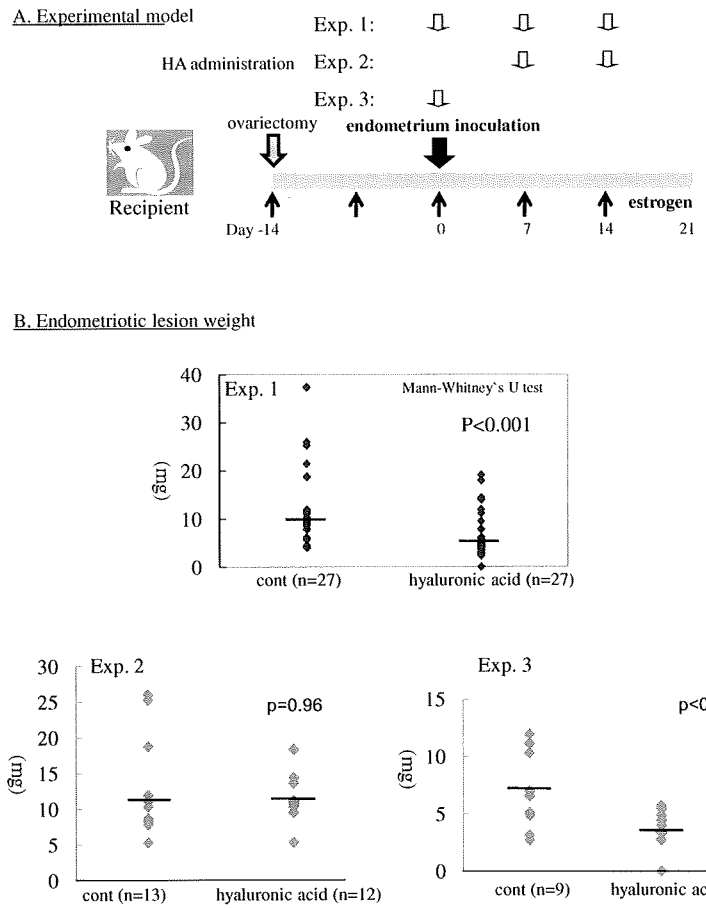
FIGURE 1

(**A**) Experimental model, on day 0, endometrium derived from donor mice was inoculated into the peritoneal cavity of recipient mice. Hyaluronic acid (HA; 100 μL) reagent was administered on days 0, 7, and 14 (Exp. 1), days 7 and 14 (Exp. 2), or only day 0 (Exp. 3). On day 21, the mice were killed and evaluated for development of endometriotic lesions. Estradiol valerate (100 μg/kg) was injected every week. (**B**) Endometriotic lesion weight per mouse was investigated in control and hyaluronic acid (HA; 100mL) administration groups. Bars represent the median values. HA reagent was administered on days 0, 7 and 14 (Exp. 1), days 7 and 14 (Exp. 2), or only day 0 (Exp. 3).



Hasegawa. Correspondence. Fertil Steril 2010.

easily (4, 5). In mice treated with HA (n = 27) or control (n = 27) on days 0, 7, and 14 (Exp. 1), the number of endometriotic lesions was significantly lower in the HA-treated group (1.1 ± 0.2) than in the control group (2.2 ± 0.2; P=.03). The total weight of the endometriotic lesions per mouse is shown in Figure 2. The weights were significantly lower in the HA-treated mice (6.7 ± 0.9 mg, median 5.1 mg) than in the control mice (12.3 ± 1.5 mg, median 9.5 mg; P<.001), whereas uterine weights were essentially the same between the HA-treated group and the control group. Also, the concentration of inflammatory cytokine interleukin (IL) 6 and monocyte chemotactic protein 1 (MCP) in the peritoneal fluid was not different between the two groups (data not shown).

To investigate when HA reagent was effective during the formation of endometriotic lesions, mice were treated with HA reagent on either days 7 and 14 (Exp. 2) or day 0 only (Exp. 3). We had previously confirmed that endometriotic lesions were established in mouse peritoneal cavity as early as day 7 (data not shown). In mice treated with HA on days 7 and 14 (Exp. 2), starting HA administration a week after endometriotic lesion induction, there was no difference in the number and the weight of endometriotic lesions per mouse between groups (number of lesions: control 1.8 ± 0.2, HA 1.7 ± 0.3 [P=.56]; weight of lesions: control 13.3 ± 2.3 mg, median 10.7 mg, HA 11.3 ± 1.1mg, median 10.7 mg [P=.96]). However, in mice treated with HA only on day 0 (Exp. 3), the number and the weight of endometriotic lesions were significantly reduced in the HA-treated group compared with the control group (number of lesions: control 1.9 ± 0.4, HA 0.9 ± 0.2 [P<.03]; weight of lesions; control 7.0 ± 1.1 mg, median 6.5 mg, HA 3.8 ± 0.6 mg, median 4.0 mg [P<.03]).

In the present study, we found that the administration of HA reagent significantly suppressed the formation of endometriotic lesions in both number and weight. This effect was found when HA treatment was conducted at the time of endometrial fragment

inoculation. Accordingly, it is plausible that exogenous HA reagent inhibited the attachment of endometrial cells to mesothelial cells, thereby suppressing the initiation of endometriosis in our mouse model.

It is known that the interaction between HA and CD44, an adhesion molecule, plays an important role in a wide variety of physiologic and pathologic processes of cell-cell attachment, including lymphocyte homing, cell migration, and cancer cell metastasis (6). Because eutopic and ectopic endometrial cells are known to express CD44 (7, 8), one possible mechanism of the present findings is that exogenous HA might bind to CD44 expressed in endometrial cells, leading to the suppressive effect on the initiation of endometriotic lesion. Further study is needed to prove this hypothesis.

Another characteristic of HA, which could be theoretically relevant to the suppression of endometriosis development, is its anti-inflammatory function. It has been reported that HA suppresses production of proinflammarory cytokines in synovium fibroblast cells (9). Because peritoneal inflammation is recognized to be associated with endometriosis (4), we investigated whether the anti-endometriotic effect of HA was exerted by suppressing the peritoneal inflammation. We thus measured IL-6 and MCP-1, typical inflammatory cytokines known to be elevated in the peritoneal fluid of endometriotic mice (4), but we failed to demonstrate an inhibitory effect of HA on the IL-6 and MCP-1 concentrations. Therefore, it is unlikely that HA inhibits the induction of endometriosis by suppressing peritoneal inflammation.

HA solutions have been used clinically in various areas, including prevention of adhesion formation after abdominopelvic surgery (3). Extrapolating our data, administration of HA solution during surgery for endometriosis might also be beneficial to prevent reattachment of spilled endometriotic cells and subsequent recurrence of the disease. Furthermore, given that implantation of endometrial tissues in retrograde menstrual flux causes endometriosis, administration of HA reagent into the uterine cavity might prevent shed endometrium from attaching to peritoneal membrane.

In conclusion, we demonstrated that administration of HA could prevent the formation of endometriotic lesions in the mouse model. Further studies are needed before using HA in clinical settings for endometriosis treatment.

Acknowledgments: The authors thank Dr. Heather Martinez for her helpful discussion and critical reading of the manuscript.

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human reproduction ORIGINAL ARTICLE ***Reproductive biology***

Progesterone decreases bone morphogenetic protein (BMP) 7 expression and BMP7 inhibits decidualization and proliferation in endometrial stromal cells

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BACKGROUND: Regulation of decidualization is decisive for proper implantation and the establishment of pregnancy. Recent studies have suggested that several bone morphogenetic proteins (BMPs) play physiological roles in reproduction. In the present study, we examined the expression of BMP7 in the endometrium and the effect of BMP7 on decidualization and proliferation of endometrial stromal cells (ESC).

METHODS: The gene expression of BMP7 in endometrial tissues collected from women with regular menstrual cycles was determined and the effect of ovarian steroid hormones on BMP7 gene expression was investigated in cultured ESC. The effect of BMP7 on the decidualization of ESC was determined by measuring the gene expression and protein secretion of insulin-like growth factor binding protein 1 (IGFBP1), a marker of decidualization. The effect of BMP7 on the proliferation of ESC was examined by the bromodeoxyuridine (BrdU) incorporation assay.

RESULTS: The gene expression of BMP7 in endometrial tissues was low at and after the mid-secretory phase of the menstrual cycle. Progesterone suppressed the gene expression of BMP7 in cultured ESC. Treatment with progesterone and estradiol for 12 days achieved decidualization of ESC, increasing the gene expression and protein secretion of IGFBP1. Addition of BMP7 protein to the culture almost completely inhibited these increases. BMP7 suppressed BrdU incorporation in ESC, which indicated an antiproliferative effect of BMP7 on ESC.

CONCLUSIONS: Progesterone-induced suppression of BMP7 and BMP7-induced inhibition of decidualization and proliferation of ESC suggest an elaborate regulatory mechanism for decidualization through BMP7 in the endometrium.

Key words: BMP7 / IGFBP1 / progesterone / decidualization / proliferation

Introduction

The endometrium undergoes dynamic changes during the menstrual cycle. Proper endometrial changes are essential for successful implantation, and aberrant endometrial status may lead to implantation failure. In addition to ovarian steroids, which have a central role in the regulation of morphological and functional changes to the endometrium, there are many local factors that modulate endometrial status (Kayisli *et al.*, 2004; Dimitriadis *et al.*, 2005).

Bone morphogenetic proteins (BMPs), together with growth differentiation factors (GDFs), comprise a subfamily of the transforming growth factor-β superfamily. BMPs and GDFs are multifunctional growth factors and their effects have been reported mainly in bone, cartilage, ligament and tendon formation (Francis-West *et al.*, 1999). However, BMPs and GDFs have also been demonstrated to control cellular proliferation, differentiation and apoptosis in reproductive tissues (Shimasaki *et al.*, 2004).

