

**FIG. 7.** Effect of IL-17A on proliferation of ESCs. **A**, The effect of IL-17A on proliferation of ESCs was examined by measuring cell number with cell-counting kit. ESCs were cultured in 1% FBS with different doses of IL-17A for 48 h. Values are the mean  $\pm$  SEM of pentaplicate cultures. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  vs. control. The result is representative of four separate experiments using samples from different patients. **B**, The effect of IL-17A on the proliferation of ESCs was examined by measuring BrdU incorporation into DNA by using a cell proliferation ELISA. ESCs were treated with IL-17A at different concentrations for 48 h. Values are the mean  $\pm$  SEM of the pentaplicate cultures. Different letters denote significant differences between groups ( $P < 0.05$ ). The data shown are representative of four independent experiments using samples from four different patients.

survival of endometrial cells (1, 9). Our previous studies suggest that expression of IL-8 in endometriotic cells is regulated by various cytokines and enzymes (15, 16, 22). Notably, these IL-8-inducing molecules are derived from macrophages, neutrophils, and mast cells, which are suggested to play important roles in the development of the disease. The present study provides evidence that IL-17A is an additional stimulant of IL-8 secretion from ESCs and strongly suggests that Th17 cells participate in the development of endometriosis.

The synergistic effect of IL-17A and TNF $\alpha$  in stimulating secretion of IL-8 from ESCs was remarkable. TNF $\alpha$  is a proinflammatory cytokine that plays multiple roles in the progression of endometriosis. The importance of TNF $\alpha$  in endometriosis is underpinned by recent findings that TNF $\alpha$ -targeted suppression by specific drugs inhibits the development of endometriosis in baboons (23, 24). During the inflammatory response TNF $\alpha$  is secreted from various cells type, such as peritoneal macrophage, endometrial epithelial, and stromal cells. IL-17A may be an accelerator of

endometriosis during chronic pelvic inflammation accompanied by increased TNF $\alpha$  production.

IL-17A-induced phosphorylation of p42/44MAPK, p38MAPK, and SAPK/JNK in ESCs, and the IL-17A-induced increase in IL-8 secretion from ESCs was suppressed by inhibitors of these kinases. This finding suggests that these enzymes are involved in the pathway of IL-17A-induced IL-8 secretion. A similar finding was reported for human airway smooth muscle cells (25). Interestingly, our previous study showed that IL-1 $\beta$  stimulated phosphorylation of these MAPKs, and IL-1 $\beta$ -induced IL-8 secretion from ESCs was inhibited by the same MAPK inhibitors (22). IL-1 $\beta$  is a well-known central player of the inflammatory condition of endometriosis, and thus, it can be speculated that cytokines promoting endometriosis, such as IL-1 $\beta$  and IL-17A, share similar pathways to induce IL-8 secretion from ESCs.

COX2, a key enzyme in prostaglandin biosynthesis, is up-regulated in endometriotic stromal cells (26–28). COX2 plays an important role in the inflammatory response associated with endometriosis and appears to function in the pathogenesis of endometriosis (29, 30). The present finding of IL-17A-induced COX2 expression in ESCs provides further evidence for a key role of IL-17A in endometriosis.

In the present study, IL-17A stimulated ESC proliferation. Human tumors show increased expression of IL-17A (31), and IL-17A promotes angiogenesis and tumor growth (32). IL-17A also enhances the proliferation of normal airway epithelial cells (33). Because IL-17A appears to exert a mitogenic effect on various cell types, IL-17A may stimulate progression of endometriosis as a result of this mitogenic effect, possibly in addition to other functions. Interestingly, IL-8 has been shown to stimulate the proliferation of endometrial stromal cells (34). Therefore, the proliferative effect of IL-17A on ESCs may be partially attributable to the increased production of IL-8 induced by IL-17A.

The proinflammatory and mitogenic action of IL-17A in ESCs demonstrated in the present study revealed possible roles of the molecule in the development of endometriosis. The prevailing medical treatment for endometriosis is suppression of ovarian hormones by GnRH analogs. The adverse effects of this treatment induced by the hypoestrogenic status often lead to a lack of compliance. IL-17A is well placed as a candidate target molecule for novel treatment strategies of endometriosis. Further investigation would benefit the development of an improved treatment option involving IL-17A.

In summary, the present study demonstrated that IL-17A stimulates inflammatory responses and proliferation of ESCs, suggesting a role for IL-17A in the pathogenesis of endometriosis.

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Address all correspondence and requests for reprints to: Yutaka Osga, M.D., Department of Obstetrics and Gynecology, Faculty of Medicine, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. E-mail: yutakaos-gky@umin.ac.jp.

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## Dienogest inhibits BrdU uptake with G<sub>0</sub>/G<sub>1</sub> arrest in cultured endometriotic stromal cells

Li Fu, M.D., Yutaka Osuga, M.D., Ph.D., Chieko Morimoto, M.D., Ph.D.,  
Tetsuya Hirata, M.D., Ph.D., Yasushi Hirota, M.D., Ph.D., Tetsu Yano, M.D., Ph.D.,  
and Yuji Taketani, M.D., Ph.D.

Department of Obstetrics and Gynecology, Faculty of Medicine, University of Tokyo, Tokyo, Japan

**Objective:** To investigate the effect of dienogest on the proliferation of endometriotic stromal cells.

**Design:** Comparative and laboratory study.

**Setting:** University of Tokyo Hospital.

**Patient(s):** Endometriotic stromal cells were isolated and cultured from ovarian endometriomas of patients undergoing surgery.

**Intervention(s):** Dienogest was added to the cultured endometriotic stromal cells.

**Main Outcome Measure(s):** 5-Bromo-2'-deoxyuridine (BrdU) incorporation into DNA of the endometriotic stromal cells was measured by ELISA. Cell cycle analysis of the cultured endometriotic stromal cells was performed by flow cytometry.

**Result(s):** Dienogest at concentration of 10<sup>-7</sup> M and 10<sup>-6</sup> M significantly inhibited BrdU incorporation into DNA at 24 and 48 hours. Dienogest significantly increased the cells in G<sub>0</sub>/G<sub>1</sub> phase and reduced the cells in S phase and G<sub>2</sub>/M phase in 24 and 48 hours.

**Conclusion(s):** The present study indicates that dienogest can inhibit the proliferation of the endometriotic stromal cells with G<sub>0</sub>/G<sub>1</sub> arrest, suggesting a possible direct effect of dienogest in the treatment of endometriosis. (Fertil Steril® 2007; ■: ■-■. ©2007 by American Society for Reproductive Medicine.)

**Key Words:** Dienogest, endometriosis, proliferation, cell cycle

Endometriosis, defined by the presence of viable endometriotic tissue outside the uterus, is an enigmatic disease. Infertility and pain are the most common symptoms that debilitate women affected with endometriosis (1, 2). Although a most prevailing medical treatment for the disease is suppression of ovarian hormones by GnRH analogue (GnRH-a), its adverse effects caused by hypoestrogen often dismiss the compliance. Accordingly, development of better drugs is anticipated.

