and activation of Sma- and Mad-related protein (Smad)-2 and Smad3 in granulosa cells (38–40). In rodents, activin-like receptor kinase-5 mRNA/protein and Smad2/3 proteins are expressed in the oocyte, granulosa, and thecal cells of both preantral and antral follicles (41), whereas BMP receptor type II mRNA expression is observed only in granulosa cells (42). These results suggest that thecal cells are not capable of responding to GDF-9 and that GDF-9 indirectly modulates thecal cell function through a granulosal factor(s). Nevertheless, one could not exclude the possibility that additional type I and type II receptors for GDF-9 might be present in thecal cells because *in vitro* studies demonstrated direct actions of GDF-9 on thecal cell androgen synthesis in rats (18) and cattle (19).

Intracellular Smad signaling molecules might also play roles in controlling CYP17A1 expression and steroid production in various types of cells. There are eight Smad proteins in total, Smad1–8. Typically, Smad2/3 are activated by members of the TGF-$\beta$/activin subfamilies (*e.g.* TGF-$\beta$, activin, and GDF-9), and Smad1/5/8 are activated by members of the BMP subfamilies (*e.g.* BMP-4, -6, -7, and -15). BMP-4, -6, and -7 suppressed basal and LH-induced CYP17A1 mRNA expression and androgen production in bovine thecal cells (43). Müllerian-inhibiting substance, which also interacts through the Smad1/5/8 pathway, inhibits the cAMP-induced expression of CYP17A1 mRNA in Leydig cells (44). In contrast, Smad2/3 signaling activated by activin increases CYP17A1 expression and steroid production in adrenal cells (45), which resembles GDF-9 action in the present results. It has also been demonstrated that Smad3 interacts with AR in prostate cancer cells (46).

In summary, we examined whether and how oocyte-derived GDF-9 controls follicular development and steroidogenesis during preantral-early antral transition by a combination of *in vitro* gene manipulation (*i.e.* intraoocyte injection of GDF-9 antisense oligos) and preantral follicle culture. Using this *in vitro* model, we have shown that GDF-9 plays an important role in promoting preantral follicle growth by up-regulating follicular androgen biosynthesis. GDF-9 is essential for CYP17A1 expression during follicular development from preantral to early antral stage.

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—Mini Review—

# Morphological Evaluation and Measurement of the Respiration Activity of Cumulus-oocyte Complexes to Assess Oocyte Quality

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**Abstract:** Scanning electrochemical microscopy (SECM) is a non-invasive and sensitive technique for measuring cellular respiration. In this paper, we review the SECM technique, to establish it as an accurate method for measuring the respiratory activity of single cumulus-oocyte complexes (COCs) and oocytes in animals as well as in humans. Oxygen consumption rates of COCs are influenced by the surrounding cumulus volume and the mitochondrial activity of the cumulus cells. An increase in the oxygen consumption rate was found in bovine oocytes, whereas the oxygen consumption of human oocytes tends to decrease during in vitro maturation (IVM). To analyze the metabolic activity of mitochondrial respiration, ATP content and mitochondrial distribution in bovine oocytes have been examined. An electron microscopic study confirmed mitochondrial reorganization in bovine oocytes during oocyte maturation. These results show that the respiratory activity of oocytes changes with maturation status during IVM and mitochondrial reorganization may partly influence respiratory activity. The SECM procedure is therefore a useful technique for evaluating the metabolic activity and quality of oocytes and cumulus cells in the IVM process.

**Key words:** Cumulus cells, Oocyte maturation, Mitochondria, Oxygen consumption, Electrochemical measurement

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## Introduction

The *in vitro* maturation (IVM) of human oocytes is an attractive technique that provides a patient-friendly approach to assisted reproductive technology. IVM is relatively simple with a shorter period of treatment and lower costs than conventional *in vivo* fertilization (IVF). For anovulatory patients with polycystic ovaries (PCO), a decrease in the dose of ovarian stimulating drugs lowers the risk of ovarian hyperstimulation syndrome. IVM has been successfully applied to animals [1, 2]. Cha *et al.* were the first group to show the success of IVM in human beings using immature donor oocytes retrieved from antral follicles [3]. Recent studies have demonstrated that the results from IVM are comparable to those achieved with contemporary IVF [4, 5]. The applicability and development of IVM technology is dependent on the improvement of *in vitro* culture systems. During *in vitro* culture, cumulus cells play an important role in oocyte maturation. If provided with the several factors that are essential for normal nuclear and cytoplasmic maturation, oocytes can mature and develop to an embryo after fertilization [6]. Therefore, an appropriate evaluation of cumulus-oocyte complexes (COCs) is indispensable for evaluating the quality of oocytes and improving of the results of IVM.

Over the years, several approaches have been used to evaluate COCs. Morphological evaluation is the main technique used to assess COC quality and to predict the subsequent maturation of oocytes in the IVM process. However, morphological evaluations are subjective and difficult, especially for COCs with

intermediate morphological qualities. Therefore, more objective evaluation criteria are needed. Previous studies have suggested that a greater understanding of the metabolic respiration of cumulus cells might yield new strategies for evaluating the quality of bovine oocytes [7, 8]. In this paper we describe the morphological evaluation of COCs and the application of a novel cell respiration measuring system using scanning electrochemical microscopy (SECM) to the assessment of the metabolic activity of cumulus cells and oocytes in bovine and human specimens.

