GDF-9; 4) the nonaromatizable androgen DHT rescued the follicular growth arrest by GDF-9 down-regulation; and 5) the specific AR antagonist flutamide suppressed GDF-9-induced preantral follicle growth in vitro. These results suggest that GDF-9 controls ovarian follicular development from the preantral stage to early antral stage by up-regulating follicular androgen biosynthesis.

The exact role of GDF-9 on follicular differentiation during preantral-early antral transition is not clear (19). Nevertheless, it is possible that GDF-9 stimulates thecal cell recruitment, proliferation, and differentiation and induces the formation of thecal cell layer during this early stage of the follicular development. Ovaries from GDF-9 null mice exhibit a developmental block at the primary follicle stage, which is characterized by failed thecal layer formation in early follicles (22). GDF-9 is believed to be more important for the differentiation than the recruitment of thecal cell because the double-mutant (GDF-9 and inhibin-α) mouse exhibits morphological thecal cells surrounding the preantral follicles without detectable selective thecal markers, CYP17A1 and LH receptor (23). GDF-9 treatment increases androgen production in cultured rat theca-interstitial cells (18) and promotes murine ovarian expression of the specific thecal cell marker CYP17A1 (22). A recent study also indicated that GDF-9 increases thecal cell number and DNA synthesis in thecal cells of small bovine follicles (19). In the present studies, we demonstrated that GDF-9 augments androgen production and CYP17A1 mRNA expression in the preantral follicles, whereas GDF-9 down-regulation suppressed this response, indicating that GDF-9 is involved in the thecal cell differentiation during preantral-early antral transition. Whether the increased follicular CYP17A1 content is a result of increased thecal cell number and/or CYP17 levels per cell remains to be elucidated.

Ovarian androgens, primarily androstenedione and testosterone, are produced by thecal cells and act via receptors (ARs) localized to granulosa cells, stromal cells, and oocytes (14). Inactivation of AR in female mice results in premature ovarian failure, indicating that normal folliculogenesis requires AR-mediated androgen action (17). AR expression is highest in granulosa cells of rat small preantral and early antral follicles (24), raising the possibility that androgens are important paracrine regulators of follicular growth during preantral to early antral transition. Although androgens have long been implicated as an inhibitor of antral follicular development (25, 26), recent evidence suggests that the effect of androgens on follicular growth is dependent on the stage of follicular development and that androgens also have a growth promoting role in early folliculogenesis. Administration of testosterone or DHT to adult rhesus monkeys significantly increased the number of preantral and small antral follicles as well as granulosa and thecal cell proliferation (27). In vitro studies have shown that androgens (e.g. testosterone, DHT, androstenedione) stimulate preantral follicle growth and granulosa cell mitosis in mice (28), the transition of primary follicle to secondary follicle in cattle (29), and follicular survival in humans (30). An AR antagonist, but not an aromatase inhibitor, inhibited this growth response, indicating that the conversion of androgens to estrogens was not responsible for the follicle growth (31). DHT has also been shown to enhance por-

cine granulosa cell proliferation by facilitating the action of GDF-9 in vitro (32). In the present study, the nonaromatizable androgen DHT, but not estradiol, rescued the follicular growth arrest by GDF-9 down-regulation. The specific AR antagonist flutamide suppressed GDF-9-induced preantral follicle growth in vitro. These findings suggest that androgens exert a direct stimulatory action on the follicular development, especially during the preantral-early antral stage transition.

Androgens enhance the FSH action in the follicles by increasing FSH receptor expression, FSH-induced granulosa cell aromatase activity and proliferation, and follicular growth (33). GDF-9 augments FSH-induced preantral follicle growth (4, 17), whereas GDF-9 down-regulation suppressed FSH-stimulated follicular development. Although GDF-9 is required for the expression of FSH receptor in rat preantral follicles (4), whether these growth responses are modulated through thecal androgen actions awaits further investigation.

Tetsuka et al. (24) reported that a gradient of AR immunostaining existed in large follicles of the rat ovary, with cumulus cells and antral granulosa cells strongly expressing more AR protein than do peripheral layers. Nevertheless, the present result suggests that GDF-9 is not the oocyte-secreted factor that influences AR expression in follicles because GDF-9 did not alter AR mRNA levels in the cultured preantral follicles.

Previous study showed that GDF-9 stimulates basal estradiol synthesis in rat undifferentiated granulosa cells but suppresses FSH-induced progesterone and estradiol production (12). Because granulosa cells often undergo luteinization in culture (34) and excess FSH induces premature granulosa cell differentiation in vitro (35), it is possible that some of the granulosa cell responses in vitro might be more related to a potential role for GDF-9 in inhibiting premature luteinization rather than to its effect on normal follicular function (13, 18). Nevertheless, the present studies indicate that GDF-9 stimulates preantral follicle production of estradiol, but not progesterone, although whether GDF-9 enhances the expression of aromatase in granulosa cells and/or increases the synthesis of androgen substrate for aromatization remains to be elucidated. In the GDF-9 MO-injected follicles, FSH stimulated progesterone and estradiol production in the preantral follicles irrespective of the presence of GDF-9, which might be related to the FSH-induced expression of steroidogenic acute regulatory (36), CYP11A1 (36), and CYP19A1 (37) genes in granulosa cells. Solovyeva et al. (18) reported that GDF-9 enhanced forskolin-stimulated androstenedione production in rat theca-interstitial cells, whereas Spicer et al. reported that GDF-9 inhibits LH and IGF1-induced steroidogenesis by bovine thecal cells (19). Whether thecal cells also undergo luteinization in vitro and GDF-9 modulates this process, is not known.

Although the present results suggest that GDF-9 controls preantral follicle growth by up-regulating thecal CYP17Al expression and androgen biosynthesis, whether the observed effect of GDF-9 is mediated through a direct action on thecal cells or indirectly on granulosa cells remains to be investigated. GDF-9 signals through a complex of type I (activin-like receptor kinase-5) and type II (BMP receptor type II) membrane serine/threonine kinase receptors (38), resulting in the phosphorylation

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-β/activin subfamilies (*e.g.* TGF-β, 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.