Dienogest (17 $\alpha$ -cyanomethyl-17 $\beta$ -hydroxy-estra-4,9-dien-3-one) is a synthetic progestogen derived from 19-norsteroids. It has high progestational and significant antiandrogenic activity, but only moderate antigonadotrophic activity (3). Dienogest significantly decreased the volume of the endometrial implants in a rat experimental model, suggesting its effect on endometriosis (4). In humans, dienogest was highly effective on endometriotic lesions and symptoms, showing

an objective endoscopic and subjective symptomatic improvement in 80% and 83%, respectively (5, 6). Furthermore, efficacy of dienogest was confirmed to be comparable with triptorelin in the treatment of endometriosis after laparoscopic surgery (7). A recent pilot study demonstrated an efficacy of long-term high-dose dienogest treatment for endometriosis (8).

The main antiendometriotic effect of dienogest has been suggested to be attributable to a central inhibition of ovulation. However, its peripheral effects are also indicated. The direct antiproliferative effect of dienogest has been shown in human eutopic endometrial stromal cells (9). In contrast, its effect on ectopic endometriotic cells remains to be elucidated. It is well known that ectopic endometrium is different from eutopic endometrium in its gene expression (10, 11). Likewise, the expression of steroid receptors differ between eutopic and ectopic endometrial tissues (12, 13). In view of these findings, the effects of dienogest may differ between eutopic and ectopic endometrial tissues.

In the present study, therefore, we examined the effect of dienogest on the proliferation of endometriotic stromal cells from ovarian endometrioma. We also examined the effect of dienogest on the cell cycle by flow cytometric analysis.

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Reprint requests: Yutaka Osuga, M.D., Ph.D., Department of Obstetrics and Gynecology, Faculty of Medicine, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo, 113-8655, Japan (FAX: 81-3-3816-2017; E-mail: yutakaos-ky@umln.ac.jp).

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## MATERIALS AND METHODS

### Tissue Collection

Endometriotic tissues were collected from the walls of ovarian endometriomas during surgery. A total of 14 patients (aged  $37.6 \pm 4.4$  years, mean  $\pm$  SD) with endometriosis were recruited in this study. All the patients had regular menstrual cycles, and none had received hormonal treatment at least 6 months before surgery. Informed consent in writing was obtained from each patient for use of the ovarian endometriotic lesion; consent forms and experimental protocols were approved by the institutional review board (IRB) of the University of Tokyo. Tissues were placed in Dulbecco's minimum essential medium (DMEM)/F12 medium (GIBCO, Grand Island, NY) on ice and transported immediately to the laboratory for separation and culture of endometriotic stromal cells. Cells obtained from each patient were used for separate experiments.

### Isolation and Culture of Human Endometriotic Stromal Cells

Endometriotic stromal cells (ESC) were purified from ovarian endometriotic tissue and cultured as described previously (14, 15). The fresh endometriotic lesion collected in sterile medium was dissected free from underlying parenchyma, minced into small pieces, incubated in DMEM/F12 with 2.5 mg/mL type 1 collagenase (Sigma, St. Louis, MO) and 15 U/mL deoxyribonuclease 1 (Takara, Tokyo, Japan) for 2 hours at 37°C with agitation. The resultant suspension was separated by serial filtration. Debris was removed with a 100- $\mu$ m nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ), and some epithelial glands were eliminated with a 70- $\mu$ m nylon cell strainer (Becton Dickinson). Stromal cells remaining in the filtrate were collected by centrifugation, resuspended in DMEM/F12, and plated onto 100-mm dishes (Iwaki, Chiba, Japan) and allowed to adhere at 37°C for 30 minutes, after which nonadherent epithelial cells and blood cells were removed with phosphate-buffered saline (PBS) rinses. The cells were cultured in DMEM/F12 reconstituted with 10% charcoal-stripped fetal bovine serum (FBS) (Hyclone, Logan, UT) and antibiotics (Sigma). The purity of ESC population was more than 95%, as confirmed by positive immunocytochemical staining for vimentin. When the cells reached confluence, they were passed into a 60- by 15-mm dish and 96-well culture plates (Becton Dickinson) at the densities of  $10^6$  cells/dish and  $3 \times 10^3$  cells/well, respectively, in medium supplemented with 10% charcoal-stripped FBS. After 48 hours of culture, the cells were sustained with serum starvation for 24 hours and then used for experiments with 1.5% charcoal-stripped FBS in the medium.

### 5-Bromo-2'-Deoxyuridine Incorporation

The effect of dienogest on the proliferation of ESC was evaluated by measuring the incorporation of 5-bromo-2'-deoxyuridine (BrdU) into DNA. The BrdU incorporation was

detected by using the Biotrak cell proliferation ELISA system (Amersham Pharmacia Biotech), as we reported previously (16, 17). Briefly, ESC were seeded into Falcon 96-multiwell plates at a density of  $3 \times 10^3$  cells per well in 100  $\mu$ L of culture medium containing 10% FBS and cultured for 48 hours. After 24 hours of serum starvation, the medium were replaced with fresh medium supplemented with 1.5% charcoal-stripped FBS containing the control vehicle or dienogest, which was dissolved in dimethyl sulfoxide and then diluted with the medium. The final concentration of dimethyl sulfoxide in the medium never exceeded 0.1%. During the last 4 hours of 24-hour and 48-hour of culture, 10  $\mu$ L of BrdU solution was added and incubated at 37°C. After removing the culture medium, the cells were fixed and the DNA denatured by the addition of 200  $\mu$ L/well fixative. The peroxidase-labeled anti-BrdU bound to the BrdU incorporated in the newly synthesized, cellular DNA. The immune complexes were detected by the subsequent substrate reaction, and the resultant color was read at 450 nm in the DigiScan Microplate Reader (ASYS Hitech GmbH, Eugendorf, Austria).

### Flow Cytometry

The ESC were seeded into a 60- by 15-mm culture dish (Becton Dickinson) at a density of  $1 \times 10^6$  cells/dish in 2 mL of the culture medium. After 48 hours of culture and later 24 hours of serum starvation, the medium was replaced with fresh medium containing 1.5% FBS and  $10^{-6}$  M dienogest or control vehicle, and the culture was continued for an additional 24 or 48 hours. The cells were then harvested with trypsin (0.05%)/EDTA (0.02%), washed twice with ice-cold PBS (pH 7.4), and fixed with 70% ethanol at -20°C overnight. After washing twice with ice-cold PBS, the cells were incubated in 0.25 mg/mL ribonuclease solution (QIAGEN GmbH, Hilden, Germany) for 30 minutes at 37°C and stained with 50  $\mu$ L/mL propidium iodide for 30 minutes, on ice, in the dark, followed by filtration through a 40- $\mu$ m nylon mesh (Becton Dickinson) to remove cell clumps. A total of  $2 \times 10^5$  stained cells per treatment were analyzed in the EPICS XL Flow Cytometry (Beckman Coulter, Inc., Fullerton, CA).