Gene expression of BMP2 (Ying and Zhao, 2000), BMP4 (Ying and Zhao, 2000), BMP6 (Lyons et al., 1989), BMP7 (Ozkaynak et al., 1997; Paria et al., 2001), GDF9 (Fitzpatrick et al., 1998) and GDF10 (Zhao et al., 1999) has been reported in the mouse uterus. These BMPs are expressed in a different spatiotemporal pattern and are thus speculated to have specific functions in the uterus. Mice deficient in ALK6, the receptor for these BMPs, have an abnormal endometrium and are infertile (Yi et al., 2001). A recent study has further demonstrated the presence of BMP2, BMP4, BMP7, GDF5, GDF8 and GDF11 in the human endometrium (Stoikos et al., 2008). BMP7 is unique among these BMPs in that its mRNA is lost from the uterine epithelium shortly after implantation in mice (Ozkaynak et al., 1997). In the human, gene expression of BMP7 has been reported in cultured endometrial stromal cells (ESC), with the expression level not being changed by cAMP-induced decidualization (Stoikos et al., 2008). In addition, immunostaining of human biopsied specimens have shown that BMP7 can be detected in highly decidualized cells with a vesicle staining pattern but not in first trimester deciduas (Stoikos et al., 2008).

Although these findings imply a functional role for BMP7 in endometrial physiology, to date there have been no studies examining the effects of BMP7 on the endometrium. To determine the possible roles of BMP7 in the human endometrium, in the present study, we first examined the gene expression of BMP7 in the endometrium. We then studied the effects of BMP7 on decidualization of ESC, measuring insulin-like growth factor binding protein 1 (IGFBP1) as a marker of decidualization (Harada et al., 2006). We also examined the effects of BMP7 on proliferation of ESC.

Materials and Methods

Patients and samples

Endometrial tissue was obtained from 39 women, either by curettage under sterile conditions or from women undergoing hysterectomy for benign gynecologic disease. The mean (±SD) age of the women was 37.8 ± 8.2 years. All women had regular menstrual cycles and none had received hormonal treatment within the 6 months prior to surgery. The specimens were dated according to the women's menstrual history. In order to avoid contamination with trophoblast cells, decidual tissues were collected from five women with ectopic pregnancy but without uterine bleeding, by dilation and curettage according to previous studies (Koga et al., 2001; Hirota et al., 2005). The experimental procedures were approved by the institutional review board of the University of Tokyo, and all women provided written informed consent for the use of their endometrial tissue.

Isolation and culture of human ESC

ESC were isolated and cultured as described previously (Koga et al., 2001; Yoshino et al., 2003). Fresh endometrial biopsy specimens collected in sterile medium were rinsed to remove blood cells. Tissues were minced into small pieces and incubated in DMEM/F-12 containing type I collagenase (0.25%; Sigma, St Louis, MO, USA) and deoxynuclease I (15 U/ml; Takara, Tokyo, Japan) for 60 min at 37°C. The resulting dispersed endometrial cells were separated by filtration through a 40-μm nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ, USA). Any intact endometrial epithelial glands that remained were retained by the strainer, whereas dispersed ESC passed through the strainer into the filtrate. ESC in the filtrate were collected by centrifugation at 250g and resuspended in phenol red-free DMEM/F-12 containing 5% charcoal-stripped fetal bovine serum (FBS), 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.25 μg/ml amphotericin B. The ESC were seeded in a 100-mm culture plate and kept at 37°C in a humidified atmosphere of 5%CO_2–95% air. At the first passage, cells were plated at a density of 1×10^5 cells/ml into 12- or 96-well culture plates (Becton Dickinson) and used for further treatments.

Treatment of ESC

To determine the effects of estrogen and progesterone on the gene expression of BMP7 in ESC, ESC were treated with 2.5% charcoal/dextran-treated (stripped) FBS (HyClone, Logan, UT, USA) in the presence of estradiol (10 ng/ml) or progesterone (100 ng/ml) for 6, 12 and 24 h. To examine the effect of BMP7 on decidualization, *in vitro* decidualization was achieved as described previously (Koga et al., 2001). Briefly, after cells had reached 70% confluence in 12-well culture plates, they were rinsed and treated with 2.5% charcoal/dextran-treated (stripped) FBS in the presence of estradiol (10 ng/ml) plus progesterone (100 ng/ml) or 0.1% ethanol vehicle (control) for 12 days. BMP7 (0, 10 or 100 ng/ml; R&D Systems, Minneapolis, MN, USA) was also added to the culture medium. Culture media were collected and replenished every 3 days.

RNA extraction, reverse transcription and real-time quantitative PCR

Total RNA was extracted from endometrial tissues and ESC using an RNeasy Mini Kit (Qiagen, Hilden, Germany). After reverse transcription, real-time quantitative PCR and data analysis were performed using a LightCycler (Roche Diagnostic, Mannheim, Germany), as reported previously (Harada et al., 2006). Expression of BMP7 and IGFBP1 mRNA was normalized for RNA loading for each sample using human glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Toyobo) mRNA as an internal standard. The BMP7 prrimers chosen (sense: 5′-GCCTACTACTGTGA GGGGGAG -3′; antisense: 5′-GAAGTAGAGGACGGAGATGGC-3′) amplified a 163-bp fragment. The IGFBP1 primers chosen (sense: 5′-GA GAGCACGGAGATAACTGAGG-3′; antisense: 5′-TTGGTGACATGGA GAGCCTTCG-3′) amplified a 131-bp fragment. The PCR conditions were as follows: for BMP7, 40 cycles of: 95°C for 10 s, 64°C for 10 s and 72°C for 4 s; for IGFBP1, 40 cycles of: 95°C for 10 s, 67°C for 10 s and 72°C for 5 s; for GAPDH, 30 cycles of: 95°C for 10 s, 64°C for 10 s, 72°C for 18 s. All PCR conditions were followed by melting curve analysis.

Measurement of IGFBP1 protein

Concentrations of IGFBP1 in the conditioned media were determined using a specific ELISA kit (R&D Systems, Minneapolis, MN, USA). The limit of sensitivity of the kit was 31.3 pg/ml. The concentrations measured were normalized against the total protein of cell lysates from each well of the culture plates.

5-Bromo-2′-deoxyuridine proliferation assay

The bromodeoxyuridine (BrdU) proliferation assay was performed as described previously (OuYang et al., 2008) using the Biotrak Cell Proliferation ELISA System (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions. Briefly, after incubation of ESC in serum-free medium for 24 h in 96-well plates, cells were treated for a further 24 h with serum-free medium containing either BMP7 (0, 10, 100 ng/ml) or 20% charcoal-stripped FBS as a positive control. After the 24 h incubation, 100 μl BrdU solution was added and cells were incubated at 37°C for an additional 2 h.

Statistical analysis

Expression of BMP7 mRNA in endometrial tissues was analyzed by the Kruskal–Wallis test, whereas other data were analyzed by ANOVA. Both tests were followed by *post hoc* analysis for multiple comparisons. $P < 0.05$ was considered significant.

Results

Expression of BMP7 mRNA in endometrial tissue throughout the menstrual cycle and in progesterone- and estradiol-treated ESC

As shown in Fig. 1, expression of BMP7 mRNA in endometrial tissues was significantly lower in the mid- and late secretory phases and in the decidua compared with expression in the mid-proliferative phase. In cultured ESC, treatment with progesterone, but not estradiol, decreased BMP7 mRNA expression at 12 and 24 h, compared with 0 h, in a time-dependent manner (Fig. 2A). Long-term culture of ESC in the presence of progesterone and estradiol remarkably decreased BMP7 mRNA expression on Day 3 and later, and distinctly induced IGFBP1 mRNA expression on Day 12 (Fig. 2B).

Effect of BMP7 on gene expression and secretion of IGFBP1 from ESC

Treatment with estradiol and progesterone for 12 days induced IGFBP1 mRNA expression in ESC. However, the addition of 10 and 100 ng/ml BMP7 to the culture medium markedly decreased the expression of IGFBP1 mRNA induced by the hormonal treatment in ESC (Fig. 3A). Figure 3B shows secretion of IGFBP1 protein from ESC, which was induced by estradiol and progesterone treatment on Day 9 and was increased to higher levels on Day 12. The addition of BMP7 to the culture medium markedly reduced IGFBP1 protein secretion, to almost undetectable levels in the presence of 100 ng/ml BMP7.

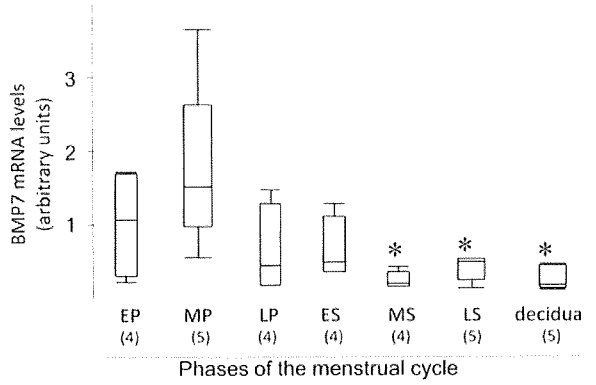

Figure 1 Expression of BMP7 mRNA in human endometrial tissues throughout the menstrual cycle and in early pregnant decidua.

Total RNA extracted from endometrial tissues and decidual tissues of ectopic pregnancies was reverse transcribed and then amplified by real-time PCR using primers for BMP7. Values were calculated by subtracting data for signal threshold cycles (Ct) of the internal standard (GAPDH) from Ct values for BMP7. The boxes represent the 25th and 75th percentiles. The median is denoted by the line that bisects the boxes. The whiskers indicate the extent of the data on the 1.5× interquartile range. *$P < 0.05$ compared with the MP. EP, early proliferative phase; MP, mid-proliferative phase; LP, late proliferative phase; ES, early secretory phase; MS, mid-secretory phase; LS, late secretory phase. The number of samples is shown in parentheses.