Acknowledgments

References

1. McGee EA, Hsueh AJ 2000 Initial and cyclic recruitment of ovarian follicles. Endocr Rev 21:200–214
2. Cattanach BM, Iddon CA, Charlton HM, Chiappa SA, Fink G 1977 Gonadotrophin-releasing hormone deficiency in a mutant mouse with hypogonadism. Nature 269:338–340
3. Kumar TR, Wang Y, Lu N, Matzuk MM 1997 Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. Nat Genet 15:201–204
4. Orisaka M, Orisaka S, Jiang JY, Craig J, Wang Y, Kotsuji F, Tsang BK 2006 Growth differentiation factor 9 is antiapoptotic during follicular development from preantral to early antral stage. Mol Endocrinol 20:2456–2468
5. Eppig JJ, Wigglesworth K, Pendola FL 2002 The mammalian oocyte orchestrates the rate of ovarian follicular development. Proc Natl Acad Sci USA 99:2890–2894
6. Vanderhyden BC, Telfer EE, Eppig JJ 1992 Mouse oocytes promote proliferation of granulosa cells from preantral and antral follicles *in vitro*. Biol Reprod 46:1196–1204
7. Chang H, Brown CW, Matzuk MM 2002 Genetic analysis of the mammalian transforming growth factor-β superfamily. Endocr Rev 23:787–823
8. Shimasaki S, Moore RK, Otsuka F, Erickson GF 2004 The bone morphogenetic protein system in mammalian reproduction. Endocr Rev 25:72–101
9. McGrath SA, Esquela AF, Lee SJ 1995 Oocyte-specific expression of growth/differentiation factor-9. Mol Endocrinol 9:131–136
10. Aaltonen J, Laitinen MP, Vuojolainen K, Jaatinen R, Horelli-Kuitunen N, Seppä L, Louhio H, Tuuri T, Sjoberg J, Butzow R, Hovata O, Dale L, Ritvos O 1999 Human growth differentiation factor 9 (GDF-9) and its novel homolog GDF-9B are expressed in oocytes during early folliculogenesis. J Clin Endocrinol Metab 84:2744–2750
11. Dong J, Albertini DF, Nishimori K, Kumar TR, Lu N, Matzuk MM 1996 Growth differentiation factor-9 is required during early ovarian folliculogenesis. Nature 383:531–535
12. Vitt UA, Hayashi M, Klein C, Hsueh AJ 2000 Growth differentiation factor-9 stimulates proliferation but suppresses the follicle-stimulating hormone-induced differentiation of cultured granulosa cells from small antral and preovulatory rat follicles. Biol Reprod 62:370–377
13. Hayashi M, McGee EA, Min G, Klein C, Rose UM, van Duin M, Hsueh AJ 1999 Recombinant growth differentiation factor-9 (GDF-9) enhances growth and differentiation of cultured early ovarian follicles. Endocrinology 140:1236–1244
14. Drummond AE 2006 The role of steroids in follicular growth. Reprod Biol Endocrinol 4:16
15. Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery Jr CA, Shyamala G, Conneely OM, O'Malley BW 1995 Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. Genes Dev 9:2266–2278
16. Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O 1993 Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. Proc Natl Acad Sci USA 90:11162–11166
17. Hu YC, Wang PH, Yeh S, Wang RS, Xie C, Xu Q, Zhou X, Chao HT, Tsai MY, Chang C 2004 Subfertility and defective folliculogenesis in female mice lacking androgen receptor. Proc Natl Acad Sci USA 101:11209–11214
18. Solovyeva EV, Hayashi M, Margi K, Barkats C, Klein C, Amsterdam A, Hsueh AJ, Tsafriri A 2000 Growth differentiation factor-9 stimulates rat theca-interstitial cell androgen biosynthesis. Biol Reprod 63:1214–1218
19. Spicer LJ, Aad PY, Allen DT, Mazerbourg S, Payne AH, Hsueh AJ 2008 Growth differentiation factor 9 (GDF9) stimulates proliferation and inhibits steroidogenesis by bovine theca cells: influence of follicle size on responses to GDF9. Biol Reprod 78:243–253
20. Nasevicius A, Ekker SC 2000 Effective targeted gene 'knockdown' in zebrafish. Nat Genet 26:216–220
21. Heasman J, Kofron M, Wylie C 2000 β-Catenin signaling activity dissected in the early Xenopus embryo: a novel antisense approach. Dev Biol 222:124–134
22. Elvin JA, Yan C, Wang P, Nishimori K, Matzuk MM 1999 Molecular characterization of the follicle defects in the growth differentiation factor 9-deficient ovary. Mol Endocrinol 13:1018–1034
23. Wu X, Chen L, Brown CA, Yan C, Matzuk MM 2004 Interrelationship of growth differentiation factor 9 and inhibin in early folliculogenesis and ovarian tumorigenesis in mice. Mol Endocrinol 18:1509–1519
24. Tetsuka M, Whitelaw PF, Bremner WJ, Millar MR, Smyth CD, Hillier SG 1995 Developmental regulation of androgen receptor in rat ovary. J Endocrinol 145:535–543

25. Bagnell CA, Mills TM, Costoff A, Mahesh VB 1982 A model for the study of androgen effects on follicular atresia and ovulation. Biol Reprod 27:903–914

26. Billig H, Furuta I, Hsueh AJ 1993 Estrogens inhibit and androgens enhance ovarian granulosa cell apoptosis. Endocrinology 133:2204–2212

27. Vendola KA, Zhou J, Adesanya OO, Weil SJ, Bondy CA 1998 Androgens stimulate early stages of follicular growth in the primate ovary. J Clin Invest 101:2622–2629

28. Murray AA, Gosden RG, Allison V, Spears N 1998 Effect of androgens on the development of mouse follicles growing *in vitro*. J Reprod Fertil 113:27–33

29. Yang MY, Fortune JE 2006 Testosterone stimulates the primary to secondary follicle transition in bovine follicles *in vitro*. Biol Reprod 75:924–932

30. Otala M, Makinen S, Tuuri T, Sjoberg J, Pentikainen V, Matikainen T, Dunkel L 2004 Effects of testosterone, dihydrotestosterone, and 17β-estradiol on human ovarian tissue survival in culture. Fertil Steril 82(Suppl 3):1077–1085

31. Wang H, Andoh K, Hagiwara H, Xiaowei L, Kikuchi N, Abe Y, Yamada K, Fatima R, Mizunuma H 2001 Effect of adrenal and ovarian androgens on type 4 follicles unresponsive to FSH in immature mice. Endocrinology 142:4930–4936

32. Hickey TE, Marrocco DL, Amato F, Ritter LJ, Norman RJ, Gilchrist RB, Armstrong DT 2005 Androgens augment the mitogenic effects of oocyte-secreted factors and growth differentiation factor 9 on porcine granulosa cells. Biol Reprod 73:825–832

33. Weil S, Vendola K, Zhou J, Bondy CA 1999 Androgen and follicle-stimulating hormone interactions in primate ovarian follicle development. J Clin Endocrinol Metab 84:2951–2956

34. Juengel JL, McNatty KP 2005 The role of proteins of the transforming growth factor-β superfamily in the intraovarian regulation of follicular development. Hum Reprod Update 11:143–160

35. Eppig JJ, O'Brien MJ, Pendola FL, Watanabe S 1998 Factors affecting the developmental competence of mouse oocytes grown *in vitro*: follicle-stimulating hormone and insulin. Biol Reprod 59:1445–1453

36. Eimerl S, Orly J 2002 Regulation of steroidogenic genes by insulin-like growth factor-1 and follicle-stimulating hormone: differential responses of cytochrome P450 side-chain cleavage, steroidogenic acute regulatory protein, and 3β-hydroxysteroid dehydrogenase/isomerase in rat granulosa cells. Biol Reprod 67:900–910

37. Parakh TN, Hernandez JA, Grammer JC, Weck J, Hunzicker-Dunn M, Zeleznik AJ, Nilson JH 2006 Follicle-stimulating hormone/cAMP regulation of aromatase gene expression requires β-catenin. Proc Natl Acad Sci USA 103:12435–12440

38. Mazerbourg S, Klein C, Roh J, Kaivo-Oja N, Mottershead DG, Korchynskyi O, Ritvos O, Hsueh AJ 2004 Growth differentiation factor-9 signaling is mediated by the type I receptor, activin receptor-like kinase 5. Mol Endocrinol 18:653–665

39. Roh JS, Bondestam J, Mazerbourg S, Kaivo-Oja N, Groome N, Ritvos O, Hsueh AJ 2003 Growth differentiation factor-9 stimulates inhibin production and activates Smad2 in cultured rat granulosa cells. Endocrinology 144:172–178

40. Kaivo-Oja N, Bondestam J, Kämäräinen M, Koskimies J, Vitt U, Cranfield M, Vuojolainen K, Kallio JP, Olkkonen VM, Hayashi M, Moustakas A, Groome NP, ten Dijke P, Hsueh AJ, Ritvos O 2003 Growth differentiation factor-9 induces Smad2 activation and inhibin B production in cultured human granulosa-luteal cells. J Clin Endocrinol Metab 88:755–762

41. Juneja SC, Chegini N, Williams RS, Ksander GA 1996 Ovarian intrabursal administration of transforming growth factor β1 inhibits follicle rupture in gonadotropin-primed mice. Biol Reprod 55:1444–1451

42. Erickson GF, Shimasaki S 2003 The spatiotemporal expression pattern of the bone morphogenetic protein family in rat ovary cell types during the estrous cycle. Reprod Biol Endocrinol 1:9