### Statistical Analysis

The data were expressed as the mean  $\pm$  SEM. Data were analyzed by Student's *t*-test for paired comparison of cell cycle analysis and one-way ANOVA with post hoc test for multiple comparisons of BrdU incorporation experiments using Stat-View software (SAS Institute Inc., Cary, NC). A *P* value less than .05 was accepted as statistically significant.

## RESULTS

### Effect of Dienogest on the Proliferation of ESC

The effect of dienogest on the proliferation of ESC was examined by measuring BrdU uptake in cultured ESC. As shown in Figure 1, dienogest at concentration of  $10^{-7}$  M and  $10^{-6}$  M induced significant suppression of BrdU incorporation into DNA at 24 and 48 hours of treatment.

FIGURE 1

Effect of dienogest on the proliferation of endometriotic stromal cells. The effect was examined by 5-bromo-2'-deoxyuridine (BrdU) incorporation using cell proliferation ELISA system. (A) Treatment with dienogest at concentrations between  $10^{-8}$  M and  $10^{-6}$  M for 24 hours. (B) Treatment with dienogest at concentrations between  $10^{-8}$  M and  $10^{-6}$  M for 48 hours. Results shown are the mean percentage of the untreated control  $\pm$  SEM (bars) of hexaplicate cultures of four independent experiments. \* $P < .05$  vs. control.

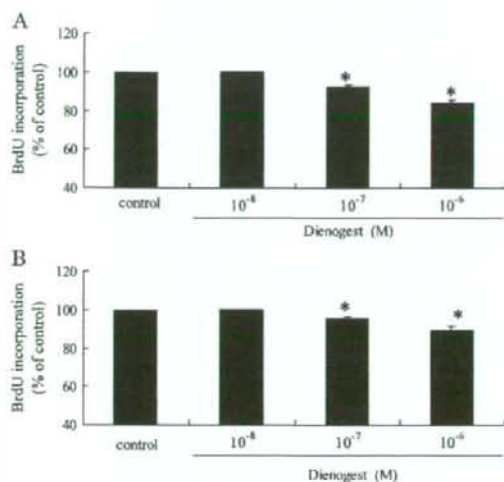


Fig. Antimetastatic effect of dienogest. *Fertil Steril* 2007.

### Effect of Dienogest on Cell Cycle in ESC

The effect of  $10^{-6}$  M dienogest on the cell cycle in ESC was determined by flow cytometric analysis. Table 1 listed the combined data from four independent experiments. Dienogest significantly increased the cells in  $G_0/G_1$  phase and reduced the cells in S phase and  $G_2/M$  phase in 24 and 48 hours.

### DISCUSSION

In the present study, we demonstrated that dienogest inhibited the proliferation of endometriotic stromal cells in vitro. The inhibitory effect was significant from a dose of  $10^{-7}$ , which was similar to that reported in eutopic endometrial stromal cells. It has been shown that maximum serum dienogest concentrations were reached within  $\sim 2$  hours and the mean maximum serum concentrations were  $0.9 \times 10^{-7}$ ,  $1.7 \times 10^{-7}$ ,  $3.2 \times 10^{-7}$ , and  $6.8 \times 10^{-7}$  M after therapeutic doses of 1, 2, 4, and 8 mg, respectively (3). In addition to our finding that dienogest inhibited ESC proliferation at a dose of  $10^{-7}$  M, dienogest at therapeutic doses conceptually yields direct suppression of the growth of endometriosis.

In recent framework of concept for etiology of endometriosis, a theory of P resistance is advocated (18). Progestins, such as medroxyprogesterone acetate (MPA) and dienogest, has been shown to inhibit the proliferation in eutopic endometrial stromal cells (9, 19, 20). However, it has also been reported that P-induced molecular changes in eutopic endometrium and ectopic endometriotic tissues of women with endometriosis are either blunted or undetectable (21–23). These observations, indicative of P resistance, were partially explained by an overall reduction of P receptors and the absence of P receptor B in the endometriotic tissue (24). In this context, it is remarkable that dienogest directly inhibited the proliferation of endometriotic cells in the present study. The present finding underscores the specific property of dienogest among progestins for endometriosis treatment.

Although antiproliferative effect of dienogest on eutopic endometrial cells has been reported, the mechanism has not been understood. Concerning this, a notable finding in the present study was that antiproliferative effect of dienogest on endometriotic stromal cells could be attributable to arrest of cell cycle in  $G_0/G_1$ . This finding seems to be similar to that P-inhibited proliferation of rat aortic smooth muscle cells with arrest of cell cycle in  $G_0/G_1$  (25). Interestingly, P suppresses  $E_2$ -induced cell proliferation, increasing p27 protein expression in endometrial glandular cells (26). It can be thus speculated that the inhibition of the proliferation of eutopic endometrial stromal cells is also associated with  $G_0/G_1$  arrest. Recent studies have shown that  $G_0/G_1$  arrest of ESC was also induced by other drugs (27, 28).

TABLE 1

Effect of dienogest on the number of endometriotic stromal cells in the respective phase of the cell cycle.

	$G_0/G_1$	S	$G_2/M$
24 h	102.04% $\pm$ 0.89% <sup>a</sup>	92.90% $\pm$ 3.21% <sup>a</sup>	89.39% $\pm$ 3.91% <sup>a</sup>
48 h	101.88% $\pm$ 0.75% <sup>a</sup>	89.51% $\pm$ 5.64% <sup>a</sup>	88.69% $\pm$ 3.55% <sup>a</sup>

Note: Values are the mean  $\pm$  SEM of the control from four independent experiments using samples from different women.  
<sup>a</sup>  $P < .05$  vs. control.

Fig. Antimetastatic effect of dienogest. *Fertil Steril* 2007.

Except for the antiproliferative effect demonstrated in the present study, direct effect of dienogest on endometriotic stromal cell has been hitherto poorly understood. There is only one report that dienogest attenuated tumor necrosis factor- $\alpha$ -induced interleukin-8 production (29). Further study would be warranted to understand additional direct effects of dienogest on endometriotic lesion.

In summary, we demonstrated that dienogest inhibited proliferation of endometriotic stromal cells in vitro with G<sub>0</sub>/G<sub>1</sub> cell cycle arrest. The finding subserves the understanding of the antiendometriotic effect of dienogest, a new therapeutic alternative to GnRH-a and conventional progestins.

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## High soluble CD44 concentration in peritoneal fluid in endometriosis

The concentration of the soluble form of CD44, a competitive inhibitor for CD44 and hyaluronic acid binding, is higher in the peritoneal fluid of patients with endometriosis than in the peritoneal fluid of patients without endometriosis. (Fertil Steril® 2007; ■:■-■. ©2007 by American Society for Reproductive Medicine.)