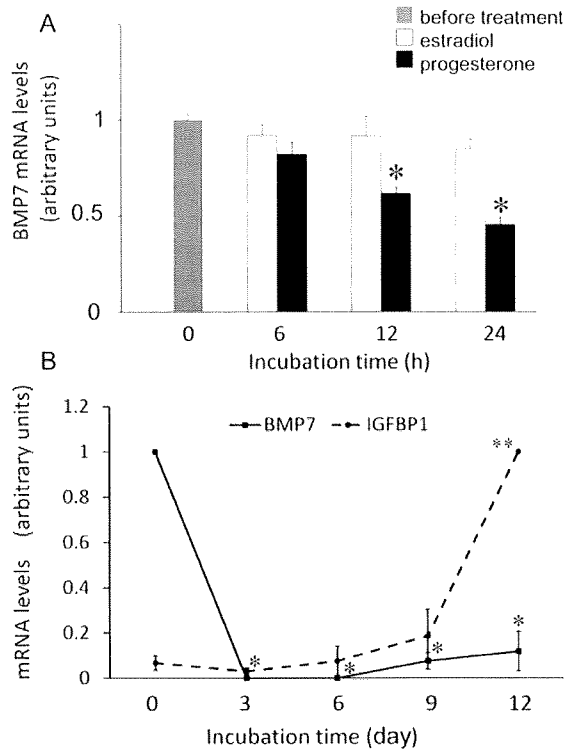

Figure 2 (**A**) Expression of BMP7 mRNA in ESC treated with estrogen (10 ng/ml) or progesterone (100 ng/ml) for 24 h. Data are the mean ± SEM of combined data from three independent experiments using different ESC from three patients. (**B**) Expression of BMP7 and IGFBP1 mRNA in ESC. *In vitro* decidualization of ESC was achieved by culturing ESC in the presence of estrogen (10 ng/ml) and progesterone (100 ng/ml) for 12 days. Data are the mean ± SEM of combined data from three independent experiments using different ESC from three patients. Total RNA isolated from ESC was reverse transcribed and then amplified by real-time PCR using primers for BMP7, IGFBP1 and GAPDH. Values were calculated by subtracting data for signal threshold cycles (Ct) of the internal standard (GAPDH) from Ct values for BMP7 or IGFBP1. (A) *$P < 0.05$ compared with 0 h. (B) *$P < 0.05$ compared with Day 0 (BMP7); **$P < 0.05$ compared with Day 0 (IGFBP1).

Effect of BMP7 on ESC proliferation

BMP7 at 10 and 100 ng/ml decreased BrdU incorporation in ESC by 20.5 ± 4.1 and 29.9 ± 4.2% (mean ± SEM of six replicate cultures) of the untreated controls, respectively (both $P < 0.05$ compared with the control), although 20% charcoal-stripped FBS increased BrdU incorporation by 134.8 ± 11.2%.

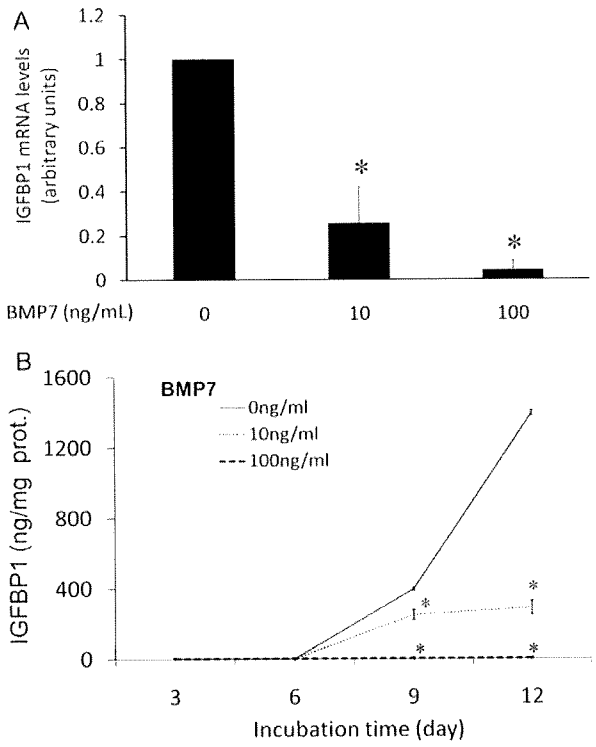

Figure 3 Effects of BMP7 on gene expression and protein secretion of IGFBP1 from ESC. (**A**) Effects of 10 and 100 ng/ml BMP7 on IGFBP1 mRNA expression in ESC treated with a combination of 10 ng/ml estradiol plus 100 ng/ml progesterone (EP) for 12 days. Total RNA isolated from ESC was reverse transcribed and then amplified by real-time PCR using primers for IGFBP1. Values were calculated by subtracting data for signal threshold cycles (Ct) of the internal standard (GAPDH) from Ct values for IGFBP1. Values are the mean ± SEM of four independent experiments using samples from four different patients. *$P < 0.05$ versus 0 ng/ml. (**B**) IGFBP1 concentrations in culture media of ESC treated with EP, with or without BMP7 (10 and 100 ng/ml), for 3, 6, 9 and 12 days. IGFBP1 concentrations were determined using a specific ELISA and normalized against the total protein of cell lysates from each well. Data are the mean ± SEM of duplicate cultures. *$P < 0.05$ compared with the respective control on each day. The result is representative of three separate experiments using samples from three different patients.

Discussion

In the present study, we demonstrated that gene expression of BMP7 in the endometrium was lower in the mid- and late secretory phases and in early pregnancy than in the mid-proliferative phase. Progesterone, but not estradiol, decreased BMP7 gene expression in ESC, which was significant after 12 h. Long-term incubation with progesterone and estradiol induced IGFBP1 protein secretion from ESC, which was inhibited by BMP7. BMP7 also decreased ESC proliferation.

In parallel with dynamic changes in the endometrium, the expression of many molecules in the endometrium changes spatiotemporally. Because embryos are accepted by the endometrium only during the 'implantation window', which corresponds to the mid-secretory phase, those substances for which levels in the endometrium change during the mid-secretory phase may have a role in preparing the receptive endometrium. In this context, the decrease in the gene expression of BMP7 in the mid-secretory phase may contribute to the development of the receptive endometrium.

Decidualization is a process in which remarkable structural and functional changes occur in ESC to prepare an appropriate environment for embryo implantation and maintenance of pregnancy. Decidualization is regulated by the ovarian steroid hormones estradiol and progesterone. In addition, the importance of other factors in the induction of decidualization has been demonstrated recently. For example, we found that mechanical stretch augments decidualization (Harada *et al.*, 2006), and others have found that paracrine factors are involved in decidualization (Tang *et al.*, 1994; Fazleabas and Strakova, 2002). The results of the present study, showing that BMP7 suppresses secretion of IGFBP1 protein from decidualizing ESC, suggest that BMP7 may act as an antidecidualization factor in the endometrium.

The antidecidualization activity of BMP7 is in marked contrast with the actions of BMP2, which increases the secretion of IGFBP1 and prolactin, another marker of decidualization, in decidualized ESC (Li *et al.*, 2007; Stoikos *et al.*, 2008). The expression patterns of BMP2 and BMP7 in the endometrium also appear to be different because *in-vitro* decidualization increases the expression of BMP2 in ESC (Li *et al.*, 2007). Thus, as a result of their different spatiotemporal expression, it is possible that the opposing actions of these two BMPs support decidualization and the subsequent establishment of pregnancy. From a therapeutic perspective, therapies targeted for BMP7 and BMP2 could be applicable for the treatment of implantation failure caused by impaired decidualization. Interestingly, the opposing functions of BMP7 and BMP2 have been demonstrated recently in adipogenesis, with BMP7 contributing to the development of brown adipocytes and BMP2 contributing to the development of white adipocytes (Tseng *et al.*, 2008).

The decrease in BMP7 expression in the decidualized endometrium may also be important for the successful development of the placenta. It has been shown that BMP7 suppresses the production of human chorionic gonadotrophin and progesterone from the trophoblast (Martinovic *et al.*, 1996). Because these hormones are tremendously important for the maintenance of pregnancy, the presence of BMP7 in the endometrium would be problematic for invading trophoblasts. Therefore, reduced BMP7 expression may be necessary not only for the development of a receptive endometrium, but also for the invading trophoblasts to establish pregnancy.

Progesterone inhibited BMP7 gene expression in ESC. This suggests that the decreased expression of BMP7 in the endometrium from the mid-secretory phase is due to the effects of progesterone. Notably, the inhibition of BMP7 gene expression by progesterone was clearly observed as early as 12 h. In addition, the decrease in BMP7 expression evidently preceded the increase in IGFBP1 expression during decidualization with progesterone and estradiol. This result, however, appears to be inconsistent with the findings by Stoikos *et al.* (2008) which showed that BMP7 gene expression was not altered by *in vitro* decidualization with cAMP. This difference may indicate that progesterone is prerequisite for down-regulation of BMP7 expression in the process of decidualization. Collectively,

progesterone may suppress BMP7 gene expression in the early stage to facilitate subsequent decidualization. Another apparently inconsistent finding of Stoikos *et al.* (2008) was the vesicular staining for BMP7 in decidual cells in mid–late secretory endometrium although staining patterns were not shown in other phases of the menstrual cycle. The decrease in BMP7 gene expression by progesterone might be involved in the change, if any, of intracellular localization of BMP7. Another possible explanation for the inconsistency may be any cross-reactivity of the antibody used in that study.