## Morphological Evaluation of Cumulus-oocyte Complexes and Oocytes

An appropriate evaluation of COCs is indispensable for the improvement of the IVM system, because cumulus cells play an important role in oocyte quality. For a morphologically precise evaluation of human COCs, size of an oocyte is an important parameter. The precise evaluation of human COCs is needed to predict the competence of oocyte maturation. Prior research has indicated that the diameter of immature oocytes is one of most reliable parameters for predicting oocyte quality. Eppig and Schroeder reported that mice oocytes isolated from females younger than 13 days of age are capable of spontaneous break down of the germinal vesicle (GVBD) when the mean diameter is greater than 60 $\mu$m [9]. In a study of porcine immature oocyte, progression to metaphase II was observed in 40% of oocytes that were over 110 $\mu$m in diameter, whereas no oocyte less than 90 $\mu$m in diameter resumed meiosis [10]. In rhesus monkey oocytes, in which meiotic competence occurs late during oocyte development, oocyte diameters appear relatively constant as the competence to undergo GVBD increases. These phenomena suggest that there is no association between oocyte diameter and maturation [11].

In comparison to animal systems, little is known about humans. Based on data from unstimulated polycystic ovary syndrome (PCOS) patients, Cavilla *et al.* deduced that an oocyte diameter of 81 $\mu$m at the time of retrieval was the threshold for GVBD, whereas oocytes of more than 103 $\mu$m would mature to metaphase II [12]. They also noted that, during *in vitro* human oocyte maturation, an increase in the average diameter of only 3 $\mu$m (from 106 to 109) represents a large change in the cytoplasmic volume (increasing an astonishing 8% during culture), suggesting that oocyte diameter provided valuable information about oocyte potential during IVM. In a proposal for the precise evaluation of human immature oocytes, the two-dimensional area of a depiction image was calculated using image analysis software "Image J".

Another important parameter of oocyte morphological quality is the volume of the human cumulus mass. During IVM, cumulus cells are known to maintain the oocyte nucleus at the GV stage [13]. Expanded human cumulus-oocyte complex patterns have a higher expression of LH receptor mRNA and are associated with more efficient oocyte maturation [14]. Early reports supported the idea that cumulus expansion during IVM improves the developmental capacity [15, 16]. In mice, the fertilization rate of IVM matured oocytes is correlated with the quantity and quality of the expanded cumulus mass [17]. The mechanical loss or spontaneous loss of cumulus cells from COCs has been shown to correlate with a loss of fertilizability [18]. Therefore, the quantity of cumulus mass is a factor influencing the success of IVM. For the morphological classification of cumulus mass, some researchers have separated the cumulus patterns into multilayered and expanded [19, 20].

In this review, we have estimated the multilayered and expanded cumulus mass as the consecutive change and made objective evaluations utilizing an image analysis software program. The COC area was calculated by tracing the edge of the cumulus mass. If the edge was not clear (usually observed in the expanded cumulus mass), the image was analyzed using an edge enhancement mode. The multiple layer formation of the cumulus mass was presented as the C-ratio (area of COC / area of immature oocyte). For the morphological evaluation of human COCs, forty-two human COCs, retrieved from eight women with the PCOS during an IVM program, were used. All COCs were aspirated 36 hours post-hCG between the 10th and 12th day of the menstrual cycle and cultured for 26 hours in TCM199 medium with 10% patient serum, 100 IU/L human chorionic gonadotropin and 75 IU/L follicle stimulating hormone under an atmosphere of 5% $CO_2$, 5% $O_2$, and 90% $N_2$. The parameters analyzed were (1) *Area*; area of the immature oocyte and (2) *Layer*; multiple layer formation of the cumulus mass, presented as the C-ratio. As shown in Fig. 1, these two morphological parameters were compared between the immature (germinal vesicle: GV or metaphase I: MI) and mature (metaphase II: MII) oocyte groups after *in vitro* culture. In the *Area* comparison, the mean level of *Area* was significantly higher in the mature group (8,886 ± 184 vs. 9,806 ± 161, Mean ± SEM, $P < 0.05$). In the

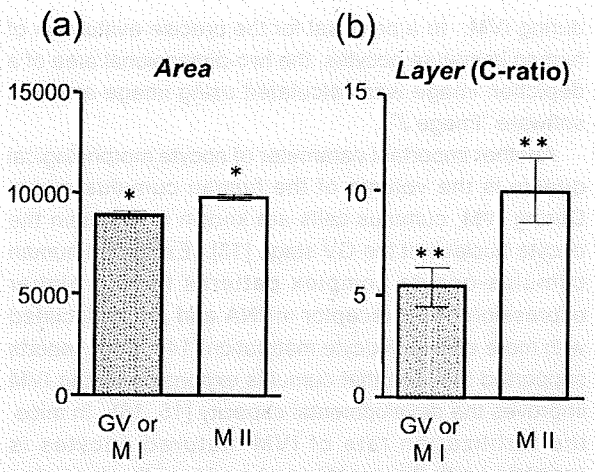


**Fig. 1.** Comparison of morphological parameters of human immature oocyte and COCs. a: *Area*, Area of immature oocyte; b: *Layer*, Multiple layer formation of cumulus mass was evaluated as C-ratio (Area of COC / Area of immature oocyte). Area and Layer were analyzed before *in vitro* culture and compared prospectively between mature (MII) and immature (MI or GV) group. *, **: significantly different ($P < 0.05$).

Layer comparison, the mean level of the C-ratio was significantly higher in the mature group (5.8 ± 0.8 vs. 10.3 ± 1.6, Mean ± SEM, $P < 0.05$). These results suggest the C-ratio is a useful parameter for predicting the maturation status of oocytes in the IVM process.