Endometriosis is an enigmatic disease that affects women of reproductive age, causing a decline in health and often resulting in infertility (1). Implantation of endometrial tissues in retrograde menstrual flux is a widely accepted etiology of the disease. From this perspective, adhesion molecules that form connections between endometrial and mesothelial cells may play a pivotal role in the pathogenesis of endometriosis. The adhesion molecule CD44 is a type I transmembrane protein that interacts with hyaluronic acid (HA) in a wide variety of physiological and pathological processes including lymphocyte homing, cell migration, and cancer cell metastasis (2). The soluble form of CD44 (sCD44), which consists of the ectodomain of CD44, has recently emerged as a key regulator of CD44 action. Soluble CD44 can also bind to HA, acting as a competitive inhibitor for membrane-bound CD44. Although there is a growing body of evidence that CD44 is involved in endometriosis (3, 4), the importance of sCD44 is still unknown. As a first step, we investigated the sCD44 expression in the peritoneal fluid (PF) of patients with endometriosis.

A total of 63 women of reproductive age with ( $n = 46$ ; age,  $34.4 \pm 5.4$  years) and without ( $n = 17$ ; age,  $33.9 \pm 6.0$  years) endometriosis participated in this study. Informed consent was obtained from each woman before laparoscopy. All of the women had regular menstrual cycles. None of the women took medications that affect hormonal and/or immunological status in the 3 months before laparoscopy, and no patient had undergone surgical treatment for endometriosis within 1 year. At the time of surgery, 35 women were in the proliferative phase, and 28 were in the secretory phase. The stage of endometriosis was evaluated according to the revised American Society for Reproductive Medicine (r-ASRM) classification (5). The distribution of the stage of endometriosis was as follows: stage I,  $n = 12$ ; stage II,  $n = 5$ ; stage III,  $n = 12$ ; stage IV,  $n = 17$ . PF was collected via a laparoscopic cannula introduced into the cul-de-sac before any manipulative procedure. The fluid was centrifuged at 400 g for 10 minutes, and the superna-

tants were frozen and stored at  $-80^{\circ}\text{C}$  until assay. Soluble CD44 concentrations in the PF were measured using a specific enzyme-linked immunosorbent assay for sCD44 (Kamiya Biomedical, Seattle, WA). The data were described as median and interquartile range (IQR). The Mann-Whitney test was used for the statistical analysis.

The sCD44 concentrations in PF showed no significant difference between the proliferative phase and the secretory phase (data not shown). The data were then combined and analyzed regardless of the menstrual phases. The sCD44 concentrations in the PF of the women with endometriosis (median, 423 ng/mL; IQR, 292–638) were significantly higher than those of the women without endometriosis (257 ng/mL, 196–337;  $P = .0004$ ). As shown in Figure 1A, a positive correlation between the sCD44 concentrations of PF and r-ASRM score was observed ( $r = 0.536$ ;  $P < .0001$ ). The women with endometriosis were then subdivided into those with stage I/II and those with stage III/IV endometriosis. As shown in Figure 1B, the concentrations of sCD44 in stage III/IV endometriosis (460 ng/mL, 311–758) were significantly higher than those in stage I/II endometriosis (310 ng/mL, 261–442;  $P = .0144$ ) and those in non-endometriosis (257 ng/mL, 196–337;  $P < .0001$ ). The sCD44 concentrations in stage I/II endometriosis were also significantly higher than those in non-endometriosis ( $P = .0439$ ).

This study is the first to demonstrate the presence of sCD44 in the PF of patients with endometriosis. We found a significant increase in sCD44 concentration in the PF of patients with endometriosis compared with patients without endometriosis. Furthermore, the expression of sCD44 was positively correlated with the stage of endometriosis.

It has been reported that CD44 is expressed not only in endometrial cells but also in endometriosis lesions (3, 4). Moreover, Dechaud et al., using the *in vitro* adhesion model, reported that interaction between endometrial cell-associated CD44 and mesothelial cell-associated HA might be crucial for the initiation of endometriosis (6).

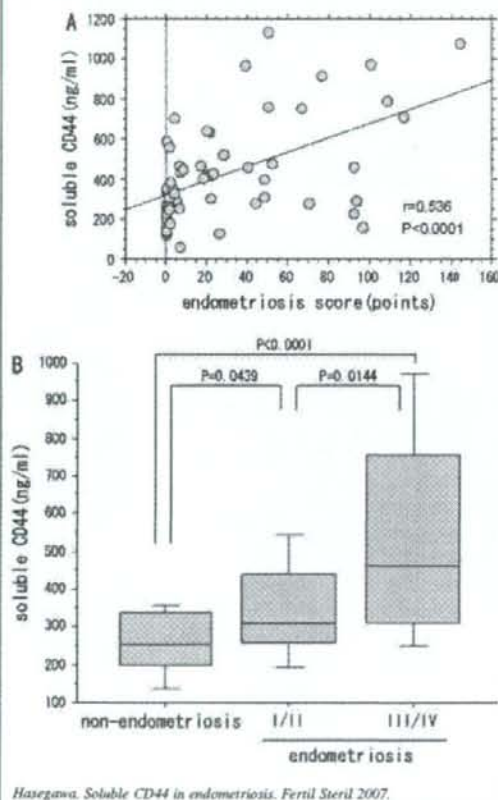
In the light of recent evidence on the importance of soluble receptors in various physiological settings and in the pathophysiology of various diseases (7–9), it is tempting

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Reprint requests: Osamu Yoshino, M.D., Ph.D., 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan (FAX: 81-3-3186-2017; E-mail: oyoshino624@hotmail.co.jp).

FIGURE 1

(A) Correlation between endometriosis score and soluble CD44 concentration in PF. Simple regression analysis showed that endometriosis score was positively correlated with the soluble CD44 concentration;  $r = 0.536$ ,  $P < .0001$ ,  $N = 63$  ( $n = 17$  patients without endometriosis;  $n = 46$  patients with endometriosis). (B) Concentration of soluble CD44 in PF of women without endometriosis ( $n = 17$ ), women with I/II endometriosis ( $n = 17$ ), and women with III/IV endometriosis ( $n = 29$ ). Boxes represent the distance between the first (25%) and third (75%) quartiles, and horizontal lines in the boxes represent medians.



Hasegawa. Soluble CD44 in endometriosis. *Fertil Steril* 2007.

to speculate that sCD44 in PF may have a role in the pathogenesis of endometriosis. CD44 expressing endometrial cells is delivered into peritoneal cavity via retrograde menstruation. Given that sCD44 can act as a competitive

inhibitor for the interaction of membrane-bound CD44 and HA, up-regulation of sCD44 in women with endometriosis may inhibit attachment of influxed endometrial cells to the peritoneal mesothelial cells as a defense mechanism against disease progression. Because CD44 is expressed in most human cell types (2), the source of sCD44 in PF is unclear. Nevertheless, in the PF, which reflects the peritoneal environment, sCD44 is expressed at a high concentration in patients with endometriosis. In view of this finding, one can hypothesize that the relative proportion of CD44 and sCD44 in PF might be important for initiation and progression of the disease. Further study is needed to elucidate the mechanism of sCD44 up-regulation in patients with endometriosis and to understand the action of sCD44 in the pathogenesis of endometriosis.