BMP7 appears to stimulate or inhibit proliferation depending on the cell type; for example, BMP7 stimulates proliferation of ovarian granulosa cells (Lee *et al.*, 2001) and Sertoli cells (Puglisi *et al.*, 2004), but inhibits proliferation of aortic smooth muscle cells (Dorai *et al.*, 2000), renal mesangial cells (Otani *et al.*, 2007) and prostate cancer cells (Miyazaki *et al.*, 2004). In the present study, BMP7 inhibited the proliferation of ESC. Thus, the decrease in BMP7 expression in the decidualized endometrium may contribute to the proliferation of decidual cells during pregnancy.

The present study has some limitations. First, the decidual tissues of ectopic pregnancies used in this study have advantages in that they are free from contamination with trophoblast cells, but they may have different characteristics from deciduas of normal pregnancies. Second, we measured mRNA levels but not protein levels of BMP7. Although cellular protein levels shown by immunostaining or immunoblotting are not necessarily proportional to their functional activities, knowledge about them would help our understanding of BMP7 in the endometrium. A further study is warranted regarding this point.

In summary, the results of the present study suggest that progesterone decreases BMP7 expression in the endometrium. The decrease in BMP7 expression may facilitate decidulalization of the endometrium, thus aiding the establishment of pregnancy.

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Post-operative oral contraceptive use reduces the risk of ovarian endometrioma recurrence after laparoscopic excision

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BACKGROUND: The aim of this study was to evaluate the impact of post-operative oral contraceptives (OCs) use on the rate of recurrence after laparoscopic excision of ovarian endometrioma.

METHODS: In May 2005, we introduced a 'post-operative OC recommendation' for patients treated with laparoscopic excision of endometrioma. That is, at the time of the operation, we provided each patient with information about OC, known and possible benefits and risks and let her decide whether to take OC. A retrospective cohort study included 87 patients who underwent a laparoscopy after May 2005. The endometrioma recurrence rate at 24 months was compared between those who used OC for the entire follow-up period OC ($n = 34$) and all of the others ($n = 53$). We also performed logistic regression analysis to identify variables associated with recurrence. A before–after study included another 224 patients who underwent a laparoscopy before May 2005 and compared the recurrence rate before and after introduction of the 'post-operative OC recommendation'.

RESULTS: The recurrence rate in those who used OC for the entire period was significantly lower than in the 'others' group (2.9 versus 35.8%, relative risk 0.082, 95% CI 0.012–0.58, $P < 0.001$). Post-operative OC was determined as an independent variable associated with lower recurrence (OR 0.054, 95% CI 0.007–0.429, $P < 0.001$). The overall recurrence rate in patients who underwent laparoscopy after the introduction of the 'post-operative OC recommendation' was significantly lower than that in patients who received laparoscopy before the introduction (18.6 versus 33.1%, relative risk 0.56, 95% CI 0.32–0.97, $P < 0.05$).

CONCLUSIONS: Post-operative OC use reduces the risk of ovarian endometrioma recurrence after laparoscopic excision. This information will help in appropriate planning of pre- and post-operative management.

Key words: endometriosis / laparoscopy / recurrence / oral contraceptives / ovary

Introduction

Ovarian endometrioma accounts for 55% of reported endometriosis cases (Jenkins *et al.*, 1986). Most cases are treated conservatively since endometrioma is commonly diagnosed in women of reproductive age (Giudice and Kao, 2004). Currently, laparoscopic excision is considered to be the 'gold standard' for the conservative treatment of endometrioma (Beretta *et al.*, 1998) and the European Society of Human Reproduction and Embryology (ESHRE) guidelines recommend that laparoscopic ovarian cystectomy can be considered if an ovarian endometrioma >3 cm in diameter is present (to confirm the diagnosis histologically) and for the purpose of reducing the risk of infection, improving access to follicles and possibly improving ovarian response (Kennedy *et al.*, 2005).

However, a frustrating aspect of laparoscopic excision of endometrioma is the disease recurrence after surgery. We have previously reported as high as 30.4% recurrence within 2 years after surgery (Koga *et al.*, 2006). Although the definition of recurrence can vary, other recent studies also indicate a 30% recurrence rate after 3–5 years of follow-up (Kikuchi *et al.*, 2006; Liu *et al.*, 2007), which is higher than previously believed.

Whereas laparoscopic excision is known to improve fertility (Chapron *et al.*, 2002), recurrence and repeated surgery can cause significant ovarian damage, thus reducing future reproductive

performance. Given that the majority of women who undergo this operation seek pregnancy in the future, a dilemma arises when a physician plans laparoscopy, with the expectation that fertility should improve but with concerns that future recurrence can place fertility at risk. It is therefore crucial to prevent recurrence after laparoscopic excision in order to maintain 'improved' fertility for long as possible.

Despite the necessity, a gold standard treatment modality of preventing recurrence does not yet exist. According to ESHRE guidelines (Kennedy *et al.*, 2005) and the Cochrane library (Yap *et al.*, 2004), post-operative hormonal treatment does not significantly reduce symptoms at 12 or 24 months, and has no effect on disease recurrence. However, the Cochrane library added 'there is insufficient evidence to conclude that hormonal treatment in association with surgery is associated with a significant benefit' (Yap *et al.*, 2004). As a result, it has remained controversial as to whether post-operative medical treatment or different drug treatments can reduce disease recurrence.

Previously, we analyzed risk factors that are associated with the recurrence of endometrioma after laparoscopic excision and identified post-operative pregnancy as a favorable prognostic factor (Koga *et al.*, 2006). The benefit of pregnancy on recurrence has also been observed in other reports (Busacca *et al.*, 2006; Porpora *et al.*, 2008). This prompted us to hypothesize that post-operative administration of oral contraceptive (OC) pills, which inhibit ovulation and create a pregnancy-like hormonal condition, may reduce the incidence of endometriosis recurrence. When we completed our previous study, only one randomized controlled trial had attempted to evaluate the efficacy of post-operative OC use for reducing recurrence (Muzii *et al.*, 2000). However, the duration of OC administration in this study (Muzii *et al.*, 2000) was limited to 6 months, consequently the long-term effect of OC on recurrence has been remained unclear.

Given that OC may reduce the recurrence after laparoscopic endometrioma excision, in May 2005, our clinic introduced a 'post-operative OC recommendation' for patients who underwent a laparoscopic excision of endometrioma. That is, at the time of the operation, we provided each patient with information about OC, and known and possible benefits and risks and let her decide whether or not to take OC. In this study, we conducted two analyses, a 'retrospective cohort study' and a 'before–after study'. In the retrospective cohort study, we compared recurrences between OC users and non-users to evaluate the 'effect' of OC use on endometriosis recurrences. On the other hand, the before–after study is a historical study which compares recurrences between before and after the introduction of the 'post-operative OC recommendation' in a clinical setting to define the efficiency of the 'post-operative OC recommendation' for the overall population.

Materials and Methods

Subjects

This investigation consists of two studies (i) a retrospective cohort study and (ii) a before–after study. For the retrospective cohort study, a total of 137 patients who underwent a laparoscopic excision of ovarian endometrioma performed at the University of Tokyo Hospital between May 2005 and August 2006 were enrolled. Of the 137 subjects originally enrolled, a total of 87 women who had a minimum of 24 months of post-operative follow-up were studied. The clinical characteristics of these patients are summarized in Table I.

For the before–after study, we included another 332 patients who underwent the same procedures in the same hospital but between January 1995 and December 2002 (before the introduction of 'post-operative OC recommendation'). Of the 332 subjects originally enrolled, a total of 224 who had a minimum of 24 months of post-operative follow-up were compared with the above-mentioned 87 patients (after the introduction of 'post-operative OC recommendation').

We confirmed that there was no significant difference in clinical characteristics between patients who were lost to follow-up and who were not, both before and after the introduction of the 'post-operative OC recommendation'. Institutional Review Board approval was not requested because laparoscopic excision is a standard procedure for the treatment of ovarian endometrioma. All patients gave written informed consent before surgery.

Surgery and the 'post-operative OC recommendation'

The method of laparoscopic excision of ovarian endometrioma was performed as previously described (Koga *et al.*, 2006). Briefly, after the ovary was freed from any adhesion, the capsule of the cyst was completely stripped away from the normal ovarian tissue. Endometriotic peritoneal implants were excised with scissors or coagulated with bipolar electro coagulation.

In May 2005, we introduced the 'post-operative OC recommendation' for patients treated with laparoscopic excision of endometrioma, who were under the age of 40 and not seeking pregnancy. At the time of the laparoscopy, every patient was routinely provided with the following information about OC: (i) known side-effects (nausea etc.) and risks (thrombosis etc.) and their likelihoods, (ii) known benefits supported by conclusive evidence (reducing pain etc.) and (iii) the possible benefits of reducing or delaying recurrence, not supported by conclusive data but the theoretical explanations. We also provided the recurrence rate and risk factors that we analyzed in our previous study (Koga *et al.*, 2006). The decision to take OC was left up to the individual women. Women who chose to take OC, were given a cyclic, monophasic OC containing ethinyl-estradiol (0.035 mg) and norethisterone (1.0 mg) (Ortho-M 21®, Mochida, Tokyo, Japan), starting in Days 1–5 of the first menstrual cycle after the laparoscopy.

Table I Characteristics of the 87patients after the 'OC recommendation'

Factors	Number of cases (%)
Age (years)	33.4 ± 4.6[a]
Infertility	11 (12.6)
Pain	40 (46.0)
Previous medical treatment of endometriosis	35 (40.2)
Previous surgery of ovarian endometrioma	7 (7.9)
Largest cyst diameter (cm)	5.3 ± 2.1[a]
Bilateral involvement	32 (36.8)
Revised ASRM score	59 ± 37[a]
Post-operative medical treatment	59 (67.8)
Post-operative pregnancy	9 (10.3)

ASRM, American Society for Reproductive Medicine.
[a]Mean ± SD.