43. Glister C, Richards SL, Knight PG 2005 Bone morphogenetic proteins (BMP) -4, -6, and -7 potently suppress basal and luteinizing hormone-induced androgen production by bovine theca interna cells in primary culture: could ovarian hyperandrogenic dysfunction be caused by a defect in thecal BMP signaling? Endocrinology 146:1883–1892

44. Fynn-Thompson E, Cheng H, Teixeira J 2003 Inhibition of steroidogenesis in Leydig cells by Mullerian-inhibiting substance. Mol Cell Endocrinol 211:99–104

45. Wang EY, Ma EY, Woodruff TK 2003 Activin signal transduction in the fetal rat adrenal gland and in human H295R cells. J Endocrinol 178:137–148

46. Hayes SA, Zarnegar M, Sharma M, Yang F, Peehl DM, ten Dijke P, Sun Z 2001 SMAD3 represses androgen receptor-mediated transcription. Cancer Res 61:2112–2118

—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*

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 μm [9]. In a study of porcine immature oocyte, progression to metaphase II was observed in 40% of oocytes that were over 110 μm in diameter, whereas no oocyte less than 90 μm 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 μm at the time of retrieval was the threshold for GVBD, whereas oocytes of more than 103 μm 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 μm (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

(a)

Area
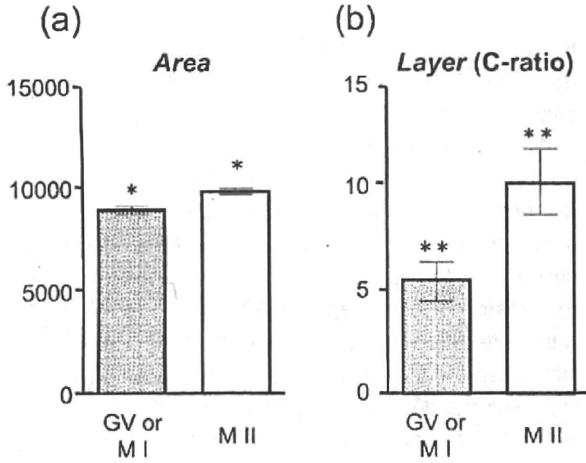

(b)

Layer (C-ratio)

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
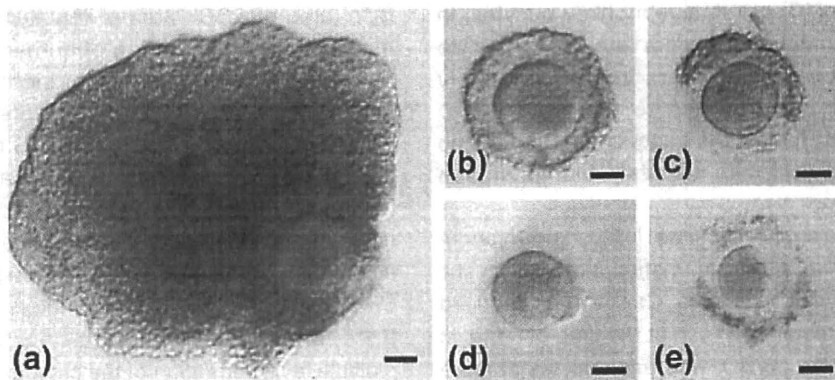

Fig. 2. Light micrographs of human COCs classified by morphological evaluation. (a) Grade 1, cumulus cells with multi-layers covering the whole oocyte, and a regular round oocyte; (b) Grade 2, cumulus cells with multi-layers (less than three layers), covering the whole oocyte and a regular round oocyte; (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 μm.

[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 ($F \times 10^{14}/\text{mol} \cdot \text{s}^{-1}$) of bovine COCs and denuded oocytes in oocyte maturation cultures

Maturation status	COC (n)	Oocyte (n)
Immature	5.48 ± 0.82 (16)[a]	0.67 ± 0.02 (16)[c]
Mature	3.15 ± 0.42 (20)[b]	1.10 ± 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 ($\times 10^{14}/\text{mol} \cdot \text{s}^{-1}$) 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.
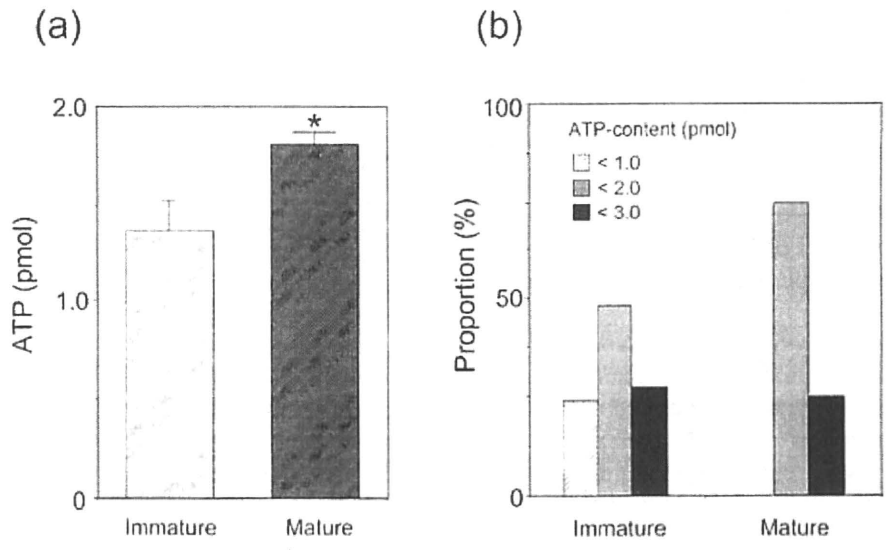
Fig. 3. (a) The ATP content and (b) proportion of oocytes categorized by ATP content: immature and mature bovine oocytes. *: significantly different ($P < 0.05$).
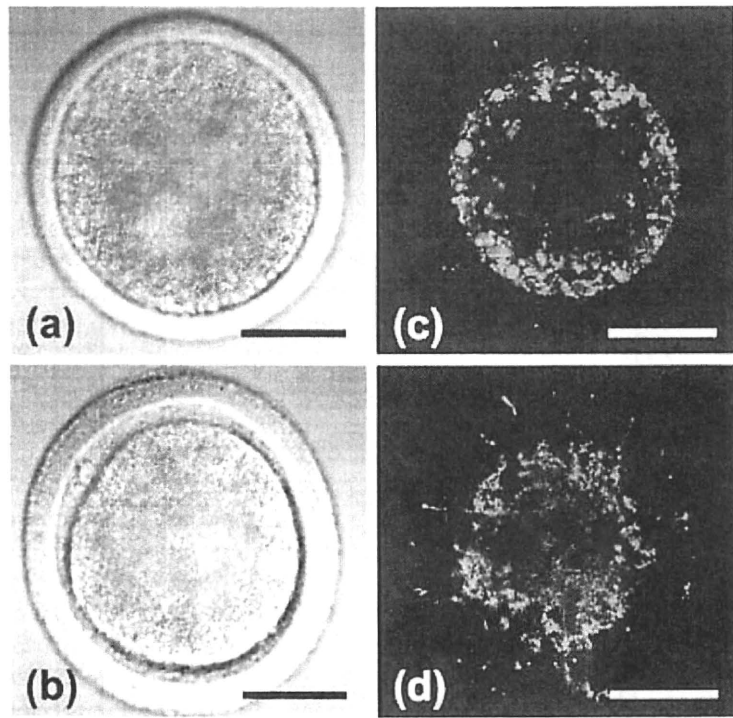