Subsequently, we examined the relationship between the cumulus mass morphology and the oocyte quality. Human COCs were classified into five grades based on cumulus mass morphology as follows: Grade 1 (G1), cumulus cells with multi-layers covering the whole oocyte, and a regular round oocyte; Grade 2 (G2), cumulus cells with multi-layers (less than three layers), covering the whole oocyte and a regular round oocyte; Grade 3 (G3), regular round oocytes with cumulus cells covering half of the domain; Grade 4 (G4), naked oocytes without cumulus mass; Grade 5 (G5), naked and irregular shaped oocytes (Fig. 2). High maturation rates of immature oocytes were detected in G1 and G2 (70.0% and 64.3%, respectively) in contrast to the lower maturation rate of 17.7% (mean percentage from G3 to G5).

Cumulus cells are a production site of steroids, growth factor, proteins and other compounds that contribute to cytoplasmic maturation of oocytes. Beneficial effects of cumulus cells on microtubule dynamics and/or chromatin stability, oocyte maturation and early embryonic development have been reported in many species, including humans [21–23]. Cumulus cells are also known to play an important role in the regulation of the meiotic progression of oocytes. During the growth and development of meiotic competence of an oocyte, the cumulus cells are responsible for maintenance of nuclear arrest at the germinal vesicle (GV) stage by transfer of an inhibitory signal through gap junctions which elevates the intracellular cyclic adenosine monophosphate (cAMP) level in the oocytes

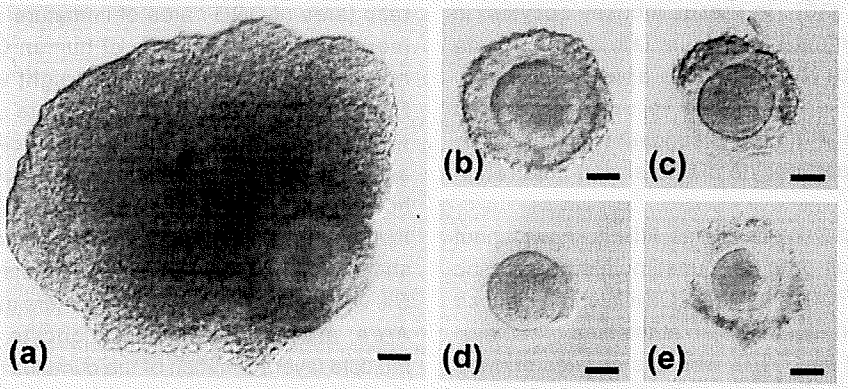


**Fig. 2.** Light micrographs of human COCs classified by morphological evaluation. (a) Grade 1, cumulus cells with multi-layers covering the whole oocyte, and a regular round oocyte; (b) Grade 2, cumulus cells with multi-layers (less than three layers), covering the whole oocyte and a regular round oocyte; (c) Grade 3, regular round oocytes with cumulus cells covering half of the domain; (d) Grade 4, naked oocytes without cumulus mass; (e) Grade 5, naked and irregular shaped oocytes. Bars = 20 $\mu$m.

[24, 25]. Although the precise regulation mechanism of meiotic progression is still controversial [26], it has been suggested that well-developed cumulus cells have the capacity to regulate appropriate maturation and the development of immature oocytes.

## Evaluating the Quality of Embryos and Oocytes with Measurement of Respiration Activity with an Electrochemical Measuring Technique

The metabolic activity of embryos and oocytes has been determined from the consumption of nutrients, such as glucose, pyruvate and amino acids [27–30]. Oxygen consumption is an idea indicator of overall metabolic activity because adenosine triphosphate (ATP) is predominantly generated by oxidative phosphorylation, a process in which oxygen plays an essential role [31–33]. Oxygen consumption by embryos and oocytes has been studied with various measuring techniques, such as the Cartesian diver [34, 35], spectrophotometry [36, 37], ultrafluorescence measurements [38, 39], and self-referencing microelectrodes [40–43].

Electrochemical measurement using scanning electrochemical microscopy (SECM) is a technique in which the tip of a microelectrode monitors the local distribution of electro-active species, such as oxygen near the sample surface [44]. This technique can measure the concentration profile of a metabolic product around a spherical sample, such as an embryo, with a probe microelectrode. We have employed the SECM technique to examine oxygen consumption by single embryos [45]. Using a modified SECM measuring procedure, we quantified the respiration activity of embryos in several animal species including humans [46]. SECM can non-invasively measure the respiration activity of single embryos from livestock, such as cattle and pigs, as well as those from small rodents, all with high reproducibility. We recently demonstrated that bovine embryos with high oxygen consumption are better candidates for further development into good quality embryos and yielded higher pregnancy rates after embryo transfer. The respiration activity correlates with the embryo quality. SECM is a highly sensitive and non-invasive method for measuring cellular respiration and may be a valuable tool for accurately assessing the quality of embryos, which could contribute to improved outcomes in assisted reproduction, including human IVF. On the other hand, an accurate method for evaluating the respiratory activity of oocytes remains to be developed.

**Table 1.** Oxygen consumption rates ($F \times 10^{14}$/mol · s$^{-1}$) of bovine COCs and denuded oocytes in oocyte maturation cultures

| Maturation status | COC (n) | Oocyte (n) |
|---|---|---|
| Immature | $5.48 \pm 0.82$ (16)[a] | $0.67 \pm 0.02$ (16)[c] |
| Mature | $3.15 \pm 0.42$ (20)[b] | $1.10 \pm 0.05$ (20)[d] |

Values with different superscripts in each column differ significantly ($P < 0.05$).