Post-operative follow-up and diagnosis of recurrence

Post-operative follow-up was performed by transvaginal ultrasonography. The initial check up was conducted on Day 4 and Day 30 after the operation, to exclude residual cysts. Patients were then followed-up every 3 months for 24 months following the laparoscopy. The diagnosis of endometrioma recurrence was made as described in our previous study (Koga *et al.*, 2006). Briefly, a recurrence was defined as the presence of cysts with a typical aspect detected by transvaginal ultrasonography (Exacoustos *et al.*, 2003) more than 2 cm in diameter. The diagnosis of recurrence endometrioma was made when the cyst was indistinguishable from a corpus luteum cyst or intraovarian hematoma, and the cyst had not disappeared after several successive examinations. Recurrence was defined by the diagnosis of ovarian endometrioma at anytime within 24 months. Pain recurrence was defined as requiring analgesia at least once a month for dysmenorrhea or chronic pelvic pain at 24 months after the laparoscopy.

Statistical analysis

For the retrospective cohort study, we compared patients' characteristics between OC users and non-users by Mann–Whitney *U*-test and Fisher's exact test. Relative risk of OC use for endometrioma recurrence was calculated. Ten variables: age, presence of infertility, pain, previous medical treatment of endometriosis, previous surgery for ovarian endometrioma, the diameter of the largest cyst, unilateral or bilateral involvement, revised American Society of Reproductive Medicine (revised ASRM) score, post-operative OC use and post-operative pregnancy were evaluated to assess their independent effects on the recurrence rate using univariate analysis followed by a step-wise selection and logistic regression analysis.

For the before–after study, we used the Mann–Whitney *U*-test and Fisher's exact test to compare patients' characteristics between patients who underwent laparoscopy before and after the introduction of the 'post-operative OC recommendation'. The relative risk of the introduction of 'post-operative OC recommendation' for endometrioma recurrence was calculated.

Statistical analyses were performed by Statview for windows Ver. 5.0 (SAS Institute Inc., Cary, NC, USA). A *P*-value of <0.05 was considered statistically significant.

Results

Retrospective cohort study

Fig. 1 shows the flowchart of the 87 patients who underwent laparoscopic excision of endmetrioma after May 2005. All received the 'post-operative OC recommendation'. Of the 87 patients, 48 started to take OCs, but 39 did not. The reasons for not commencing OC were seeking pregnancy ($n = 25$) and age over 40 ($n = 6$) although 8 women chose not to take OC without a particular reason. Of the 48 patients who had started OC, 34 continued OC for the entire study period (24 months), whereas 14 discontinued. The reasons for discontinuing OC were desire for pregnancy ($n = 5$), and side effects ($n = 9$). This group suffered minor side effects e.g. headache and nausea; major complications such as thrombosis did not occur. The mean length of OC taken in this group was 9.7 ± 6.1 months.

Endometrioma recurrence was detected in 20 out of 87 subjects (23.0%) in all study groups. The mean diameter of the recurrent cyst at diagnosis was 33.1 ± 12.0 (SD) mm. Among patients who had recurrence, one underwent second-line surgery due to the large size of the recurrent cyst.

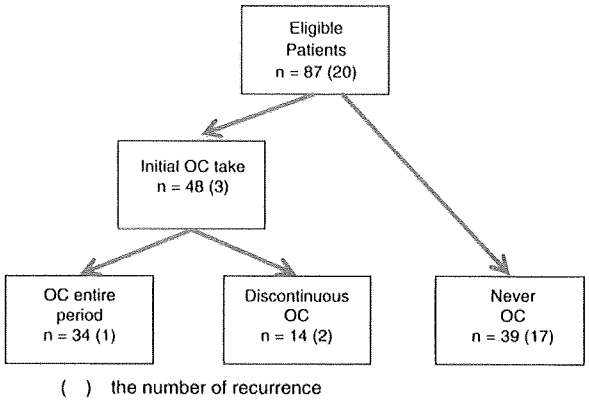

Figure 1 Flowchart of the patients who underwent laparoscopic excision of endometrioma after May 2005, for the retrospective cohort study.

A total of 87 patients were followed up for 24 months. Of the 87 patients, 48 started to take OCs, but 39 did not. Of the 48 patients who had started OC, 34 continued OC for the entire study period (24 months), whereas 14 discontinued. Recurrence was detected in 20 out of 87 subjects (23.0%) in all study groups. To determine the effect of OC use on recurrence, we compared recurrence rates with patients who used OC for the entire study period (entire period OC group), patients who discontinued OC use (discontinuous OC group) and patients who never used OC (never OC group). The recurrence rate was 2.9% (1/34), 14.3% (2/14) and 43.5% (17/39) in the entire period OC, discontinuous OC and never OC groups, respectively.

We then calculated recurrence rates with patients who used OC for the entire study period (entire period OC group), patients who discontinued OC (discontinuous OC group) and patients who never used OC (never OC group). The recurrence rates were 2.9% (1/34), 14.3% (2/14) and 43.5% (17/39) for the entire period OC, discontinuous OC and never OC groups, respectively.

We then combined the discontinuous OC group and never OC group together ('others') and compared that with the entire period OC group ('entire period OC group'). Table II shows the comparison of patients' characteristics between these two groups. Age, percentages of patients who have pain, previous medical treatment of endometriosis, previous surgery of ovarian endometrioma, the diameters of the largest cyst at laparoscopy, the percentage of patients who had bilateral involvement and the revised ASRM scores were not significantly different between groups. The percentage of patients who were infertile and patients who achieved pregnancy in the post-operative period were significantly lower in the 'entire period OC group' ($P < 0.05$ for both).

We then compared the recurrence rate between the groups. The recurrence rate in the 'entire period OC group' was significantly lower than in the 'others' (2.9 versus 35.8%, relative risk 0.082, 95% CI 0.012–0.58, $P < 0.001$). This indicates that taking OC without cessation reduces the risk of endometrioma recurrence by a factor of 12.

In order to confirm whether post-operative OC use is independently associated with the lower recurrence rate, we further performed univariate analysis followed by logistic regression analysis

Table II Comparison of characteristics between those who used OC for the entire period and the others who did not

Factors	OC entire period ($n = 34$)	Others ($n = 53$)	*P*-value
Age (years)	33.4 ± 4.8[a]	33.4 ± 4.3[a]	NS
Infertility	0%	11.0%	<0.05
Pain	55.9%	39.6%	NS
Previous medical treatment of endometriosis	38.0%	28.0%	NS
Previous surgery of ovarian endometrioma	3.0%	11.0%	NS
Largest cyst diameter (cm)	5.2 ± 2.4[a]	5.4 ± 2.8[a]	NS
Unilateral involvement	59.0%	66.0%	NS
Revised ASRM score	61 ± 37[a]	56 ± 38[a]	NS
Post-operative pregnancy	0%	17.0%	<0.05

ASRM, American Society for Reproductive Medicine; NS, not significant.
[a]Mean ± SD.

Table III Univariate and logistic regression analysis of factors related to the recurrence of ovarian endometrioma

Factors	Univariate analysis *P*-values	Logistic regression analysis *P*-values	Odds ratio (95% confidence interval)
Age (years)	NS	NS	0.951 (0.853–1.061)
Infertility	NS		
Pain	NS		
Previous medical treatment of endometriosis	NS		
Previous surgery of ovarian endometrioma	NS		
Largest cyst diameter (cm)	NS		
Bilateral involvement	NS		
Revised ASRM score	<0.05	<0.005	1.018 (1.003–1.034)
Post-operative OC for the entire study period	<0.01	<0.0001	0.054 (0.007–0.429)
Post-operative pregnancy	NS		

ASRM, American Society for Reproductive Medicine; NS, not significant.

(Table III). Ten variables were evaluated by univariate analysis and three variables (age, revised ASRM score and post-operative use of OC) were selected for logistic regression analysis by a forward stepwise variable selection. Post-operative use of OC was determined as an independent variable which is associated with lower recurrence (OR 0.054, 95% CI 0.007–0.429, $P < 0.001$). A higher revised ASRM score was significantly associated with higher recurrence (OR 1.018, 95% CI 1.003–1.034, $P < 0.01$). Neither infertility nor post-operative pregnancy influenced the rate of recurrence.

Before–after study

To evaluate the impact of the 'post-operative OC recommendation' on the endometrioma recurrence rate and pain recurrence rate in the overall population who underwent laparoscopic excision, we performed a historical study, the before–after study. Among patients who had a minimum of 24 months of post-operative follow-up, patients who were over the age of 40 and/or were infertile were excluded since these patients would not take OC regardless the recommendation. In total, 133 received laparoscopy before the introduction of 'post-operative OC recommendation' and 70 underwent laparoscopy after the introduction of the 'post-operative OC recommendation'. As indicated in Table IV, clinical characteristics of both groups were not different except for the patient's age. Among patients who received laparoscopy before the introduction of the 'OC recommendation', 15.8% actually used post-operative medical therapy including OC, GnRH analogue etc., whereas among patients who underwent laparoscopy after the introduction of 'OC recommendation', 78.6% started post-operative medical therapy, and most of the cases in this group was OC.

We then compared the recurrence rate between the groups. The overall endometrioma recurrence rate in patients who underwent laparoscopy after the introduction of the 'post-operative OC recommendation' was significantly lower than that in patients who received laparoscopy before the 'post-operative OC recommendation' (18.6 versus 33.1%, relative risk 0.56, 95% CI 0.32–0.97, $P < 0.05$). As for pain recurrence, the recurrence rate was not significantly different between the groups (58.7 versus 53.1% in before versus after the introduction of the 'post-operative OC recommendation', relative risk 0.90, 95% CI 0.62–1.31, $P = 0.6739$).