Fig. 4. Midline confocal sections of (a, b) immature and (c, d; cultured in IVMD101 medium) mature bovine oocytes stained by MitoTracker orange. Bars = 50 μm.
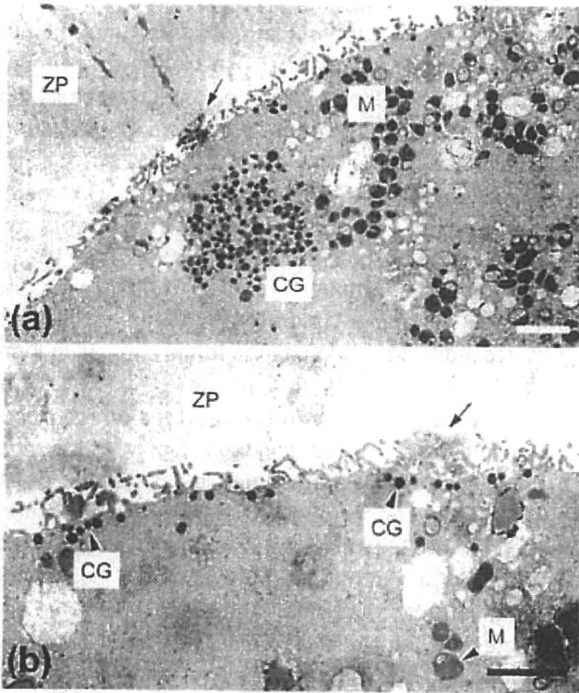
Fig. 5. Electron micrographs of (a) immature and (b) cultured in serum-free maturation medium mature oocytes. a: Many aggregates of mitochondria (M) and cortical granules (CG) were present in the cortex cytoplasm. b: Cortical granules were distributed in the periphery of the cytoplasm, but aggregates of mitochondria were not found. ZP, zona pellucida. Arrows: microvilli. Bars = 2 μm.

Table 2. Oxygen consumption rates ($F \times 10^{14}$/mol $\cdot$ 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)
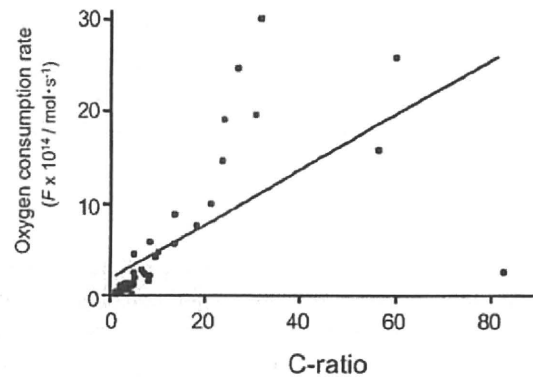
Fig. 6. Correlation between oxygen consumption rate and C-ratio.

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,

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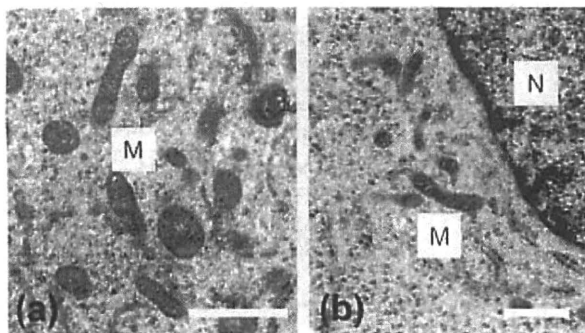
Fig. 7. Electron micrographs of human cumulus cells. Many well-developed mitochondria (M) are present in the cumulus cells of the Grade 1 COC (left image). In contrast, mitochondria showing small size are scattered in the cumulus cells of the Grade 3 COC. N: Nucleus. Bars = 1 μm.

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},
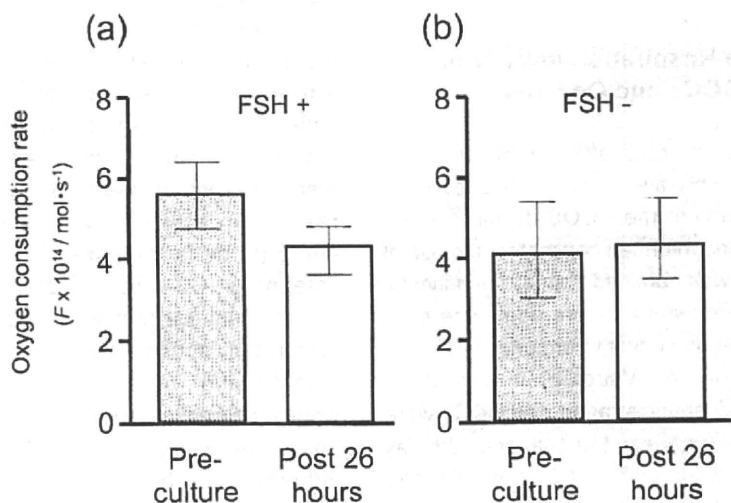

Fig. 8. Comparison of oxygen consumption with or without FSH administration at the pre-culture stage and after 26 h of culture. (a) oxygen consumption rate in the FSH administered group and (b) in the non-FSH administered group.

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 × 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.

Acknowledgements

References

1) Blondin, P., Bousqent, D., Twagiramungu, H., Barnes, F. and Sirand, M.A. (2002): Manipulation of follicular development to produce developmentally competent bovine oocyte. Biol. Reprod., 66, 38–43.

2) Barnes, F.L. and Sirard, M.A. (2000): Oocyte maturation. Semin. Reprod. Med., 18, 123–131.

3) Cha, K.Y., Koo, J.J., Ko, J.J., Choi, D.H., Han, S.Y. and Yoon, T.K. (1991): Pregnancy after in vitro fertilization of human follicular oocytes collected from nonstimulated cycles, their culture in vitro and their transfer in a donor oocyte program. Fertil. Steril., 55, 109–113.

4) Le Du, A., Kadoch, I.J., Bourcigaux, N., Doumerc, S., Bourrier, M.C., Chevalier, N., Fanchin, R., Chian, R.C., Tachdjian, G., Frydman, R. and Frydman, N. (2005): In vitro maturation for the treatment of infertility associated with polycystic ovarian syndrome: the French experience. Hum. Reprod., 20, 420–424.

5) Soderstrom-Anttila, V., Makinen, S., Tuuri, T. and Suikkari, A.M. (2005): Favorable pregnancy results with insemination of in vitro matured oocytes from unstimulated patients. Hum. Reprod., 20, 1534–1540.

6) Nagai, T. (2001): The improvement of in vitro maturation systems for bovine and porcine oocytes. Theriogenology, 55, 1291–1301.

7) Abe, H., Saito, T., Shiku, H., Yokoo, M., Itoh-Sasaki, T., Hoshi, H. and Matsue, T. (2006): Analysis of respiratory activity of single bovine oocytes by scanning electrochemical microscopy. In: Proceeding of the 4th International Forum on Post-genome Technologies, pp. 19–22.

8) Abe, H., Shiku, H., Aoyagi, S., Matsue, T. and Hoshi, H. (2006): Oxygen consumption of bovine cumulus cells and oocytes cultured in different culture systems for oocyte maturation. Reprod. Fertil. Dev., 18, 267.

9) Eppig, J.J. and Schroeder, A.C. (1989): Capacity of mouse oocytes from preantral follicles to undergo embryogenesis and development to live young after growth, maturation and fertilization in vitro. Biol. Reprod., 41, 268–276.

10) Hirao, Y., Nagai, T., Kubo, M., Miyano, T., Miyake, M. and Kato, S. (1994): In vitro growth and maturation of pig oocytes. J. Reprod. Fertil., 100, 333–339.

11) Schramm, R.D., Tennier, M.T., Boatman, D.E. and Bavister, B.D. (1993): Chromatin configuration and meiotic competence of oocytes are related to follicular diameter in non-stimulated rhesus monkeys. Biol. Reprod., 48, 349–356.

12) Cavilla, J.K., Kennedy, C.R., Byskov, A.G. and Hartshorne, G.M. (2008): Human immature oocytes grow during culture for IVM. Hum. Reprod., 23, 37–45.