Oocyte quality could be the most important factor in determining successful fertilization and embryo development. Therefore, we attempted to establish an evaluation system for oocyte quality based on the respiratory activity of oocytes.

In previous studies, we evaluated the SECM technique, to establish an accurate method for measuring the respiratory activity of single bovine and porcine oocytes [8, 48]. With the SECM procedure, oxygen consumption of bovine COCs and denuded oocytes was monitored (Table 1). Oxygen consumption rates ($\times 10^{14}$/mol · s$^{-1}$) of immature COCs and oocytes (immediately after recovery from an ovary) were 5.48 and 0.67, respectively. Although the respiration rate of denuded oocytes was lower than that of cumulus cells, the oxygen consumption rate by a single bovine oocyte was quantitatively measured by SECM.

Oxygen consumption has been monitored in COCs and oocytes cultured in serum-free medium for oocyte maturation. An increase in the oxygen consumption rate was found in oocytes [1, 10], whereas the oxygen consumption by COCs [3, 15] decreased during IVM. To analyze the metabolic activity of mitochondrial respiration, the ATP content and mitochondrial distribution in oocytes were examined. The ATP content of oocytes after maturation culture was significantly higher than that of immature oocytes (Fig. 3). In immature oocytes, staining with MitoTracker Orange revealed mitochondrial clumps with a strong signal in the periphery of the cytoplasm (Fig. 4). After IVM, the mitochondrial clumps were located more toward the center of the cytoplasm. An electron microscope study confirmed mitochondrial reorganization in bovine oocytes during oocyte maturation (Fig. 5). These results show that the respiratory activity of bovine oocytes increases during IVM and mitochondrial reorganization may thus be partly due to the respiratory activity. Therefore, we consider the SECM procedure is a useful technique for evaluating the metabolic activity and quality of single oocytes.

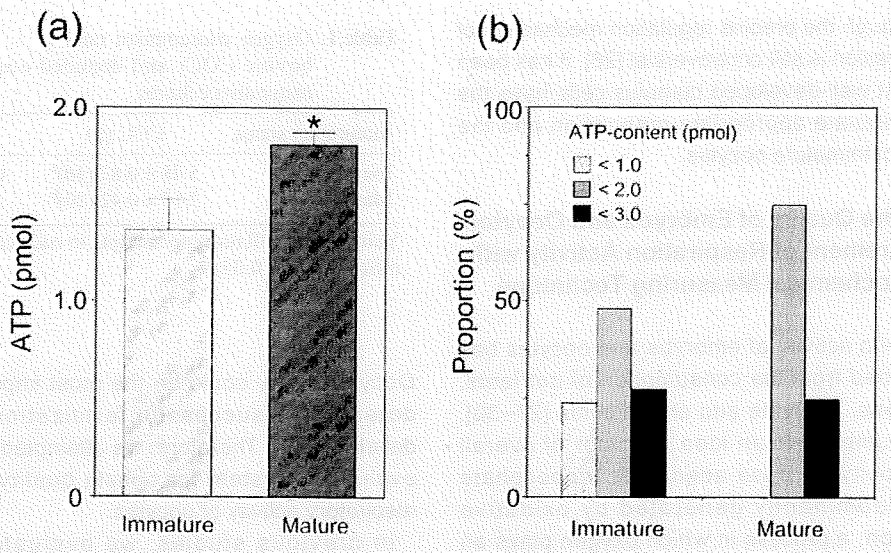


**Fig. 3.** (a) The ATP content and (b) proportion of oocytes categorized by ATP content: immature and mature bovine oocytes. *: significantly different ($P < 0.05$).

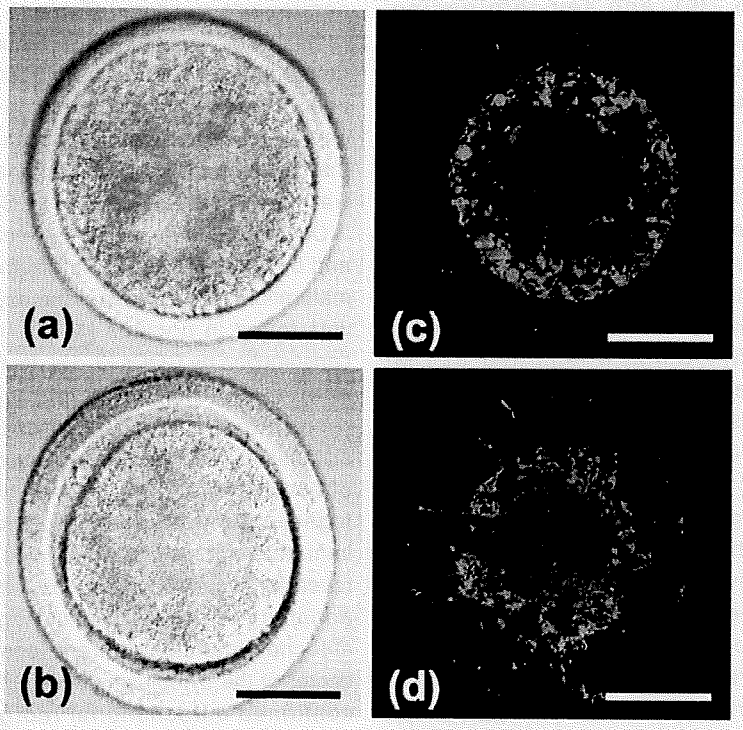


**Fig. 4.** Midline confocal sections of (a, b) immature and (c, d; cultured in IVMD101 medium) mature bovine oocytes stained by MitoTracker orange. Bars = 50 $\mu$m.