Discussion

In this study, we demonstrated the 'effect' of post-operative OC use on the recurrence of ovarian endometrioma following laparoscopic excision in a retrospective cohort study. Furthermore, we showed the 'efficiency' of our intervention; the introduction of the 'post-operative OC recommendation', for reducing the overall recurrence rate in a before–after study.

In the retrospective cohort study, we demonstrated that post-operative OC use significantly reduces the recurrence of ovarian endometrioma within 24 months following laparoscopic excision. The recurrence rate after surgery in patients who used OC for the entire study period (24 months), in patients who discontinued OC and in patients who never used OC was 2.9, 14.3 and 43.5%, respectively. The relative risk of recurrence was 0.082 in patients who used OC for the entire study period compared with patients who did not, in other words, patients who took post-operative OC without cessation were12 times less likely to have recurrence. We further showed that post-operative use of OC is an independent variable

Table IV Comparison between patients' characteristics before and after the 'OC recommendation'

Factors	Before 'OC recommendation' (n = 133)	After 'OC recommendation' (n = 70)	P-value
Age (years)	30.7 ± 4.8[a]	32.7 ± 4.3[a]	<0.01
Pain	60.2%	47.1%	NS
Previous medical treatment of endometriosis	47.4%	41.4%	NS
Previous surgery of ovarian endometrioma	9.0%	4.3%	NS
Largest cyst diameter (cm)	5.8 ± 1.8[a]	5.5 ± 1.9[a]	NS
Bilateral involvement	33.8%	35.7%	NS
Revised ASRM score	52 ± 30[a]	55 ± 31[a]	NS
Post-operative medical treatment of endometriosis	15.8%	78.6%	<0.001
Post-operative pregnancy	14.3%	8.6%	NS

ASRM, American Society for Reproductive Medicine; NS, not significant.
[a]Mean ± SD.

that is associated with lower recurrence. Subsequent to our previous study (Koga *et al.*, 2006) and commencement of the 'post-operative OC recommendation' in our clinic in 2005, two studies have been conducted to investigate the efficacy of post-operative OC in preventing endometrioma recurrence following laparoscopic excision. Vercellini *et al.* (2008a, b) evaluated the recurrence within 36 months after excision and reported that 94% were recurrence-free in the always OC users compared with 51% in the never users, with an incidence rate ratio of 0.10. Furthermore, in a study comparing cyclic and continuous OC use versus non-use, the recurrence rates within 24 months were 14.7, 8.2 and 29%, respectively (Seracchioli *et al.*, 2008). Our current results corroborate with these observations and strongly support the proposal that post-operative OC administration reduces the endometrioma recurrence rate after laparoscopic excision.

In addition to the 'effect' of post-operative OC use, we further demonstrated the 'efficiency' of the introduction of the 'post-operative OC recommendation' for reducing the endometrioma recurrence rate in the overall population who underwent laparoscopic excision, as demonstrated by the before–after study. The overall recurrence rate declined significantly from before to after the introduction of 'post-operative OC recommendation'. This is a unique observation that showed the impact of 'post-operative OC recommendation' in a daily clinical context. As a consequence of informing patients of the 'possible' benefits of OC in preventing recurrence and leaving the decision whether to take and continue OC to individuals, not many patients chose OC. However, the introduction of a 'post-operative OC recommendation' significantly contributed to the reduction of recurrence among the overall population who underwent laparoscopic excision. This encourages us to continue the 'post-operative OC recommendation' with further emphasis of its benefit in reducing endometrioma recurrence.

In contrast to the efficiency of the OC recommendation for reducing endometrioma recurrence, the intervention did not decrease pain recurrence. One explanation would be that the pain does not necessarily reflect the presence of endometriosis. It is also possible that our pain evaluation did not detect a subtle change of pain severity because it was not quantitative. Further studies are needed to determine the effect of OC on pain recurrence.

This study also suggests that OC can provide a better option in terms of safety and tolerability. Despite the small study group, no patients developed major complications and all side effects reported were minor, which is consistent with a previous report which used OC for dysmenorrhea (Harada *et al.*, 2008). Moreover, although the recommendation was not absolute, most women continued OC, indicating a good tolerability that yielded high compliance. This is in contrast to other hormonal drugs for endometriosis such as GnRH analogue and danazol, which are known to cause various side effects (Vercellini *et al.*, 2008a, b).

The mechanism by which OC reduces endometrioma recurrence has not been elucidated. Previously, we found that post-operative pregnancy was associated with a low recurrence rate (Koga *et al.*, 2006), and consequently we hypothesized that pregnancy or a pregnancy-mimicking hormonal condition may reduce the risk of recurrence. Indeed, several epidemiological studies have shown that OC use reduces the risk of endometriosis development (Vercellini *et al.*, 1993; Vessey *et al.*, 1993; Missmer *et al.*, 2004). One possible explanation is the effect of OC on the eutopic endometrium. OC intake has been reported to increase apoptosis and decrease cell proliferation in the eutopic endometrium (Meresman *et al.*, 2002), which could result in lowering *de novo* endometriosis development. It is also possible that OC may decrease the risk of endometrioma development by inhibiting ovulation since ovarian endometrioma can develop from ovarian follicles (Jain and Dalton, 1999) and corpus luteum (Vercellini *et al.*, 2009). Additionally, OC intake diminishes the amount of retrograde menstruation and reduces the chance of recurrence in the same manner as endometrial ablation can prevent endometriosis recurrence (Bulletti *et al.*, 2001; Osuga, 2008). If this is the case, it would be interesting to compare the effect of OC between cyclic and continuous administration since the latter yields less frequent bleeding. Intriguingly, Seracchioli *et al.* (2008) addressed this point and reported that the recurrence rate was slightly lower in continuous OC users than in cyclic users, although the difference was not statistically significant. Further studies will be needed to confirm a benefit of continuous OC.

Our study arouses debate in regard to the benefits of OC administration; whether it 'prevents' recurrence or 'delays' it. The first study

to evaluate the efficacy of post-operative OC demonstrated that using OC for a period of 6 months reduced the recurrence rate at 12 months, compared with controls, but then by 24 months this benefit was eroded and recurrence was similar to non-user controls (Muzii *et al.*, 2000). Consequently this group concluded that post-operative OC does not affect the long-term recurrence rate but achieves a delay in recurrence. Focusing on the disease recurrence after the cessation of OC, this observation seems consistent with ours and others (Vercellini *et al.*, 2008a, b), that OC users have an extremely small chance of recurrence (2.9% in our study, 6% in others) whereas patients who discontinued OC were at higher risk. These findings suggest that OC administration may only 'delay' the chance of recurrence rather than 'prevent'. From the clinical point of view, however, this 'delay' provides a significant benefit especially for patients who seek pregnancy several years after the laparoscopy.

There are several limitations in the current study. Firstly, the numbers of patients in both the retrospective cohort study and the before–after study were not sufficient enough to generalize the results to the general population, although the difference in each study was statistically significant. Secondly, since the retrospective cohort study was designed in a clinical context rather than in an experimental context, the allocation of subjects to OC groups was not random but based on patient preference. Therefore, the result might be affected by selection bias. This may explain why the magnitude of OC-use benefit in our study was comparable with the one designed in a clinical context (Vercellini *et al.*, 2008a, b), but was slightly different from the one conducted in more experimental context (Seracchioli *et al.*, 2008). Lastly, we cannot exclude the presence of confounding factors in our before–after study, such as surgical technique and surgeons' experience, although we tried to minimize them. Further studies will be necessary to overcome these limitations.

Several questions remain unanswered. Firstly, how long should patients continue using OC? Given that most of studies set the OC administration period at <3 years, it will be interesting to see whether OC can yield 'preventive' effects if the period of treatment is prolonged. Secondly, up to what age should OC is recommended? In the current study, we did not offer 'post-operative OC recommendation' for patients over the age of 40, since OC is considered to be relatively contraindicated for women over the age 40, in regard to promoting thrombotic disease (1981). However, given that an increasing number of women seek pregnancy in their early 40s and that the presence of endometriosis in the 40s has a high risk of cancer development (Kobayashi *et al.*, 2008), we must weigh the benefits of OC in preventing the recurrence against the possible adverse effects of OC. Further evidence is necessary to evaluate both efficacy and safety.

In summary, this study demonstrates that post-operative OC use reduces the risk of ovarian endometrioma recurrence after laparoscopic excision. Having this information will help gynecologists and patients plan pre- and post-operative management appropriately to prevent recurrence and consequently to maintain fertility for long as possible.

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Bone morphogenetic protein-6 stimulates gene expression of follicle-stimulating hormone receptor, inhibin/activin β subunits, and anti-Müllerian hormone in human granulosa cells

Immunohistochemical staining using human normal ovaries showed that bone morphogenetic protein-6 (BMP-6) was abundantly present in the granulosa cells (GC) of healthy tertiary follicles but not in atretic follicles. An in vitro study showed that BMP-6 induced gene expression of FSH receptor, inhibin/activin β subunits, and anti-Müllerian hormone (AMH) in human GCs, suggesting that BMP-6 is an important mediator to support healthy follicle growth in the human ovary. (Fertil Steril® 2009;92:1794–8. ©2009 by American Society for Reproductive Medicine.)

Folliculogenesis is the process by which primordial follicles grow and develop to the ovulatory follicle stage. Through this process, one healthy follicle is usually selected for maturation in the spontaneous menstrual cycle. It has been reported that granulosa cells (GCs) of healthy follicles express activins and FSH receptor (1, 2). Activins support GC survival and cell proliferation and maintain the functional FSH receptor (3). Activation of FSH receptor triggers cytodifferentiation and proliferation of GCs (3). Although activins and FSH receptor are recognized as important factors during folliculogenesis, the precise mechanism of activin and FSH receptor expression is poorly understood.