13) Dekel, N. and Beers, W.H. (1980): Development of the rat oocyte in vitro: Inhibition and induction of maturation in the presence or absence of the cumulus oophorus. Dev. Biol., 75, 247–254.

14) Yang, S.H., Son, W.Y., Yoon, S.H., Ko, Y. and Lim, J.H. (2005): colleration between in vitro maturation and

expression of LH receptor in cumulus cells of the oocytes collected from PCOS patients in HCG-primed IVM cycles. Hum. Reprod., 20, 2097–2103.

15) Ball, G.D., Leibfried, M.L., Lenz, R.W., Ax, R.L., Bavister, B.D. and First, N.L. (1983): Factors affecting successful in vitro fertilization of bovine follicular oocytes. Biol. Reprod., 28, 717–725.

16) Naito, K., Fukuda, Y. and Toyama, Y. (1988): Effects of porcine follicular fluid on male pronucleus formation in porcine oocytes matured in vitro. Gamete. Res., 21, 289–295.

17) Chen, L., Russell, P.T. and Larsen, W.J. (1993): Functional significance of cumulus expansion in the mouse: Roles for the preovulatory synthesis of hyaluronic acid within the cumulus mass. Mol. Reprod. Dev., 34, 87–93.

18) Itagaki, Y. and Toyoda, Y. (1991): Factors affecting fertilization in vitro of mouse eggs after removal of cumulus oophorus. J. Mamm. Ova Res., 8, 126–134.

19) Smitz, J., Picton, H.M., Platteau, P., Rutherford, A., Cortvrindt, R., Clyde, J., Nogueira, D., Devroey, P., Lyby, K. and Grondahl, C. (2007): Principal findings from a multicenter trial investigating the safety of follicular-fluid meiosis-activating sterol for in vitro maturation of human cumulus-enclosed oocytes. Fertil. Steril., 87, 949–964.

20) Jurema, M.W. and Noguera, D. (2006): In vitro maturation of human oocytes for assisted reproduction. Fertil. Steril., 86, 1277–1291.

21) Cha, K.Y. and Chian, R.C. (1998): Maturation in vitro of human oocytes for clinical use. Hum. Reprod. Update., 4, 103–120.

22) Goud, P.T., Goud, A.P., Qian, C., Laverge, H., Van der Elst, J., De Sutter, P. and Dhout, M. (1998). In vitro maturation of human germinal vesicle stage oocytes: role of cumulus cells and epidermal growth factor in the culture medium. Hum. Reprod., 13, 1638–1644.

23) Ueno, S., Kurome, M., Ueda, H., Tomii, R., Hiruma, K. and Nagashima, H. (2005): Effects of maturation conditions on spindlemorphology in porcine MII oocytes. J. Reprod. Dev., 51,405–410.

24) Isobe, N. and Terada, T. (2001): Effect of the factor inhibiting germinal vesicle breakdown on the disruption of gap junctions and cumulus expansion of pig cumulus-oocyte complexes cultured in vitro. Reproduction, 121, 249–257.

25) Tanghe, S., Van Soom, A., Nauwynck, H., Coryn, M. and De Kruif, A. (2002): Minireview: Functions of the cumulus oophorus during oocyte maturation, ovulation and fertilization. Mol. Reprod. Dev., 61, 414–424.

26) Guoliang, X., Byskov, A.G. and Anderson, C.Y. (1994): Cumulus cells secrete a meiosis-inducing substance by stimulation with forskolin and cyclic adenosine monophosphate. Mol. Reprod. Dev., 39, 17–24.

27) Rieger, D. (1992): Relationship between energy metabolism and development of early mammalian embryos. Theriogenology, 37, 75–93.

28) Rieger, D., Loskutoff, N.M. and Betteridge, K.J. (1992): Developmentally related changes in the uptake and metabolism of glucose, glutamine and puruvate by cattle embryos produced in vitro. Reprod. Fertil. Dev., 4, 547–557.

29) Rieger, D. and Loskutoff, N.M. (1994): Changes in the metabolism of glucose, puruvate, glutamine and glycine during maturation of cattle oocytes in vitro. J. Reprod. Fertil., 100, 257–262.

30) Gopichandran, N. and Leese, H.J. (2003): Metabolic characterization of the bovine blastocyst, inner cell mass, trophectoderm and blastocoel fluid. Reproduction, 126, 299–308.

31) Thompson, J.G., Partridge, R.J., Houghton, F.D., Cox, C.I. and Leese, H.J. (1996): Oxygen uptake and carbohydrate metabolism by in vitro derived bovine embryos. J. Reprod. Fertil., 106, 299–306.

32) Thompson, J.G., McNaughton, C., Gasparrini, B., McGowan, L.T. and Tervit, H.R. (2000): Effect of inhibitors and uncouplers of oxidative phosphorylation during compaction and blastulation of bovine embryos cultured in vitro. J. Reprod. Fertil., 118, 47–55.

33) Trimarchi, J.R., Liu, L., Porterfield, D.M., Smith, P.J.S. and Keefe, D.L. (2000): Oxidative phosphorylation-dependent and -independent oxygen consumption by individual preimplantation mouse embryos. Biol. Reprod., 62, 1866–1874.

34) Fridhandler, I., Hafez, E.S.E. and Poncus, G. (1957): Developmental changes in the repiratory activity of rabbit ova. Experiments in Cell Research, 13, 132–139.

35) Mills, R.M. and Brinster, R.L. (1967) Oxygen consumption of preimplantation mouse embryos. Exp. Cell Res., 470, 337–344.

36) Magnusson, C., Hillensjo, T., Hamberger, L. and Nilsson, L. (1986): Oxygen consumption by human oocytes and blastocysts grown in vitro. Hum. Reprod., 1, 183–184.

37) Nilsson, B., Magnusson, C., Widehn, S. and Hillensjo, T. (1982): Correlation between blastocyst oxygen consumption and trophoblast cytochrome oxidase reaction at initiation of implantation of delayed mouse blastocysts. J. Embryol. Exp. Morphol., 71, 75–82.

38) Houghton, F.D., Thompson, J.G., Kennedy, C.J. and Leese, H.J. (2000): Oxygen consumption and energy metabolism of the early mouse embryo. Mol. Reprod. Dev., 44, 476–485.

39) Trimarchi, J.R., Liu, L., Smith, P.J.S. and Keefe, D.L. (2000): Noninvasive measurement of potassium efflux as an early indicator of cell death in mouse embryos. Biol. Reprod., 63, 851–857.

40) Land, S.C., Porterfield, D.M., Sanger, R.H. and Smith, P.J.S. (1999): The self-referencing oxygen-selective microelectrode: detection of transmembrane oxygen flux from single cells. J. Exp. Biol., 202, 211–218.

41) Smith, P.J.S., Hammar, K., Porterfield, D.M., Sanger, R.H. and Trimarchi, J.R. (1999): Self-referencing, non-invasive, ion selective electrode for single cell detection of trans-plasma membrane calcium flux. Microsc. Res. Tech., 46, 398–417.

42) Lopes, A.S., Larsen, L.H., Ramsing, N., Lovendahl, P.,

Raty, M., Peippo, J., Greve, T. and Callesen, H. (2005): Respiration rates of individual bovine in vitro-produced embryos measured with a novel, non-invasive and highly sensitive microsensor system. Reproduction, 130, 669–679.

43) Lopes, A.S., Greve, T. and Callesen, H. (2007): Quantification of embryo quality by respirometry. Theriogenology, 67, 21–31.

44) Bard, A.J. and Mirkin, M.V. (2001): Scanning Electrochemical Microscopy, Marcel Dekker Inc., New York.

45) Shiku, H., Shiraishi, T., Ohya, H., Matsue, T., Abe, H., Hoshi, H. and Kobayashi, M. (2001): Oxygen consumption of single bovine embryos probed with scanning electrochemical microscopy. Anal. Chem., 73, 3751–3758.