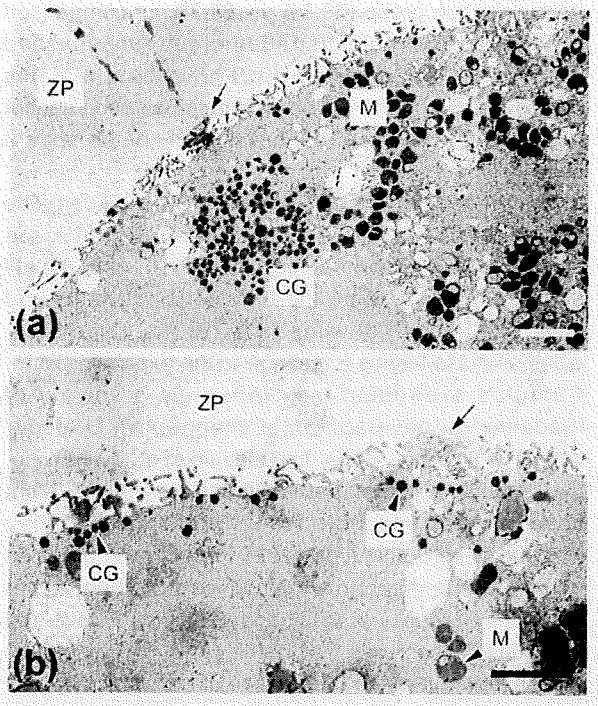


**Fig. 5.** Electron micrographs of (a) immature and (b) cultured in serum-free maturation medium mature oocytes. a: Many aggregates of mitochondria (M) and cortical granules (CG) were present in the cortex cytoplasm. b: Cortical granules were distributed in the periphery of the cytoplasm, but aggregates of mitochondria were not found. ZP, zona pellucida. Arrows: microvilli. Bars = 2 $\mu$m.

## Measuring the Respiration Activity of Human COCs and Oocytes

In this part, we review the respiration activity of human COCs and oocytes. Eighty-five human COCs retrieved from eighteen women with the PCOS during an IVM program were examined. Informed consent for the use of the COCs in this study was obtained from all the patients. Thirteen of the eighteen women were administered a short course of follicular stimulating hormone (FSH) and five women accomplished an IVM program without the use of FSH before hCG administration. All COCs were aspirated 36 h post-hCG between the 10th and 12th day of the menstrual cycle and cultured 26 h in TCM199 medium with 10% patient serum, 100 IU/L human chorionic gonadotropin and 75 IU/L follicle stimulating hormone under an atmosphere of 5% $CO_2$, 5% $O_2$, and 90% $N_2$. Cellular unevenness of the cumulus mass has an influence on SECM measurement results. Therefore,

**Table 2.** Oxygen consumption rates ($F \times 10^{14}$/mol · s$^{-1}$) of human COCs classified by morphological evaluation

| Category | Pre-culture (n) | Post-culture (n) |
|---|---|---|
| G1 | 7.79 ± 1.00 (50) | 6.11 ± 0.74 (50) |
| G2 | 1.46 ± 0.15 (25) | 1.63 ± 0.33 (25) |
| G3 | 1.26 ± 0.35 (8) | 1.60 ± 0.55 (8) |
| G4 | 0.86 ± 0.30 (2) | 0.79 ± 0.11 (2) |
| G5 | 0.77 (1) | 0.35 (1) |

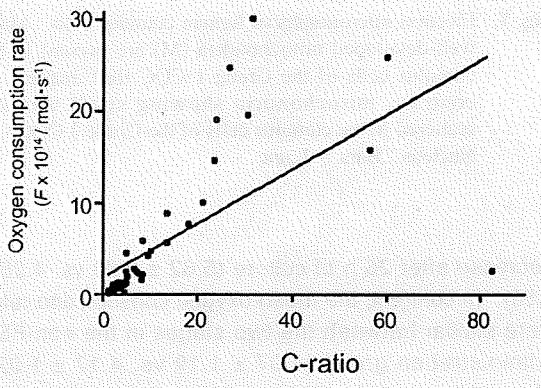


**Fig. 6.** Correlation between oxygen consumption rate and C-ratio.

the oxygen consumption rate was measured three times for each COC and the mean was used as the measured value.

Relationship between the oxygen consumption rates and morphological categories of human COCs in the pre-culture and post 26 hours-culture stages is shown in Table 2. A linear correlation between the oxygen consumption rate and the C-ratio was shown in Fig. 6 (correlation coefficient: $r^2 = 0.423$, $P < 0.01$). The respiration activity measured by SECM showed that the respiration activity of human COCs with multi-layer cumulus cells (G1) was higher than in the other categories (G2–G5). Ultrastructual studies revealed that the cumulus cells in G1 COCs, which showed high respiration activity, contained many well-developed mitochondria. In contrast, undeveloped mitochondria were scattered in the cumulus cells in G3 COCs (Fig. 7). These results suggest that respiration rates are directly influenced by the surrounding cumulus volume and mitochondrial activity in cumulus cells.