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Akiko Hasegawa, M.D.  
Osamu Yoshino, M.D., Ph.D.  
Yutaka Osuga, M.D., Ph.D.  
Tetsuya Hirata, M.D., Ph.D.  
Tetsu Yano, M.D., Ph.D.  
Yuji Taketani, M.D., Ph.D.

Department of Obstetrics and Gynecology, Tokyo University, Tokyo, Japan

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## CASE REPORT

## A case of hydrosalpinx associated with the menstrual cycle

Yutaka Osuga, M.D., Ph.D., Kaori Koga, M.D., Ph.D., Tetsuya Hirata, M.D., Ph.D.,  
Hisahiko Hiroi, M.D., Ph.D., and Yuji Taketani, M.D., Ph.D.

Department of Obstetrics and Gynecology, Faculty of Medicine, University of Tokyo, Tokyo, Japan

**Objective:** To describe a case report of hydrosalpinx that changed dramatically in size during the menstrual cycle.

**Design:** Case report.

**Setting:** University teaching hospital reproductive endocrinology and infertility practice.

**Patient(s):** A 32-year-old woman with a history of medical and surgical treatments of endometriosis who sought infertility treatment.

**Intervention(s):** Transvaginal ultrasonography performed sequentially during menstrual cycles.

**Main Outcome Measure(s):** Size of hydrosalpinx-like image.

**Result(s):** The size of the hydrosalpinx-like image in the left adnexal region varied; it peaked during the ovulatory period and then remarkably diminished in a cyclic manner. Laparoscopy revealed a dense adhesion between the left tubal fimbriated end and the posterior uterine wall, which led to terminal obstruction of the tube.

**Conclusion(s):** Change in the volume of the hydrosalpinx in this case was speculated to reflect the normal tubal fluid production regulated by ovarian hormones. (Fertil Steril® 2007; ■:■-■. ©2007 by American Society for Reproductive Medicine.)

**Key Words:** Hydrosalpinx, ultrasound, endometriosis, menstrual cycle, tubal fluid

Hydrosalpinx is usually found as a consequence of chlamydia or other infections in the fallopian tube (1). Cases of hydrosalpinx that appear during ovarian stimulation, though very rare, have also been reported (2, 3). Here we report a unique case of hydrosalpinx, which developed in the absence of infection and hormonal treatment and changed size dramatically with the menstrual cycle. No specific institutional review board approval was sought because the patient was not enrolled in a research protocol.

## CASE REPORT

The patient was a 32-year-old woman who had had endometriosis since the age of 24 years. She underwent ethanol sclerotherapy of bilateral ovarian endometrioma at age 26 years and laparoscopic cystectomy for the right endometrioma at age 30 years. She received GnRH analogue treatments at 24 and 26 years of age. She was seen for infertility treatment after 1 year of attempting to get pregnant. Her basal body temperature was biphasic, with adequate duration of high phase. Serum anti-*Chlamydia trachomatis* IgA and IgG antibodies were negative. Hysterosalpingography

conducted just after menstruation showed terminal obstruction of the left fallopian tube. During ultrasonography workups to check follicular growth and ovulation, we noticed a hydrosalpinx-like structure at the left adnexal area, which varied in size and appearance with each observation. We then observed this structure sequentially by ultrasonography during menstrual cycles. Interestingly, the size of the structure was minimal during the menstrual period but increased and reached a maximum size at ovulation, to then shrink again (Fig. 1A and B). Laparoscopy revealed a dense adhesion between the left tubal fimbriated end and the posterior uterine wall, presumably caused by previous surgery and endometriosis, which subsequently led to terminal obstruction of that tube (Fig. 2). Salpingectomy was undertaken with the hope of improving IVF-ET outcome. Histologically, the structural integrity of tubal epithelium was maintained, and endometriosis was found at the serosa of the tubal wall.

## DISCUSSION

This is a unique case of hydrosalpinx with menstrual cycle-dependent change during normal menstrual cycles. Although there are several reports of hydrosalpinx that enlarge during ovarian stimulation (2, 3), there has been no report of hydrosalpinx with enlargement and shrinking during menstrual cycles. Another unique aspect of this case is that no evidence of pathological damage was found within the tubal epithelium.

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Reprint requests: Yutaka Osuga, M.D., Department of Obstetrics and Gynecology, Faculty of Medicine, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-8655, Japan (FAX: 81-3-3816-2017; E-mail: yutakaos-ky@umin.ac.jp).

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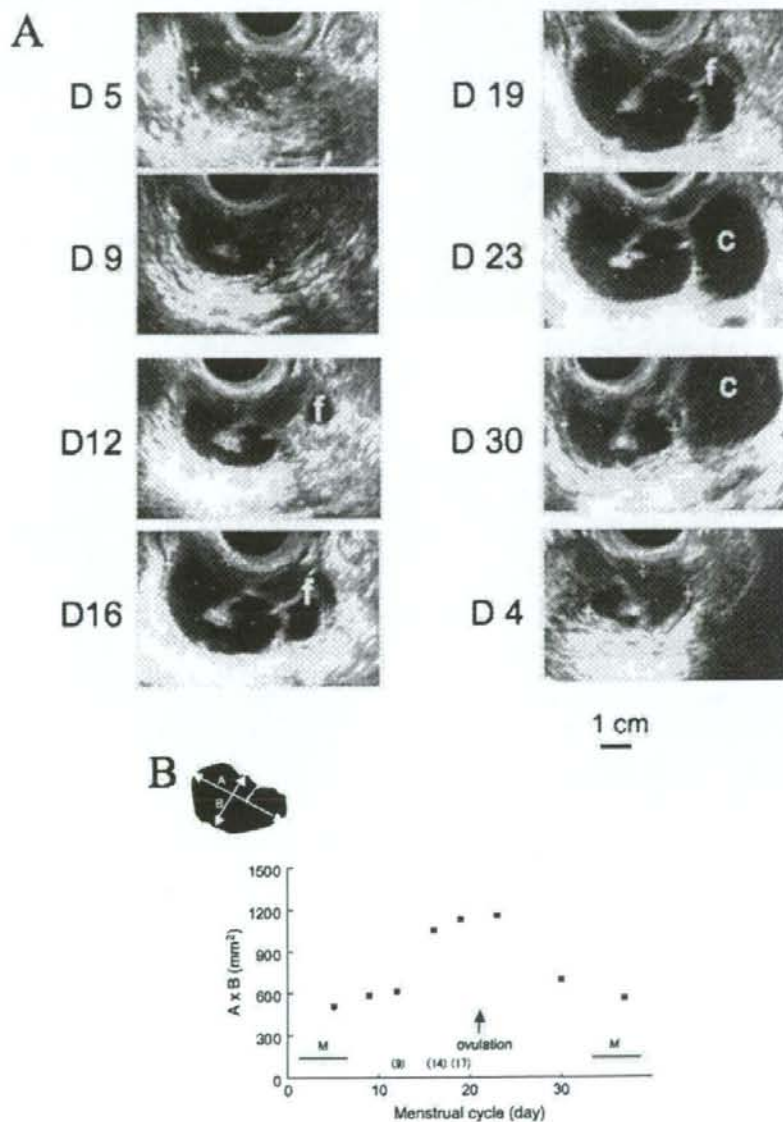
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FIGURE 1