46) Abe, H., Shiku, H., Aoyagi, S. and Hoshi, H. (2004): In vitro culture and evaluation of embryos for production of high quality bovine embryos. J. Mamm. Ova Res., 21, 22–30.

47) Abe, H. (2007): A non-invaseve and sensitive method for measureing cellular respiration with a scanning electrochemical microscopy to evaluate embryo quality. J. Mamm. Ova Res., 24, 70–78.

48) Yokoo, M., Ito-Sasaki, T., Shiku, H., Matsue, T. and Abe, H. (2008) Multiple analysis of respiratory activity in the identical oocytes by applying scanning electrochemical microscopy. Trans. Materials Res. Soc. JPN., 33, 763–766.

49) Mikkelesen, A.L., Smith, S.D. and Lindenberg, S. (1999): In vitro maturation of human oocytes from regularly menstruating women may be successful without follicle stimulating hormone priming. Hum. Reprod., 14, 1847–1851.

50) Wynn, P., Piction, H.M., Krapez, J.A., Rutherford, A.J., Balen, A.H. and Gosden, R.G. (1998): Pretreatment with follicle stimulating hormone promotes the numbers of human oocytes reaching metaphase II by in vitro maturation. Hum. Reprod., 13, 3132–3138.

51) Suto, E. and Koide, S. (1987): Biochemical transmitters regulating the arrest and resumption of meiosis in oocytes. Int. Rev. Cytol., 106, 1–33.

52) Jin, Z.X., Ock, S.A., Tan, S.L. and Chian, R.C. (2005): What is the smallest size of follicle to respond to human chorionic gonadotropin injestion? Fertl. Steril., 84, S147.

53) Chian, R.C., Buckett, W.M., Turandi, T. and Tan, S.L. (2000): Prospective randomized study of human chorionic gonadotrophin priming before immature oocyte retrieval from unstimulated women with polycystic ovarian syndrome. Hum. Reprod., 15, 165–170.

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 main-

tain 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 mono-ovulatory 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

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 $\times$ 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 $\pm$ 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

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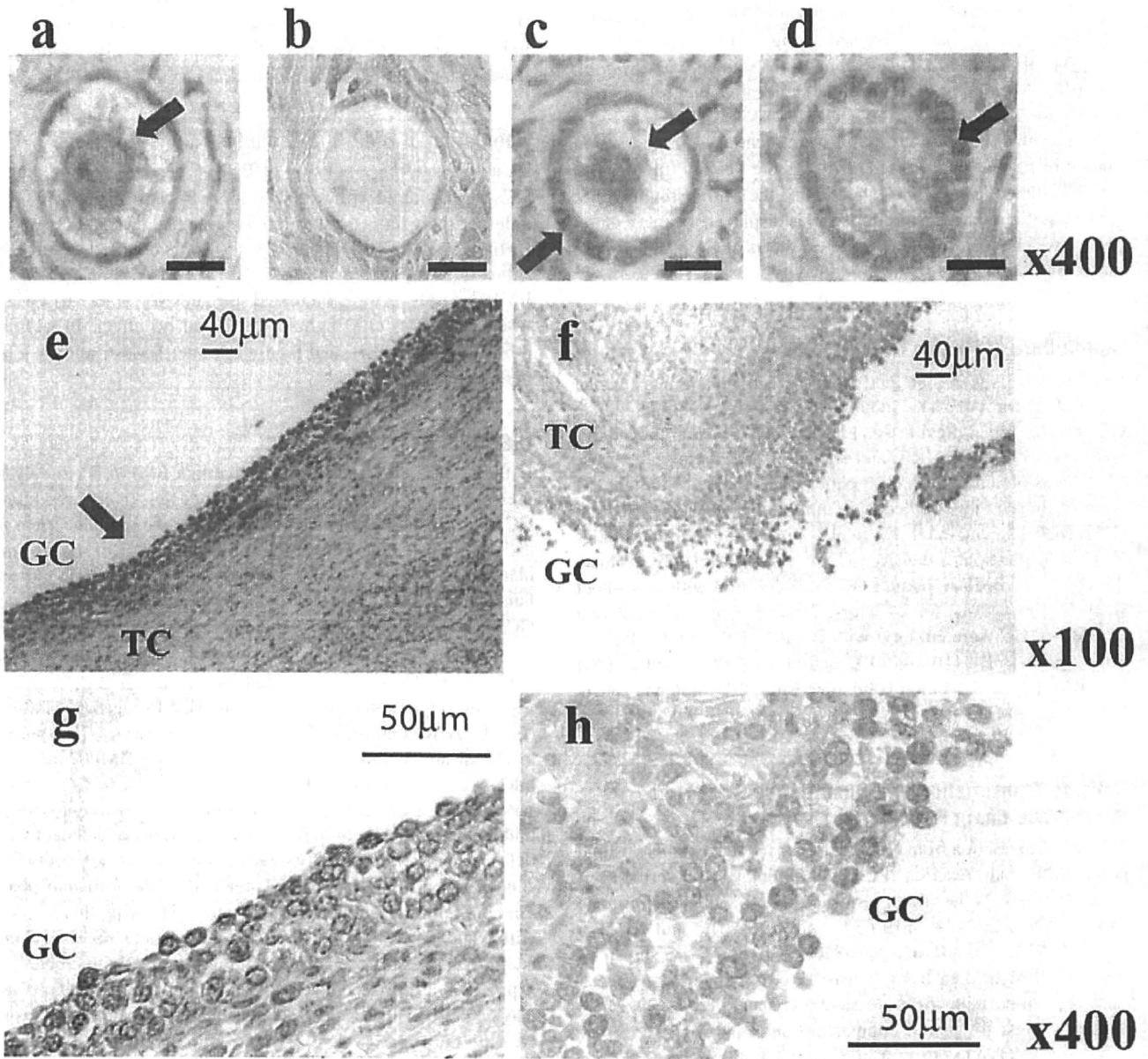
concept that BMP-15 governs ovulation quota, mono, and polyovulation (4), it is possible that the differences in BMP-6 expression and functions between species might also be related to ovulation quota.

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 $P < .05$.
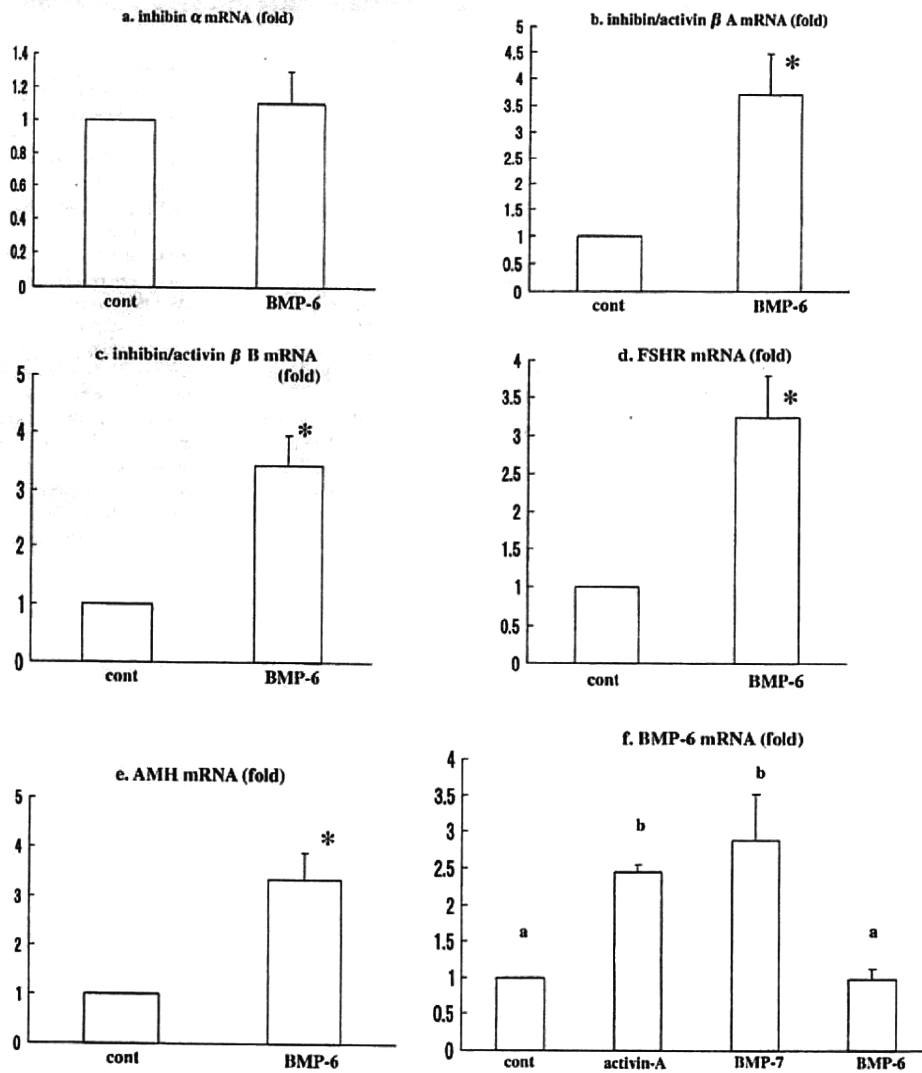
Shi. Correspondence. Fertil Steril 2009.