A comparison of the oxygen consumption fluctuation between the pre-culture stage and after 26 hours of culture is shown in Fig. 8. In the FSH administration group, the mean oxygen consumption rate tended to

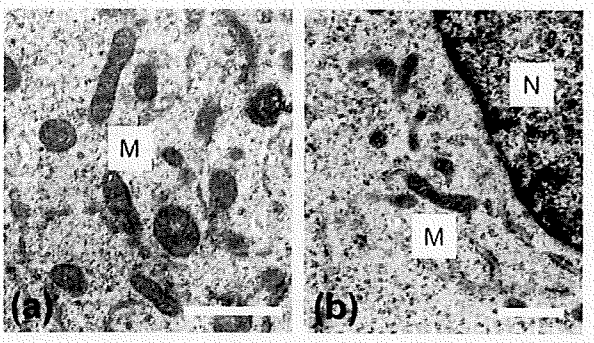


**Fig. 7.** Electron micrographs of human cumulus cells. Many well-developed mitochondria (M) are present in the cumulus cells of the Grade 1 COC (left image). In contrast, mitochondria showing small size are scattered in the cumulus cells of the Grade 3 COC. N: Nucleus. Bars = 1 $\mu$m.

decrease after 26 h of culture (5.62 ± 0.83 vs. 4.25 ± 0.58). In contrast, the mean oxygen consumption rates were similar between the two stages in the non-FSH administration group (4.07 ± 1.19 vs. 4.17 ± 1.02). There was no clinical advantage gained by extending the FSH pre-treatment from 3 to 6 days to produce follicles more than 10 mm in diameter [49]. On the other hand, Wynn *et al.* demonstrated a higher maturation rate in a FSH treatment group [50]. In their study, the maturation rate to metaphase II was higher in the FSH administration group (68.3% vs. 61.3%, in comparison to the non-FSH administration group, unpublished data). The benefit of FSH pre-treatment remains controversial and the development competence cannot be evaluated because of the limitations of the current study protocol.

The efficacy of the administration of hCG remains controversial. In hCG protocol, all patients are administered hCG before oocyte retrieval. After the LH surge, oocytes resume the first meiotic division and enter the second division [51]. At the same time, cumulus mass begins to change to the expanding form. Cumulus expansion may influence a variety of fundamental developmental changes which occur during fertilization. Regarding the use of hCG in bovine, the cumulus cells from antral follicles as small as 5 mm have mRNA transcripts for LH receptors and may respond to hCG stimulation [52]. This finding provides evidence of a mechanism by which hCG begins the maturation process of small antral oocytes *in vivo* and facilitates the completion of meiosis *in vitro*. Chian *et al.* demonstrated that the percentage of oocytes achieving maturation after 48 h *in vitro* culture was significantly higher in the hCG-primed group than in the non-hCG-primed group during human IVM-IVF [53].

Finally, the results of the respiration measurement of single human oocytes using a SECM system are listed in Table 3. The oxygen consumption rate of pre-cultured oocytes (GV stage) was 0.49 × $10^{14}$/mol · $s^{-1}$,

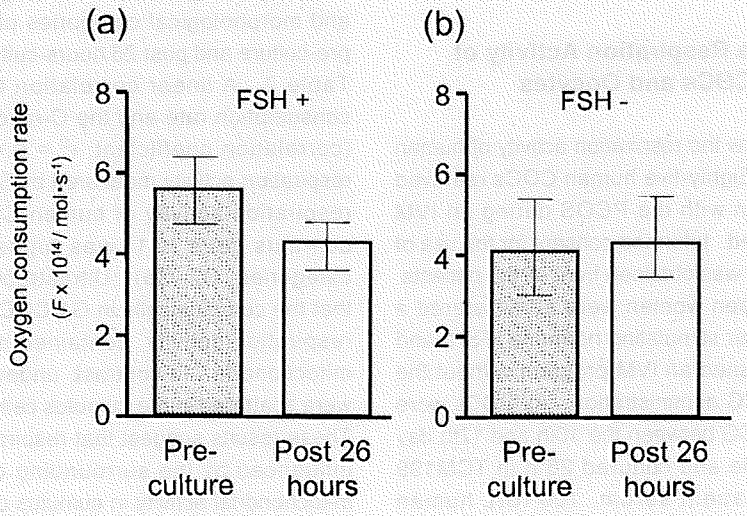


**Fig. 8.** Comparison of oxygen consumption with or without FSH administration at the pre-culture stage and after 26 h of culture. (a) oxygen consumption rate in the FSH administered group and (b) in the non-FSH administered group.

**Table 3.** Oxygen consumption rates ($F \times 10^{14}$/mol · s$^{-1}$) of human denuded oocytes in oocyte maturation cultures

| Maturation status | Oxygen consumption rate (n) |
|---|---|
| GV (Pre-culture) | 0.49 ± 0.07 (10) |
| GV or MI (Post-culture) | 0.40 ± 0.21 (19) |
| MII (Post-culture) | 0.41 ± 0.15 (30) |

whereas the oxygen consumption rate tended to decrease in matured MII oocytes ($0.41 \times 10^{14}$/mol · s$^{-1}$). These results suggest that the respiration activity of human oocytes changes with maturation status of oocytes, although the mechanism of this fluctuation needs to be confirmed with further studies.

## Conclusions

The SECM technique is a non-invasive and sensitive method for measuring the oxygen consumption of individual COCs and oocytes in animal species including humans. The respiration activity of COCs is directly influenced by the surrounding cumulus cell volume and the mitochondrial activity of cumulus cells. Biochemical and cytological studies strongly suggest that oxygen consumption is an important parameter for evaluating the competence of oocyte maturation. It may be feasible to monitor the profile of an oocyte's mitochondrial activity by measureing its oxygen consumption, and select the oocytes that can sustain fertilization and the development of embryos. Therefore, the SECM technique may have a future in clinical application as a predictor of oocyte quality which could be used for determining to develop into good quality embryos.