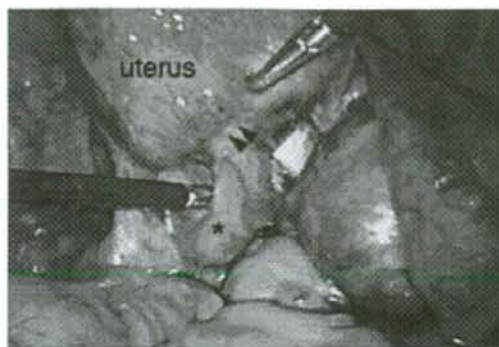
Sequential change of ultrasonographic images of the left fallopian tube in a representative menstrual cycle. (A) The left adnexal area was scanned by transvaginal ultrasonography on various menstrual days. A cystic part on the right side of the hydrosalpinx at day (D)12 and D16 are follicles (f), whereas D23 and D30 are corpus luteum (c). Ovulation occurred at D20, confirmed by basal body temperature. (B) The size of the hydrosalpinx area imaged by ultrasonography was plotted. M = menstruation. Values in parenthesis show the mean diameter (in millimeters) of the dominant follicle.



Osuga. Cyclic enlargement of hydrosalpinx. *Fertil Steril* 2007.

FIGURE 2

Laparoscopic finding of the left tube. A dense adhesion between the left tubal fimbriated end and the posterior uterine wall was found (arrowheads), which subsequently led to terminal obstruction of the left tube (asterisk).



Osuga. Cyclic enlargement of hydrosalpinx. *Fertil Steril* 2007.

Hydrosalpinx is often a sequel of chlamydial infection, which affects fallopian tube epithelial transporters and ion channels, particularly the cystic fibrosis transmembrane conductance regulator, resulting in increased epithelial secretion and decreased fluid absorption, hence an accumulation of hydrosalpinx fluid (4). In our case, however, the serologic chlamydial test was negative and the microscopic structure of the tubal epithelium was normal, suggesting that the capacity of fluid secretion and absorption in the affected tube was maintained (5). Given these observations, what was the underlying cause of the tubal fluid retention and its cyclic volume change?

It is well established in animal models that the fluid production by the fallopian tube is controlled by ovarian hormones and that the volume is highest during the ovulatory period. A recent study suggests that an ovarian hormone-

regulated water channel, aquaporin 9, located in the oviductal epithelium, is involved in tubal fluid balance (6). In the human, the tubal fluid may also be regulated hormonally, reaching maximum levels during the ovulatory period. However, the fluid constantly empties into the peritoneal cavity, so that changes are not detected by ultrasonography. Only in patients undergoing ovarian stimulations, in whom ovarian hormone levels are superphysiological, fluid production is excessive and detectable by ultrasonography, when it is not effectively discharged from the tube. The resulting fluid retention could explain the etiology of hydrosalpinx during ovarian stimulation, as reported previously. On the other hand, in the present case, obstruction of the tubal terminal end would lead to fluid accumulation or a hydrosalpinx as seen by ultrasonography. Indeed, increased volume during the ovulatory period may reflect normal tubal fluid production, regulated by ovarian hormone.

In summary, we have reported a unique case of hydrosalpinx that changed size during menstrual cycles. This change is proposed to reflect a normal tubal epithelial function, producing fluid under ovarian hormone regulation.

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1 **A case of hydrosalpinx associated with the menstrual cycle**

Y. Osuga, K. Koga, T. Hirata, H. Hiroi, and Y. Taketani  
*Tokyo, Japan*

The authors present a unique case of hydrosalpinx that changed size during menstrual cycles, peaking during the ovulatory period and then remarkably diminishing in a cyclic manner.

## The Presence of Midkine and its Possible Implication in Human Ovarian Follicles

Yasushi Hirota<sup>1</sup>, Yutaka Osuga<sup>1</sup>, Emi Nose<sup>1</sup>, Kaori Koga<sup>1</sup>, Osamu Yoshino<sup>1</sup>, Tetsuya Hirata<sup>1</sup>, Tetsu Yano<sup>1</sup>, Osamu Tsutsumi<sup>1</sup>, Sadatoshi Sakuma<sup>2</sup>, Takashi Muramatsu<sup>3</sup>, Yuji Taketani<sup>1</sup>

<sup>1</sup>Department of Obstetrics and Gynecology, Faculty of Medicine, University of Tokyo, Hongo, Bunkyo-ku, Tokyo, Japan;

<sup>2</sup>Cell Signals Inc., Ono Tsurumi-ku, Yokohama, Japan;

<sup>3</sup>Department of Health Science, Faculty of Psychological and Physical Sciences, Aichi Gakuin University, Nisshin, Aichi, Japan

### Keywords

Follicular fluid, folliculogenesis, growth factor granulosa cells

### Correspondence

Yutaka Osuga, Department of Obstetrics and Gynecology, Faculty of Medicine, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo, 113-8655, Japan.  
E-mail: yutakaos-ky@umin.ac.jp

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### Problem

Ovarian follicles undergo a dynamic change to provide a mature ovum, and the process involves angiogenesis, follicular cell proliferation and leukocyte recruitment. Midkine (MK) is a heparin-binding growth factor that has angiogenic, mitogenic, and chemotactic activities. In the present study, we investigated the presence of MK and its possible role in human ovarian follicles.

### Method of study

Follicular fluid (FF) and luteinized granulosa cells (LGC) were collected from women undergoing *in vitro* fertilization and embryo transfer. Expression of MK protein in FF was examined by Western blotting. Concentrations of MK, estradiol and oxygen in FF were measured. 5-bromo-2'-deoxyuridine (BrdU) incorporation assay was performed in LGC. Normal ovarian tissues were obtained surgically and used in in-situ hybridization of MK mRNA.

### Results

The presence of MK protein was verified in FF. MK mRNA was expressed in both granulosa cells and theca cells of large follicles. There is a significant negative correlation between the concentrations of MK and oxygen in FF, and a significant positive correlation between the concentrations of MK and estradiol. MK promoted BrdU uptake in LGC.

### Conclusion

The present findings imply that hypoxic condition, a characteristic of growing follicles, associates with the production of MK. Given that MK is involved in granulosa cell proliferation and estradiol production in developing follicles, MK may play a role as a local regulator in the human ovary.