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.

REFERENCES

1. Yamoto M, Minami S, Nakano R, Kobayashi M. Immunohistochemical localization of inhibin/activin subunits in human ovarian follicles during the menstrual cycle. J Clin Endocrinol Metab 1992;74:989–93.
2. Minegishi T, Tano M, Igarashi M, Rokukawa S, Abe Y, Ibuki Y, et al. Expression of follicle-stimulating hormone receptor in human ovary. Eur J Clin Invest 1997;27:469–74.
3. Knight PG, Glister C. Potential local regulatory functions of inhibins, activins and follistatin in the ovary. Reproduction 2001;121:503–12.
4. Shimasaki S, Moore RK, Otsuka F, Erickson GF. The bone morphogenetic protein system in mammalian reproduction. Endocrinol Rev 2004;25:72–101.
5. Knight PG, Glister C. TGF-beta superfamily members and ovarian follicle development. Reproduction 2006;132:191–206.
6. Kovanci E, Rohozinski J, Simpson JL, Heard MJ, Bishop CE, Carson SA. Growth differentiating factor-9 mutations may be associated with premature ovarian failure. Fertil Steril 2007;87:143–6.
7. Laissue P, Christin-Maitre S, Touraine P, Kuttenn F, Ritvos O, Aittomaki K, et al. Mutations and sequence variants in GDF9 and BMP15 in patients with premature ovarian failure. Eur J Endocrinol 2006;154:739–44.
8. Otsuka F, Moore RK, Shimasaki S. Biological function and cellular mechanism of BMP-6 in the ovary. J Biol Chem 2001;276:32889–95.
9. Glister C, Kemp CF, Knight PG. Bone morphogenetic protein (BMP) ligands and receptors in bovine ovarian follicle cells: actions of BMP-4, -6 and -7 on granulosa cells and differential modulation of Smad-1 phosphorylation by follistatin. Reproduction 2004;127:239–54.
10. Shi SR, Chaiwun B, Young L, Cote RJ, Taylor CR. Antigen retrieval technique utilizing citrate buffer or urea solution for immunohistochemical demonstration of androgen receptor in formalin-fixed paraffin sections. J Histochem Cytochem 1993;41:1599–604.
11. Chang RJ, Gougeon A, Erickson GF. Evidence for a neutrophil-interleukin-8 system in human folliculogenesis. Am J Obstet Gynecol 1998;178:650–7.
12. Shi J, Yoshino O, Osuga Y, Nishii O, Yano T, Taketani Y. Bone morphogenetic protein 7 increases the expression of FSH receptor in human granulosa cells. Fertil Steril. Published online December 22, 2008 [Epub ahead of print].
13. Erickson GF, Fuqua L, Shimasaki S. Analysis of spatial and temporal expression patterns of BMP family members in the rat uterus over the estrous cycle. J Endocrinol 2004;182:203–17.
14. Gruijters MJ, Visser JA, Durlinger AL, Themmen AP. Anti-Mullerian hormone and its role in ovarian function. Mol Cell Endocrinol 2003;211:85–90.
15. Baarends WM, Uilenbroek JT, Kramer P, Hoogerbrugge JW, van Leeuwen EC, Themmen AP, et al. AMH and AMH type II receptor messenger ribonucleic acid expression in rat ovaries during postnatal development, the estrous cycle, and gonadotropin-induced follicle growth. Endocrinology 1995;136:4951–62.

Interleukin (IL)-1β Stimulates Migration and Survival of First-Trimester Villous Cytotrophoblast Cells through Endometrial Epithelial Cell-Derived IL-8

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IL-1, secreted by human embryos and trophoblast cells, is important for successful implantation and pregnancy. We previously reported that IL-1β induced IL-8 production in human endometrial stromal cells (ESCs) and that induction was regulated by substances implicated in implantation. In the present study using human primary cells in culture, we measured IL-1β-induced production of IL-8 from endometrial epithelial cells (EECs) and ESCs and examined effects of the endometrium-derived IL-8 on migration and number of first-trimester villous cytotrophoblast cells (vCTs). Both basal and IL-1β-induced IL-8 levels of cell supernatants were much higher in EECs than ESCs. Addition of IL-1β to EECs increased the chemotactic activity of the supernatants to vCTs, and this effect was suppressed by immunoneutralization with anti-IL-8 antibody. Supernatants of IL-1β-stimulated EECs yielded significantly higher number of vCTs compared with those of untreated EECs, and the effect was inhibited by IL-8 antibody. These findings suggest that IL-1 promotes implantation by stimulating EECs to produce IL-8, which subsequently induces migration of vCTs and contributes to survival of vCTs. (*Endocrinology* 150: 350–356, 2009)

Migration and survival of trophoblast cells are essential for establishing a pregnancy. These phenomena are controlled elaborately by various substances that are secreted from the embryo, the endometrium, or both during the implantation process (1–5). In particular, molecular cross talk between the embryo and endometrium is indispensable for their coordinated development, which is required for successful implantation (6, 7).

IL-1 is a typical cytokine that affects the implantation process at the interface between the embryo and endometrium (8–10). In view of the findings that IL-1 increases secretion of prostaglandin E2 and leukemia inhibitory factor (LIF) and expression of the integrin β3-subunit in human endometrium (11–13), IL-1 may be one of the first signals that the blastocyst exerts on the endometrium. The notion is also supported by evidence of IL-1 expression in human embryo and trophoblast as described below. It has been shown that successful implantation after *in vitro* fertilization is correlated positively with high concentrations of IL-1α and IL-1β, two active ligands of IL-1, in the embryos'

culture media (14, 15). Immunohistochemical studies have demonstrated the localization of IL-1β in first-trimester villous cytotrophoblast cells (vCTs) (16, 17). In addition, an *in vivo* study in mice has shown that administration of IL-1 receptor antagonist significantly reduces the number of implanted embryos (18).

With the aim of gaining a better understanding of the interaction between the human endometrium and trophoblast, a recent study identified the gene expression profile of endometrial stromal cells (ESCs) cocultured with first-trimester trophoblast explants (19). The study revealed that one of the most up-regulated genes was chemokine IL-8, which was up-regulated more than 300-fold. In another study, IL-8 from decidual natural killer cells promoted invasion of first-trimester extravillous trophoblast cells (20). These findings imply that endometrial IL-8 is involved in the process of implantation under the stimulus of trophoblast.