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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-$\mu$m sections were prepared. Antigen retrieval was performed using sodium citrate buffer (10 mM, pH 6.0) (10). The sections were stained with 2 $\mu$g/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 $2 \times 10^5$ 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 $\beta$A and $\beta$B subunits, but exerted no effect on inhibin $\alpha$ 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).

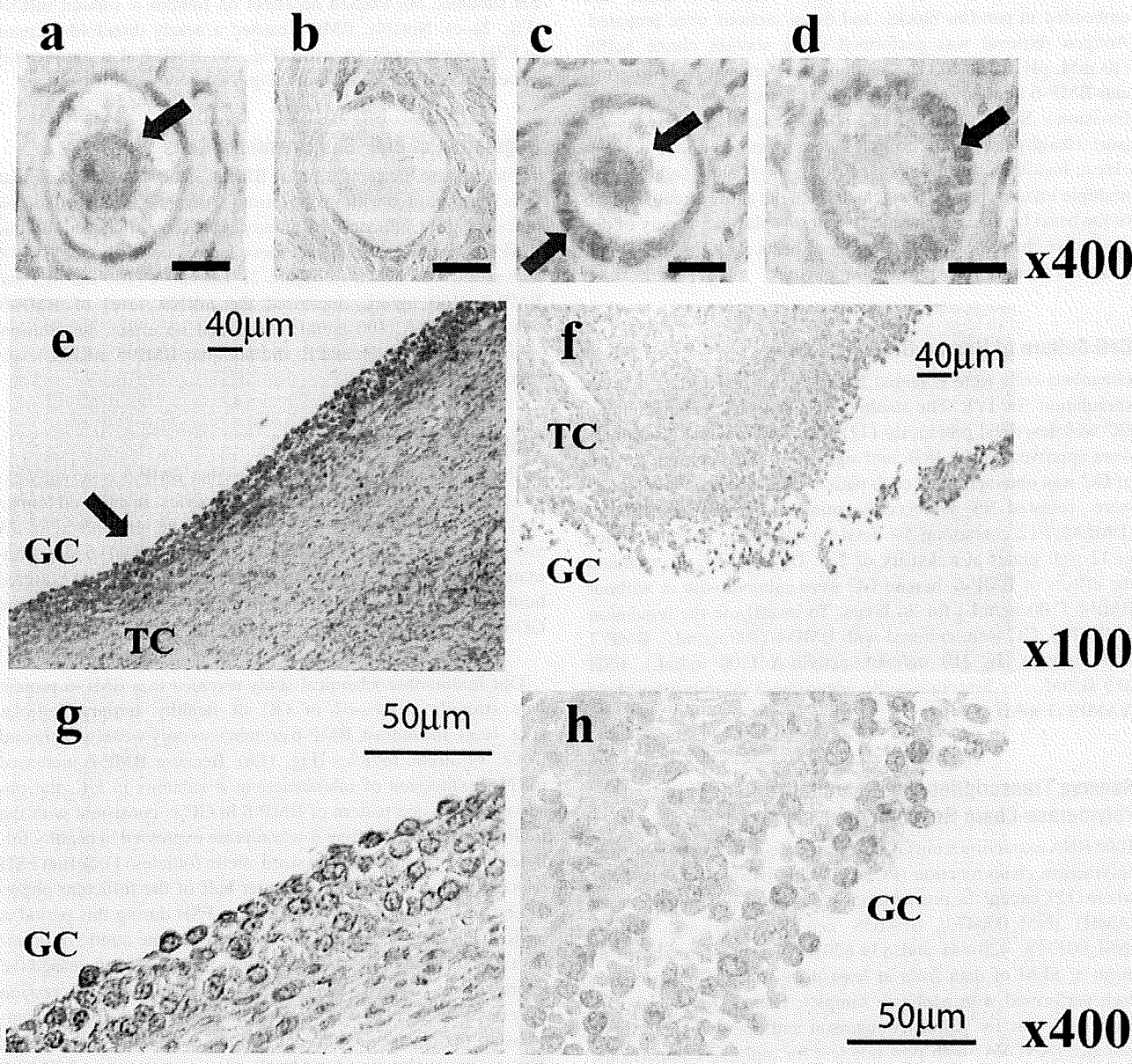
## DISCUSSION

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 $\beta$A and $\beta$B subunits, but not the inhibin $\alpha$ 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 $\beta$ subunits in GC, the observed expression pattern of BMP-6 in GC is consistent with the finding that inhibin/activin $\beta$ 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 $\beta$ 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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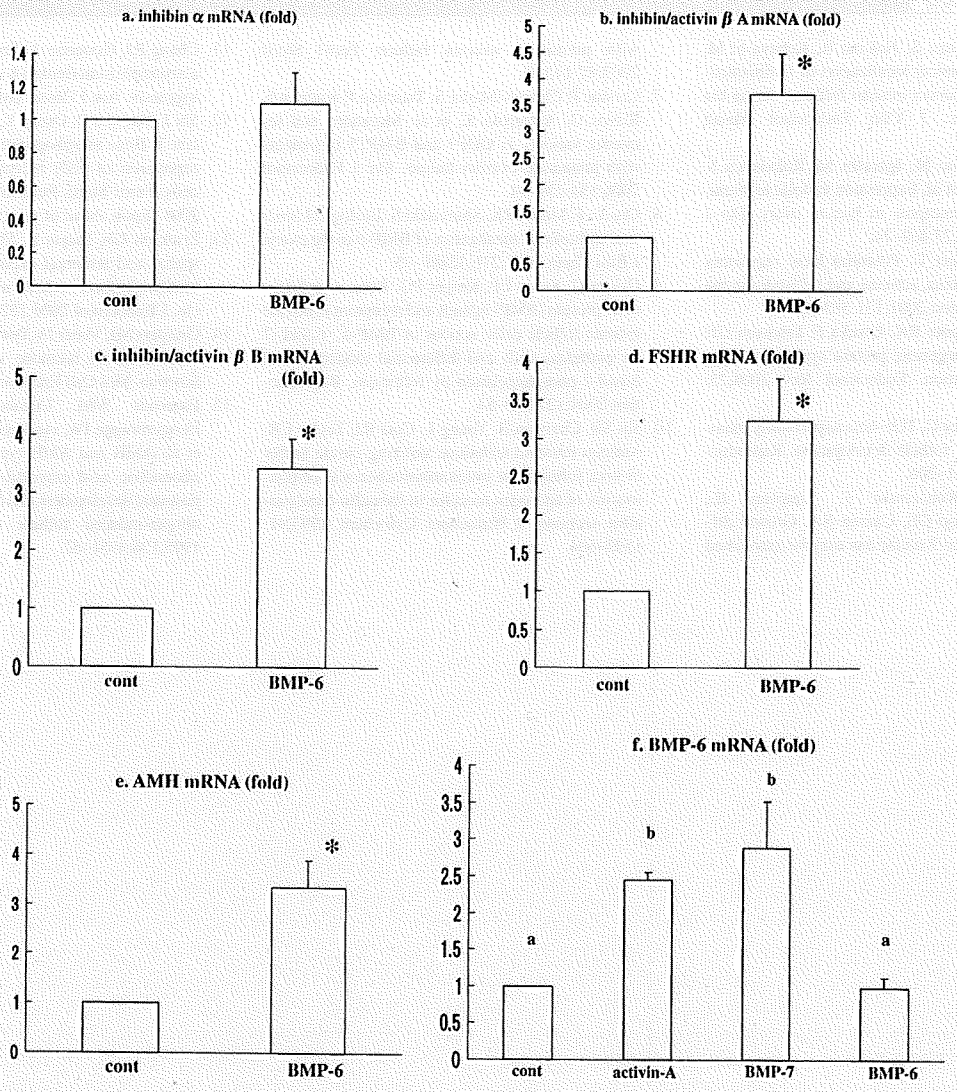
concept that BMP-15 governs ovulation quota, mono, and polyovulation (4), it is possible that the differences in BMP-6 expression and functions between species might also be related to ovulation quota.