### Introduction

Folliculogenesis is a dynamic process that involves angiogenesis, follicular cell proliferation and steroid hormone production. These phenomena are under the control of general and local regulators, such as gonadotropins, steroids, cytokines, and growth fac-

tors. Substances participating in folliculogenesis are often secreted or transported into follicular fluid (FF). Several factors have been detected in human FF, and they are suggested to play roles in the human ovary.<sup>1-4</sup>

Midkine (MK) is a heparin-binding growth factor. MK is a basic, low molecular weight non-glycosylated

protein, composed of two peptide chains held by disulfide bridges. MK is widely expressed in adult rat and human tissues, and strongly induced during oncogenesis, inflammation, and tissue repair.<sup>5</sup> MK has multiple activities such as promoting angiogenesis, cell migration, and cell proliferation.<sup>5</sup> Receptors of MK are considered to be a molecular complex containing receptor-type tyrosine phosphatase z (PTPz), low density lipoprotein receptor-related protein 1 (LRP1), anaplastic leukemia kinase (ALK), and syndecans.<sup>5</sup>

MK protein has been shown to be present in bovine FF.<sup>6</sup> In the rat ovary, MK mRNA is located in granulosa cells, and is increased by pregnant mare serum gonadotropin (PMSG) *in vivo*, and by follicle stimulating hormone (FSH) *in vitro*.<sup>7,8</sup> In addition, we have reported high concentrations of MK in FF of the human ovary.<sup>9</sup>

In view of pleiotropic functions of MK and its presence in the ovary, we hypothesized that MK is involved in the development of human ovarian follicles. In the present study, we first verified the presence of MK protein in FF. We also studied the gene expression of MK by *in-situ* hybridization. We then assayed concentrations of MK, ovarian hormones and oxygen in FF, and analyzed their relationships. Lastly, an effect of MK on proliferation of granulosa cells was studied.

## Materials and methods

### Collection of FF and Measurement of Oxygen Concentration in FF

We recruited a total of 16 women, aged between 33 and 41 years (mean, 37.5 years), undergoing the treatment of *in vitro* fertilization (IVF) for tubal factor and/or male factor. The experimental procedure was approved by the institutional review board, and signed informed consent for use of FF and luteinized granulosa cells (LGC) were obtained from each woman.

The protocol for ovarian stimulation and collection of FF was described previously.<sup>2,3</sup> Briefly, following ovarian suppression by the gonadotropin releasing hormone (GnRH) agonist (buserelin acetate; Suprecur, Aventis Pharma, Tokyo, Japan) from the midluteal phase of the preceding cycle, a dose of 150–300 IU human menopausal gonadotropin (hMG) (Nikken, Tokyo, Japan) was given daily until the diameter of the leading follicle reached 17 mm or greater. Then, human chorionic gonadotropin (hCG)

at a dose of 10,000 IU (hCG; Mochida, Tokyo, Japan) was administered and transvaginal ultrasound-guided oocyte retrieval was performed 35 hr later.

To evaluate the relationship between concentrations of oxygen, cytokines, and steroid hormones in FF, FF from each follicle was separately aspirated without flushes. Measurement of oxygen concentration was performed as reported previously.<sup>4</sup> After isolation of an oocyte, oxygen concentration in aspirated FF was immediately measured with a dissolved oxygen meter (DO meter) (Iijima Electronics Corporation, Aichi, Japan). The principle of this oxygen meter is based on a diaphragm-type Galvanic cell system, which is widely applied to measure dissolved oxygen in various fluid samples.<sup>10</sup> The gas analysis was performed within 30 s from the aspiration. The samples were confined to a constant-temperature glass bath during the analysis. Aspirated FF was then centrifuged ( $300 \times g$ ) for 5 min, and the supernatant was divided into several aliquots and stored at  $-80^{\circ}\text{C}$  until use. In total, 21 FF samples were obtained from five women.

### Isolation of LGC

Isolation and culture of LGC were processed as described previously.<sup>11</sup> To obtain LGC, FF was aspirated with repeated flushes during the oocyte pick-up procedure. FF contained mainly mural granulosa cells and very few cumulus cells. All the follicular aspirates and flush medium from each woman were mixed and centrifuged at  $300 \times g$  for 5 min to obtain a cell pellet, which was resuspended in phosphate-buffered saline (PBS) with 0.1% hyaluronidase and incubated at  $37^{\circ}\text{C}$  in a shaking water bath for 30 min. The suspension was layered onto Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden) and centrifuged at  $150 \times g$  for 30 min. Crude LGC fractions were recovered from the interface and washed with PBS. The cells were then treated with the monoclonal anti-CD45 antibody coupled with magnetic immunobeads (Dynal A.S., Oslo, Norway) to remove white blood cells. The cells were suspended in 2 mL medium containing 2% fetal bovine serum (FBS) and incubated for 20 min at  $4^{\circ}\text{C}$  with anti-CD45 immunomagnetic beads. Subsequently, the suspension was placed into a magnetic test tube rack (Dynal A.S.) for 2 min at room temperature to remove immunobeads-bound white blood cells from the cell suspension. With this method, remaining CD68 (a marker of monocyte/macrophages) positive cells were  $<1\%$  in isolated LGC by immunostaining.

### Heparin-Sepharose Chromatography

Midkine in FF was partially purified by heparin-sepharose affinity chromatography. Heparin-sepharose chromatography was performed as described previously.<sup>2</sup> The slurry of 1.0 g Heparin Sepharose CL-6B (Amersham Biosciences) was incubated with approximately 100 mL FF with gentle stirring for 3 hr at 4°C. The mixture was poured into an open column. The packed column was washed with PBS and eluted with 20 mL NaCl in stepwise concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0 M. The eluted fractions were further concentrated by Centricon YM-10 (Millipore, Billerica, MA, USA) and tested for Western blotting.

### Western Blotting

Samples were resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were blotted onto a nitrocellulose membrane and incubated with rabbit anti-MK primary antibody (1:200), which was kindly provided by Cell Signals (Yokohama, Japan), and anti-rabbit horse-radish peroxidase secondary antibody (1:1000, Amersham Biosciences). Immune complexes were visualized by use of an ECL Western blotting system (Amersham Biosciences).

### RNA Extraction and RT-PCR of MK mRNA and its Receptor LRP1 mRNA

Total RNA was extracted from isolated LGC using an RNeasy Mini Kit (Qiagen, Hilden, Germany). RT-PCR was performed using Rever Tra Dash (Toyobo, Tokyo, Japan). One microgram of total RNA was reverse-transcribed in a total volume of 20 µL, and cDNA was amplified using oligonucleotide primers based on the sequences of human MK and human LRP1. The human GAPDH primers (Toyobo) were used to ensure RNA quality and amounts. MK primers (sense, 5'-CCTGCAACTGGAAGAAGGAG-3'; antisense, 5'-AGCAGACAGAAGGCACTGGT-3') were chosen to amplify a 320 bp fragment. LRP1 primers (sense, 5'-CACCTTAACGGGAGCAATGT-3'; antisense, 5'-GTCACCCAGTCTGTCCAGT-3') were chosen to amplify a 240 bp fragment. PCR conditions for the amplifications of MK, LRP1, and GAPDH were 30 cycles at 98°C for 10 s, 60°C for 2 s, and 74°C for 20 s. PCR products were analyzed by agarose gel electrophoresis with ethidium bromide. Each PCR product was purified with a QIAEX II gel extraction kit

(Qiagen), and their identities were confirmed using an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

### Collection of Ovarian Tissues

Tissue specimens of human ovaries were obtained from four women (age range, 28–40 years) who underwent salpingo-oophorectomy for the treatment of uterine cervical cancer under signed informed consent. All patients had normal regular ovarian cycle before operation and no histological abnormality of ovarian tissues. At the time of operation, all patients were in the late follicular phase and their ovaries had large follicles (>9 mm). The experimental procedure was approved by the institutional review board.

