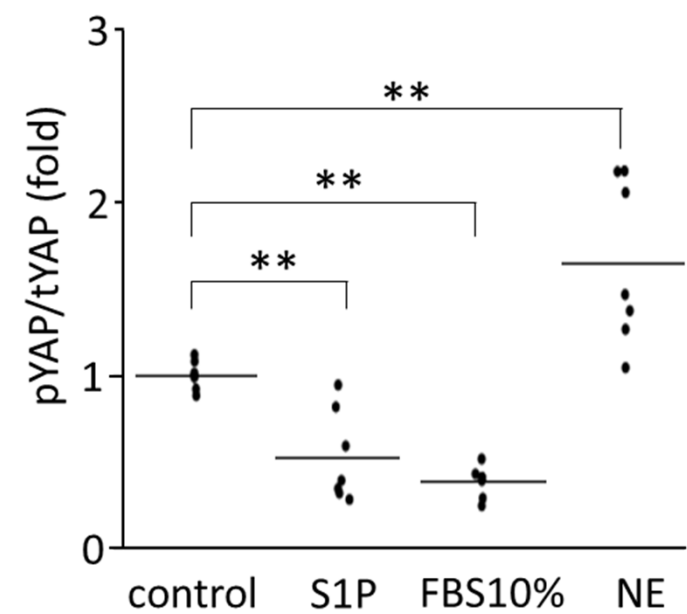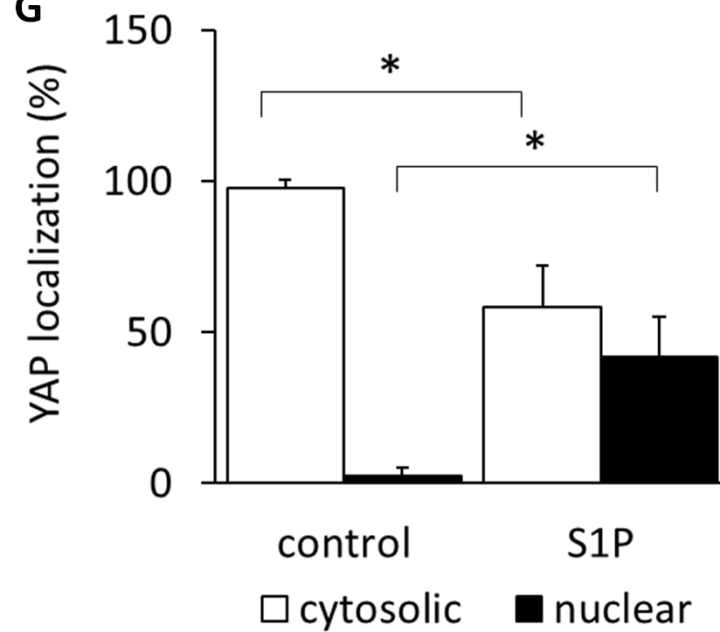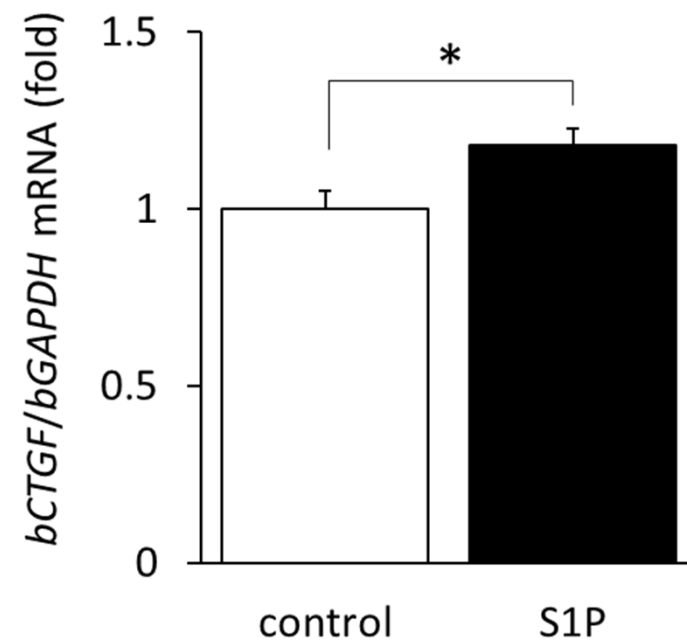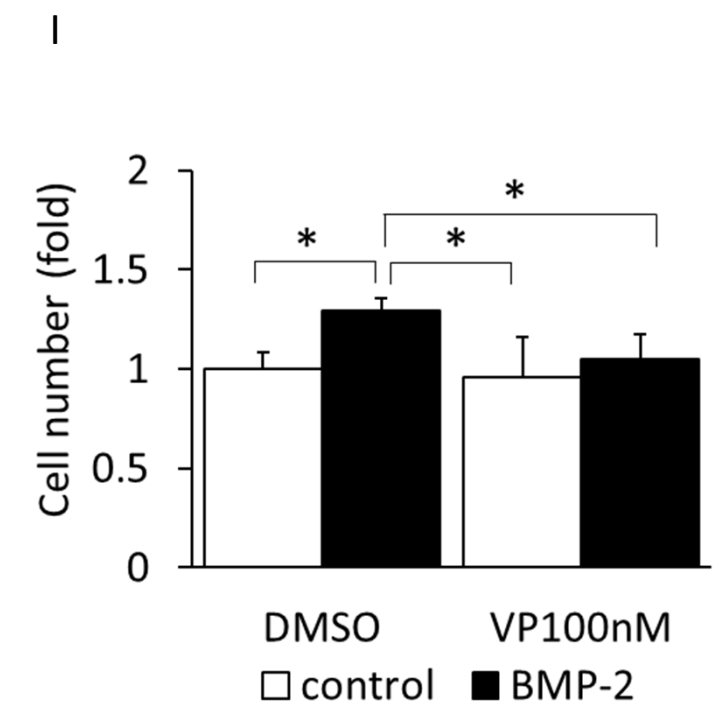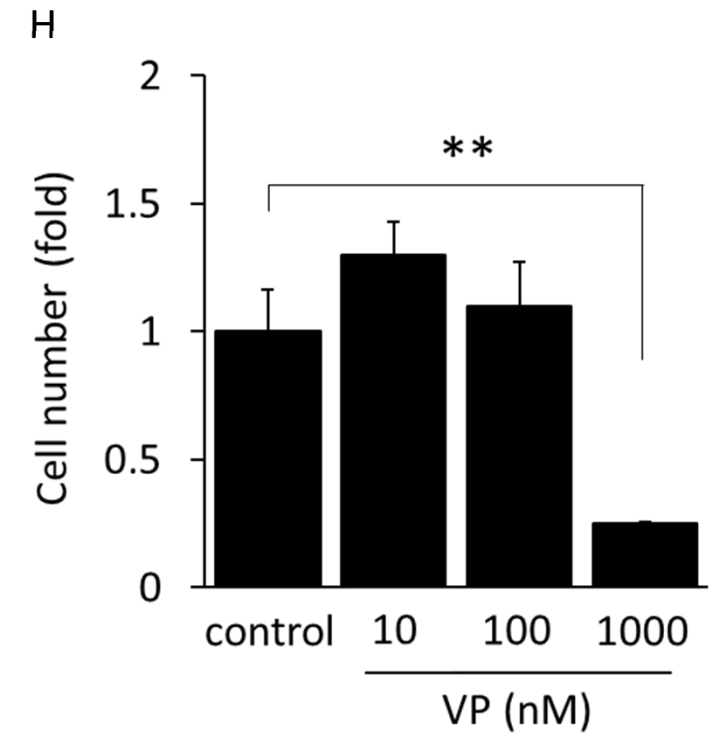
Figure 5

Figure 6

Granulosa cells (gonadotropin-independent)

Figure 7

Gn-independent

Pre-antral

Gn-sensitive

Secondary

Primary

FSHR↑
Hippo pathway↓
SPHK1↑

BMP-2

Early-antral

ovulation

Gn-dependent

Pre-ovulatory