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 $\alpha$ (**a**), inhibin/activin $\beta$A (**b**), inhibin/activin $\beta$B (**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 $\alpha$ 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 $\beta$ 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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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-$\beta$ 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 ($\pm$SD) age of the women was 37.8 $\pm$ 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 250*g* 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 $1 \times 10^5$ 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.

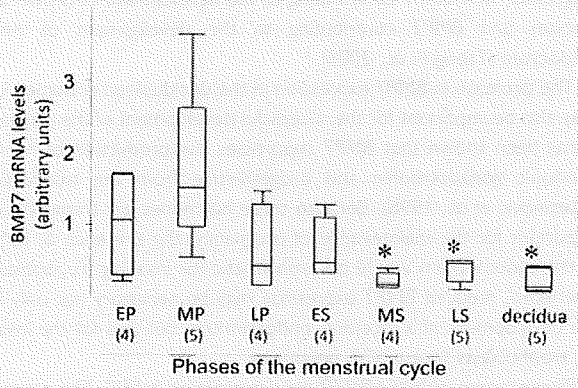


**Figure 1** Expression of BMP7 mRNA in human endometrial tissues throughout the menstrual cycle and in early pregnant decidua.

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.

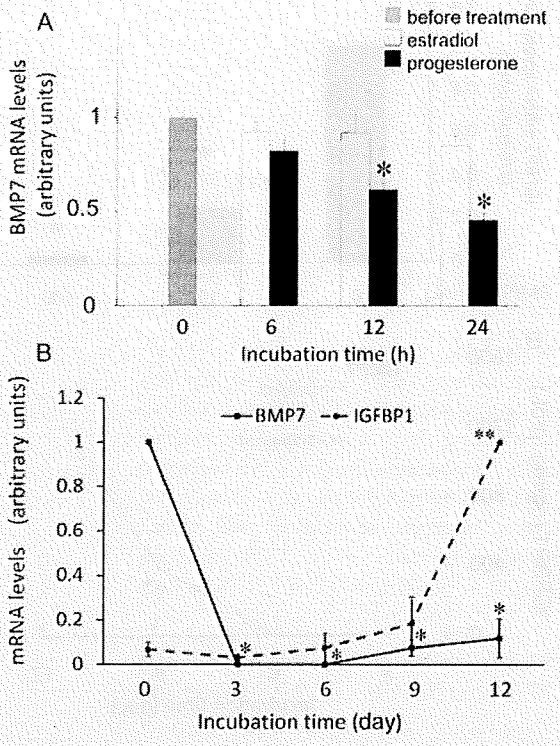


**Figure 2** (**A**) Expression of BMP7 mRNA in ESC treated with estrogen (10 ng/ml) or progesterone (100 ng/ml) for 24 h. Data are the mean ± SEM of combined data from three independent experiments using different ESC from three patients. (**B**) Expression of BMP7 and IGFBP1 mRNA in ESC. *In vitro* 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%.