Endometrial IL-8 acts as not only a chemoattractant of leukocytes but also an autocrine growth factor (21). IL-8 acts on cells through IL-8 chemokine receptors CXCR-1 and CXCR2, which are expressed in endometrium (22). In contrast to the roles

of IL-8 on endometrium, its chemotactic and proliferative effects on first-trimester vCTs still remain obscure. As for expression of the receptors, a study has shown that CXCR2 is expressed in primary first-trimester trophoblast cells and a trophoblast cell line BeWo cells but has not examined the CXCR1 expression (23).

We reported previously that IL-1 induces IL-8 production in human ESCs and the induction is modulated by substances that are implicated in implantation (24, 25). Combined with the above findings, we hypothesized that the development of trophoblast in early pregnancy is affected by endometrial IL-8, which is up-regulated by embryo-derived IL-1. To address this issue, we first examined IL-1β-induced production of IL-8 from human endometrial epithelial cells (EECs) and ESCs. We then assessed effects of the culture media from EECs stimulated by IL-1β on migration and number of human first-trimester vCTs.

Materials and Methods

Reagents and materials

Type I collagenase, antibiotics, and magnesium sulfate (MgSO₄) were purchased from Sigma (St. Louis, MO). DMEM and Ham's F12 medium, DMEM/F12, 0.25% trypsin and 0.25% trypsin/EDTA, were from Life Technologies (Rockville, MD). Charcoal-stripped fetal bovine serum (FBS) was from HyClone (Logan, UT). Ficoll-Paque Plus (1.077 g/ml) was from Amersham Biosciences (Piscataway, NJ). Mouse monoclonal antihuman IL-8 antibody (IL-8Ab), human recombinant IL-1β and human recombinant IL-8 were from R&D Systems (Minneapolis, MN). Isotype control mouse IgG1 (mIgG) and mouse monoclonal antibodies to human pan-cytokeratin (AE1/AE3), human vimentin, and human CD45 were from Dako (Glostrup, Denmark). Mouse monoclonal antibody to human cytokeratin 7 was from AbD Serotec (Oxford, UK). Deoxyribonuclease I (DNase I) was from Takara (Tokyo, Japan).

Tissue sources

Endometrial tissues were obtained from a total of 20 women (43.7 ± 4.2 yr, mean ± SD) undergoing hysterectomy for benign gynecological conditions such as uterine fibroids without endometrial pathologies. Although the relatively high reproductive age range of the subjects and the myometrium pathology results may place some limitations on the present study, we used these samples due to the unavailability of endometrial tissue in healthy young women. All subjects had regular menstrual cycles and had not received hormone therapy for at least 6 months before surgery. Placental tissues between 5 and 8 wk of gestation (7.4 ± 6.7 wk) were obtained from a total of 25 women (29.6 ± 6.7 yr) undergoing elective terminations of pregnancy for isolation of first-trimester vCTs. Peritoneal leukocytes (PLs) were used as a positive control in the experiment of RT-PCR. To obtain PL, peritoneal fluid (PF) with PL was obtained from a woman with endometriosis undergoing laparoscopy. PF was collected via a laparoscopic cannula introduced into the cul-de-sac before starting any manipulative procedures. The Institutional Review Board of the University of Tokyo approved this study, and written informed consent for use of the tissue samples was obtained from each woman. The tissues were collected under sterile conditions and were processed for primary cell cultures.

Isolation, purification, and culture of EECs, ESCs, first-trimester vCTs, and PLs

The isolation and culture of human EECs and ESCs was carried out as described previously (2, 26). Endometrial tissues were minced and incubated in DMEM/F12 containing 0.25% type I collagenase and 15 U/ml DNase I for 60 min at 37 C. The resulting dispersed endometrial cells were separated by filtration through a 40-μm nylon cell strainer

(Becton Dickinson, Lincoln Park, NJ). Intact endometrial epithelial glands were retained by the strainer, whereas dispersed ESCs passed through the strainer into the filtrate. ESCs in the filtrate were collected by centrifugation and resuspended in DMEM/F12 containing 5% FBS and antibiotics. ESCs were then plated in a 100-mm culture plate and incubated at 37 C in a humidified 5% CO₂-95% air atmosphere. At the first passage, ESCs were plated at a density of 2 × 10⁵ cells/well into 12-well culture plates for experimental use. Cells enriched with endometrial epithelial glands were collected by backwashing the strainer with DMEM/F12, plated in a 100-mm plate, and incubated at 37 C for 60 min to allow any contaminating stromal cells to attach to the plate wall. The nonattached epithelial glands formed a monolayer of EEC after attachment to culture plates. EECs at a density of 2 × 10⁵ cells/well in 12-well culture plates were used for the experiments.

The isolation and culture of first-trimester vCTs were performed as described previously (2). Briefly, the first-trimester placental tissues were washed in PBS, and the soft villous material was cut away from connective tissue and vessels. The washed tissue was incubated in sterile PBS containing 1 mM MgSO₄, 0.125% trypsin, and 30 U/ml DNase I for 30 min at 37 C with mild stirring. The suspension was then filtered through a 100-μm nylon cell strainer, and the cells were centrifuged at 200 × g for 5 min to obtain a cell pellet, which was resuspended in DMEM/F12 with 5% FBS. The cell suspension was layered onto Ficoll-Paque Plus and centrifuged at 150 × g for 15 min. The cells recovered from the interface were washed with PBS and resuspended in DMEM/F12. The remaining leukocytes were removed by plating the cells for 30 min, followed by aspiration of the supernatant enriched with cytotrophoblast cells. The cells were washed with PBS, the medium was changed to DMEM/F12 with 10% FBS, and the cells were placed in type IV collagen-coated plates (BD Biosciences, Bedford, MA) and incubated at 37 C in a humidified 5% CO₂-95% air atmosphere. The cells were plated at a density of 2 × 10⁴ cells/well in 96-well plates for cell number assays, and at a density of 4 × 10⁵ cells/well in 6-well plates for RT-PCR and in vitro migration assay. After incubation for 24 h, the cells were ready for experimental use.

PLs were collected as previously described (27). PF containing peritoneal leukocytes was centrifuged at 200 × g for 5 min, and the supernatant removed. The cell pellet was resuspended in PBS, layered onto Ficoll-Paque Plus and centrifuged at 150 × g for 30 min. PLs were recovered from the interface.

We confirmed the purity of EECs, ESCs, vCTs, and PLs by immunocytochemistry as we described previously (2). The purity of EEC preparations was greater than 95%, as judged by positive cellular staining for pan-cytokeratin (a marker for epithelial cells), negative cellular staining for vimentin (a marker for stromal cells), and CD45 (a marker for leukocytes). The purity of ESC preparations was greater than 98%, as judged by positive cellular staining for vimentin and negative cellular staining for pan-cytokeratin and CD45. The purity of vCT preparations was greater than 90%, as judged by positive cellular staining for cytokeratin 7 (a marker for trophoblast cells) and negative staining for vimentin and CD45. The purity of PLs was greater than 90%, as judged by positive cellular staining for CD45.

Treatment of endometrial cell cultures

When ESCs and EECs approached confluence, the complete media were removed and replaced with fresh media and antibiotics, and the cells were cultured for 24 h. The wells were then replenished with FBS-free media containing different concentrations of IL-1β (0, 0.1, 1, and 10 ng/ml) and incubated for an additional 24 h. After IL-1β treatment, the cell supernatants of ESCs and EECs (ESC-SN and EEC-SN, respectively) were collected, centrifuged and stored at −80 C for subsequent analysis.

Measurement of IL-8 in endometrial cell supernatants

Concentrations of IL-8 in endometrial cell culture media were measured using human IL-8 ELISA kit (R&D Systems) following the manufacturer's protocol. Absorbance was read at 450 nm with the DigiScan microplate reader (ASYS Hitech GmbH, Eugendorf, Austria). The total amount of IL-8 in the culture medium was calculated from the liquid