A growing body of evidence indicates that the bone morphogenetic proteins (BMPs), members of the transforming growth factor-β (TGF-β) superfamily, play a key role in female fertility in mammals (4, 5). Among the BMPs, BMP-15, GDF-9, BMP-7, and BMP-6 are expressed in the ovary. These four molecules maintain folliculogenesis by inhibiting early luteinization (4). In addition, each BMP has a specific role in folliculogenesis. BMP-15 and GDF-9 have been of particular interest in the study of mammalian reproduction. Mutation of the GDF-9 gene leads to arrested folliculogenesis in mice, ewes, and humans (4, 6, 7). In contrast, the mutation of BMP-15 leads to arrested folliculogenesis in monovulatory species, ewes, and humans, but not in mice (4). Furthermore, an in vitro transfection system of 293 human embryonic kidney and Chinese hamster ovary cell lines demonstrated that recombinant GDF-9 proteins of mice, ewes, and humans are readily processed. Although human and sheep BMP-15 are processed in this system, mouse BMP-15 is not produced (4). Thus, it has been proposed that GDF-9 protein is essential for early folliculogenesis in mammals, whereas the role of BMP-15 in folliculogenesis is different between species (4). Like BMP-15, the function of BMP-6 in folliculogenesis appears to differ in ruminants versus rodents (8, 9). BMP-6 decreased FSH receptor, inhibin α, inhibin/activin β subunits messenger RNA (mRNA) expressions in GC of rats (8), whereas BMP-6 enhanced inhibin-A and activin-A production in bovine GC (9). Regulation of activins and FSH receptor is crucial in folliculogenesis (1, 2), thus these findings prompted us to investigate the roles of BMP-6 in the human ovary. We studied the localization of BMP-6 in the ovary, and then examined the effects of BMP-6 on folliculogenesis-related molecules in GC. We also investigated the regulator of BMP-6 mRNA expression.

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MATERIALS AND METHODS

Except where indicated, all reagents were purchased from Sigma (St. Louis, MO). Recombinant human BMP-6 and BMP-7 were purchased from R&D Systems (Minneapolis, MN). A monoclonal antibody against BMP-6 was purchased from Chemicon International (Billerica, MA). Recombinant human FSH and activin-A were kindly provided by Nippon Organon (Tokyo, Japan) and Dr. Shunichi Shimasaki (University of California, San Diego, CA), respectively.

Collection of Ovarian Tissues and Immunohistochemistry

Tissue specimens of human ovaries were obtained under signed informed consent from eight women (age range, 28–40 years old) who underwent salpingo-oophorectomy for the treatment of

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uterine cervical cancer. All patients had normal ovarian cycles before surgery and any histologic abnormalities and malignant lesions were not observed in ovarian tissues. The experimental procedure was approved by the institutional review board (IRB). Ovarian tissues were fixed in neutral-buffered formalin and embedded in paraffin blocks, and 6-μm sections were prepared. Antigen retrieval was performed using sodium citrate buffer (10 mM, pH 6.0) (10). The sections were stained with 2 μg/mL anti-BMP-6 antibody or mouse IgG as negative control using an Envision+ System/HRP Mouse (DAB+) kit (Dako, Tokyo, Japan). Healthy and atretic follicles were identified on the basis of classic histologic features (11). Briefly, the healthy follicles had multiple intact layers of GC, which lined the entire circumference of the basal lamina. The atretic follicles displayed a variety of degenerative changes; in atretic follicles, sheets of GC had dislodged and were floating free in the antral cavity.

Cell Culture of Human Granulosa Cells

Granulosa cells were obtained from patients undergoing ovarian stimulation for IVF. The method to purify and culture human GC was described previously (12). The experimental procedures were approved by the IRB, and signed informed consent for use of GC was obtained from each patient. The collected human GC were cultured in Dulbecco's minimum essential medium (DMEM)/F12 containing 5% fetal bovine serum and antibiotics in 12-well plates at a density of 2×10^5 cells/mL. To evaluate the effects of BMP-6, human GC were cultured with or without BMP-6 (100 ng/mL) for 24 hours. To investigate the regulation of BMP-6, GC were cultured with BMP-6 (100 ng/mL), BMP-7 (100 ng/mL), E_2 (10 ng/mL), activin-A (100 ng/mL), FSH (0.5 IU/mL), or 8-bromo-cyclic adenosine 3′:5′ monophosphate (cAMP) (1 mM).

Reverse Transcription and Quantitative Real-Time Polymerase Chain Reaction Analysis

Total RNA extraction from GC, the primer sequences and real-time polymerase chain reaction (PCR) conditions were describe elsewhere (12), except the primer sequence of anti-Müllerian hormone (AMH) (NM_000479: 619-638 and 820-801) and BMP-6 (NM_001718: 420-441 and 839-820). All results are shown as mean ± SEM of data from at least three separate experiments, each performed with triplicate samples. Data were analyzed by Student's *t*-test for paired comparison and one-way analysis of variance (ANOVA) with post hoc test for multiple comparisons. A *P* value less than .05 was considered statistically significant.

RESULTS

Localization of BMP-6 in Human Ovaries

The expression of BMP-6 in human ovaries was examined by immunohistochemistry using normal human ovaries. As shown in Figure 1a,c, BMP-6 expression was clearly detected in the oocytes of primordial and primary follicles. BMP-6 was also detected in GC. The intensity of the staining of BMP-6 in GC was barely detected in primordial follicle, and low in GC of primary and secondary follicles (Fig. 1a,c,d), whereas it was high in GC of healthy antral follicles (Fig. 1e,g). In contrast, BMP-6 staining was very weak in GC of atretic follicles (Fig. 1f,h).

The Effect of BMP-6 on Gene Expression of Folliculogenesis Factors

Incubation of GC with BMP-6 (100 ng/mL) for 24 hours significantly increased the gene expression of inhibin/activin βA and βB subunits, but exerted no effect on inhibin α subunit mRNA (Fig. 2a–c). Notably, BMP-6 caused a nearly threefold increase in FSH receptor mRNA levels (Fig. 2d). BMP-6 also significantly increased AMH mRNA levels (Fig. 2e).

Regulation of BMP-6 in Granulosa Cells

To investigate the regulation of BMP-6 gene expression, human GCs were cultured with various stimuli. Because the growth of follicles is highly influenced by intraovarian factors (i.e., activins and BMP-7), we checked whether these factors could induce BMP-6 expression. As shown in Figure 2f, activin-A (100 ng/mL) and BMP-7 (100 ng/mL) increased the mRNA level of BMP-6, whereas BMP-6 (100 ng/mL) itself had no effect. In addition, 8-bromo-cAMP, FSH, and E_2 did not alter BMP-6 mRNA levels (data not shown).

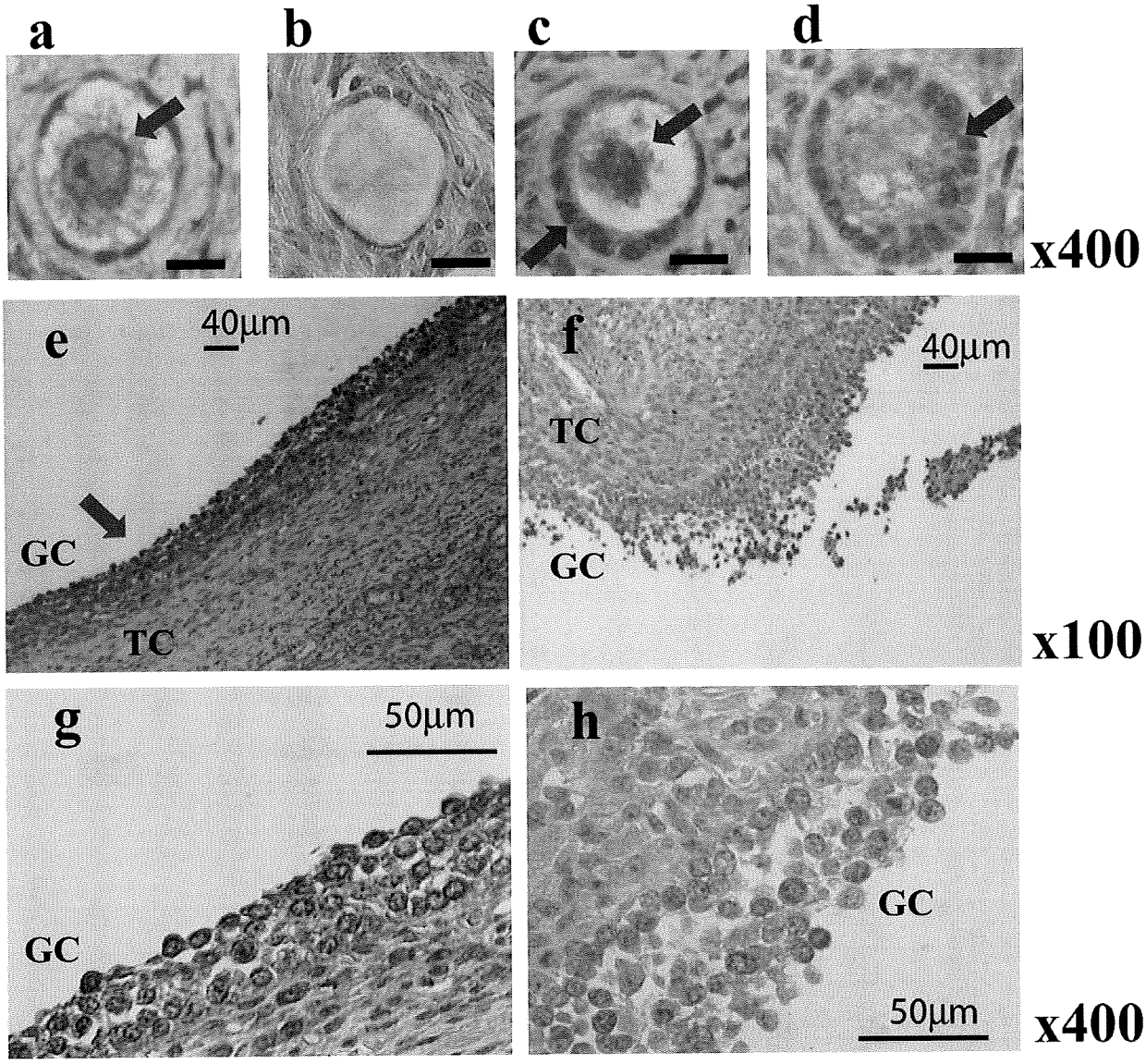
DISCUSSION

In the present study, we demonstrated that BMP-6 is strongly expressed in GC of tertiary follicles and oocytes. In cultured human GC, BMP-6 stimulated gene expression of the inhibin/activin βA and βB subunits, but not the inhibin α subunit. BMP-6 also stimulated mRNA expression of FSH receptor and AMH in cultured human GC. In addition, BMP-6 gene expression in cultured human GC was increased by activin-A and BMP-7.

Our immunohistochemical study revealed that BMP-6 protein was strongly expressed in GC of healthy tertiary follicles (Fig. 1e,g). In contrast, BMP-6 protein was only weakly expressed in GC of atretic follicles (Fig. 1f,h). Because BMP-6 increased mRNA expression of inhibin/activin β subunits in GC, the observed expression pattern of BMP-6 in GC is consistent with the finding that inhibin/activin β subunits are expressed in healthy follicles, but not in the similarly sized atretic follicles (1). Serum FSH concentration decreases in the latter half of the follicular phase. Therefore, the sensitivity of follicles to FSH during this period is critical and determines whether follicles become atretic or dominant (2). In view of the present finding that BMP-6 increased the expression of FSH receptor, follicles with high BMP-6 expression may be more likely to survive the decrease in serum FSH, thus increasing the chances of surviving to the dominant follicle stage. This notion is also supported by the strong expression of BMP-6 in GC of healthy tertiary follicles. In contrast to our immunohistochemical findings in human ovaries, the mRNA levels of BMP-6 in GC were found to decrease at the time of dominant follicle selection in rats (13). Furthermore, our findings on the in vitro effects of BMP-6 in cultured human GC are the opposite of that observed in rats, in which BMP-6 decreased FSH-induced expression of FSH receptor and inhibin/activin β subunits (8). One possible explanation may be that the different expression pattern of BMP-6 in the follicle between human and rat in vivo is due to the different effect of BMP-6 on folliculogenesis between the species. Glister et al. (9) also reported that in bovine GC, the effect of BMP-6 is different from rat GC. Because there is a growing evidence that the expression pattern of BMP-15, another BMP family cytokine, is different from in mono-ovulatory and polyovulatory species, leading to the

FIGURE 1

Localization of bone morphogenetic protein-6 (BMP-6) expression in human ovaries. The BMP-6 expression in normal human ovaries was investigated by immunohistochemistry. Primordial follicle (**a, b**), primary follicle (**c**), secondary follicle (**d**), healthy tertiary follicle (**e, g,**) and unhealthy tertiary follicle (**f, h**). (**b**) Negative control. Arrows indicate positive BMP-6 signal. GC = granulosa cells; TC = theca cells.



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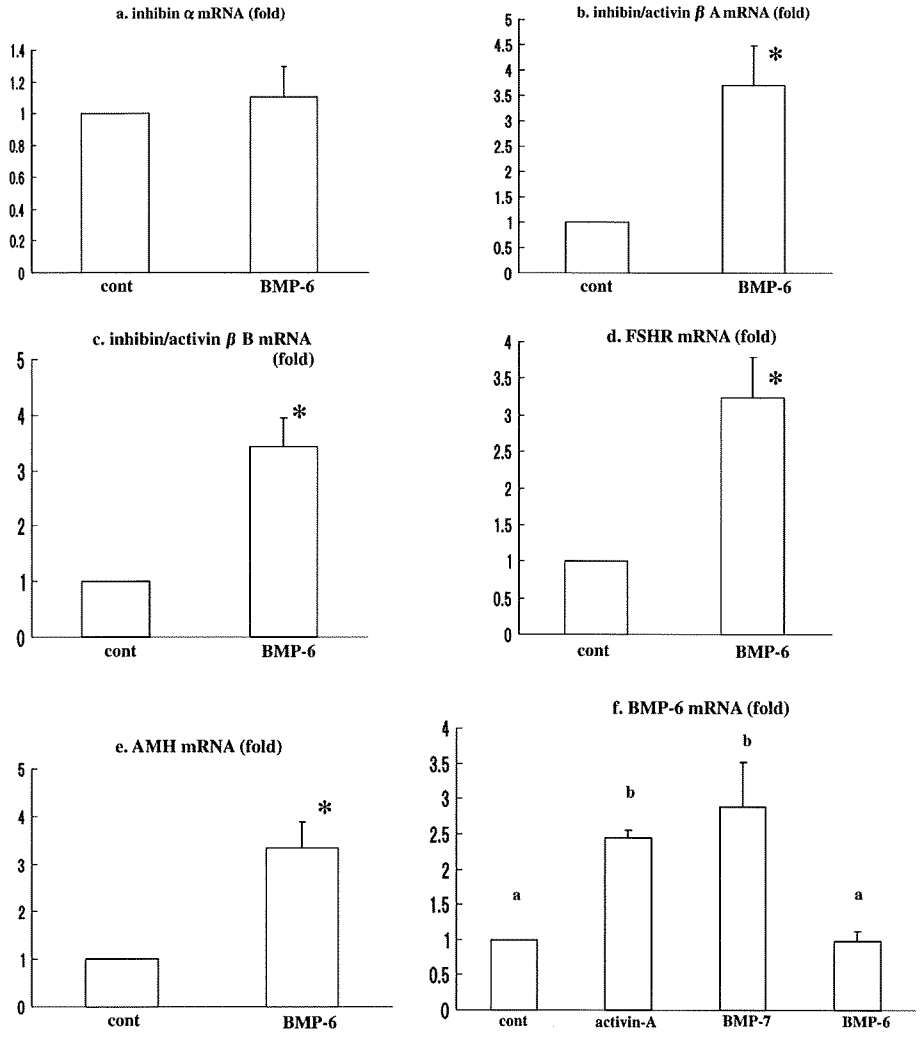
concept that BMP-15 governs ovulation quota, mono, and polyovulation (4), it is possible that the differences in BMP-6 expression and functions between species might also be related to ovulation quota.

Activin-A and BMP-7 increased mRNA level of BMP-6 in cultured human GC. Activins and BMP-7 are derived from GCs and theca cells, respectively (5), thus, an autocrine or paracrine mechanism might be working to regulate BMP-6 expression in the follicle. Interestingly, activin-A and BMP-7 are both known to induce FSH receptor (12). In view of the present finding that BMP-6 induced FSH receptor mRNA in GC, activin-A and BMP-7 might induce FSH receptor partially by up-regulation of BMP-6 expression in GC.

Our immunohistochemical study also revealed that oocytes of primordial and primary follicles strongly expressed BMP-6 protein (Fig. 1a,c). In primordial and primary follicles, which do not express FSH receptor, activins are known to be important factors for follicle growth (3), but regulation of activins in the follicles of this stage is not well understood. Given that BMP-6 secreted from oocytes of primordial and primary follicles could act on

FIGURE 2

(**a–e**) Effect of bone morphogenetic protein-6 (BMP-6) on inhibin α (**a**), inhibin/activin βA (**b**), inhibin/activin βB (**c**), FSH receptor (**d**), and anti-Müllerian hormone (AMH) (**e**) messenger RNA (mRNA) expression. The granulosa cells (GC) were cultured with BMP-6 (100 ng/mL) for 24 hours. (**f**) Effect of various stimuli on BMP-6 mRNA expression. The GCs were cultured with activin-A, BMP-7, or BMP-6 (100 ng/mL) for 24 hours. Total RNA was extracted from the GCs and subjected to real-time polymerase chain reaction (PCR) to determine the mRNA levels. Data were normalized to GAPDH mRNA levels. Data from three different experiments were combined and represented as the mean ± SEM relative to an adjusted value of 1.0 for the mean value of the each control. *$P<.05$ (vs. control). Bars with different letters indicate a significant difference at $P<.05$.



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GC, activins, rather than inhibins, might be induced preferentially in early follicles that do not express the inhibin α subunit abundantly. Namely, BMP-6 derived from oocytes might be an inducer of activins in the primordial and primary stages of folliculogenesis. We also found that activin-A induced BMP-6 expression (Fig. 2f), thus BMP-6 and activins might have a reciprocal effect on inducing one another, especially in the early follicles.

Recently, AMH has been demonstrated to play an important role in ovarian function with its inhibitory effect on follicle recruitment (14). In addition, in humans, AMH has been found to be a marker of ovarian reserve (14). Although it is reported that FSH and E_2 down-regulate AMH expression in the GC (15), no AMH up-regulators have been identified to date. The present study provided the evidence that BMP-6 increased expression of AMH. Thus, we hypothesize that BMP-6 in the healthy growing follicles up-regulates AMH expression, which, in turn, suppresses growth of the surrounding primordial follicles, thereby preserving the ovarian reserve.

In summary, BMP-6 is expressed in the GC of healthy, growing follicles, but not in atretic follicles. BMP-6 increased gene

expression of FSH receptor, inhibin/activin β subunits, and AMH, contributing to the survival of healthy growing follicles and suppressing depletion of the primordial follicle reserve. Cultured GCs used in this study may not represent the stages of growing follicles and further studies are needed. However, our findings that BMP-6 regulates folliculogenesis-related genes in human GCs are novel, and open new insights into our understanding of ovarian physiology.

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