### In Situ Hybridization

Ovarian tissues were fixed in neutral-buffered formalin and embedded in paraffin blocks. Sections were prepared from the blocks and stained with hematoxylin and eosin.

For preparation of the digoxigenin (DIG)-labeled RNA probe for MK, the 320-bp fragment of the human MK cDNA, obtained by RT-PCR with the primers described above, was subcloned into the appropriate restriction site of the PCR II-TOPO vector (Invitrogen, Carlsbad, CA, USA). After linearization of the plasmid with an appropriate restriction enzyme, the linearized vectors were used as templates for the synthesis of DIG-labeled RNA probes using SP6 or T7 RNA polymerase. In situ hybridization was performed using an ISHR starting kit (Nippon Gene, Toyama, Japan) as described previously.<sup>12,13</sup> Briefly, tissues collected for in situ hybridization were fixed by immersion in 10% neutral-buffered saline overnight at 4°C before routine paraffin embedding. The paraffin-embedded specimens were sliced at a 5-µm thickness. These sections were mounted on poly-L-lysine-treated slides, deparaffinized, and rehydrated. They were further digested with 5 mg/mL proteinase K for 10 min at room temperature, treated with acetic anhydride, and then subjected to treatment with pre-hybridization solution for 30 min at 42°C. Hybridization was carried out by applying the diluted probe to each slide section. Each section was incubated in a humidified chamber overnight at 42°C. Slides were washed and then treated with RNase for 30 min at

37°C. After being blocked with blocking solution, the sections were incubated with an anti-DIG, alkaline phosphatase-conjugated antibody (1:500; Roche, Basel, Switzerland) for 1 hr at room temperature. Color development was carried out by overlaying them with 4-nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche), and they were incubated in a humidified container in the dark for 12 hr. Sense probe hybridization was used as a control for background level.

#### Measurements of Estradiol, Progesterone and MK in FF

Concentrations of estradiol in FF were measured using an SR1 analyzer (Biochem ImmunoSystems Italia, Rome, Italy). For the intra-assay and inter-assay, coefficients of variation were <10% for this assay. Concentrations of progesterone in FF were measured using a specific enzyme immunoassay (Progesterone EIA Kit; Cayman Chemical, Ann Arbor, MI, USA). The sensitivity limit was 10 pg/mL and the intra-assay and inter-assay coefficients of variation were <10% for this assay. Concentrations of MK in FF were determined using an enzyme-linked immunosorbent assay (ELISA), kindly provided by Cell Signals. The sensitivity limit of this assay was 0.1 ng/mL per sample.

#### Treatment of LGC with MK and Cell Proliferation Assay

Isolated LGC were suspended in DMEM/Ham's F-12 medium containing 10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 µg/mL amphotericin B, then plated in 24-well plates at a density of  $5 \times 10^4$  cells/well and kept at 37°C in a humidified 5% CO<sub>2</sub>/95% air environment. After 24 hr of incubation, FBS-free media were replaced, and the cells were cultured for another 24 hr. To examine the effect of MK, the cells were further cultured in FBS-free media containing recombinant MK, which was kindly provided by Cell Signals, for an additional 24 hr.

The cell proliferation assay was performed as we have reported previously.<sup>14</sup> The effect of MK on the proliferation of LGC was examined by measuring 5-bromo-2'-deoxyuridine (BrdU) incorporation into DNA using the Biotrak cell proliferation ELISA system (Amersham Biosciences) according to the manufacturer's instructions.

#### Statistical Analysis

The data of MK in FF were described as median and interquartile range (IQR). Correlations between the concentrations of MK and those of oxygen, estradiol, and progesterone in FF were calculated by linear regression analysis. Student's *t*-test was used for the analysis of BrdU incorporation of the cultured cells. *P* < 0.05 was accepted as statistically significant.

#### Results

##### Presence of MK Protein in FF

Using Western blotting, we demonstrated the presence of MK protein in FF as a band at about 13 kDa, which was the same size as recombinant MK. MK in FF bound to heparin-sepharose was eluted in a 0.8 M NaCl fraction (Fig. 1).

##### Expressions of MK mRNA and its Receptor LRP1 mRNA in LGC

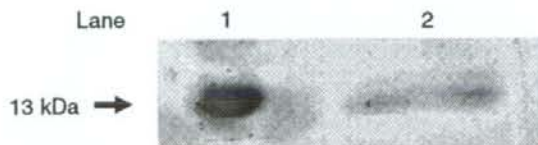
RT-PCR analysis demonstrated the expressions of MK mRNA and its receptor LRP1 mRNA in LGC as a clear band at 320 and 240 bp, respectively (Fig. 2).

##### In vivo, Expression of MK mRNA in Human Follicles

Human MK mRNA was detected in both granulosa cells and theca cells of large follicles by *in situ* hybridization (Fig. 3). No specific hybridization products were observed when using the sense riboprobes.

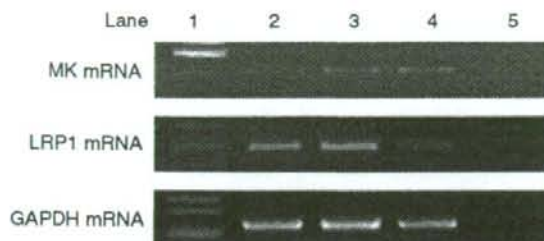
##### Correlation Between Concentrations of MK and those of Oxygen and Ovarian Steroid

The median concentrations of MK in FF were 217 ng/mL (IQR, 160–313). As depicted in Fig. 4a,b,



**Fig. 1** Midkine (MK) protein in human follicular fluid (FF). MK protein expression was examined by Western blotting in human FF. Several fractions were separated from crude FF by heparin-sepharose chromatography. Recombinant MK was used as a positive control band. Lanes 1, recombinant MK; lane 2, separated fractions eluted by 0.8 M NaCl.





**Fig. 2** Expression of midkine (MK) mRNA and low density lipoprotein receptor-related protein 1 (LRP1) mRNA in human luteinized granulosa cells (LGC). Amplification of GAPDH was used to ensure RNA quality and amounts. Lane 1, Marker; lanes 2, 3 and 4, three different samples of LGC; lane 5, negative control with water.

simple regression analysis demonstrated a significant negative correlation between the concentrations of oxygen and those of MK in FF ( $r = -0.6661$ ,  $P = 0.0013$ ) and a significant positive correlation between the concentration of estradiol and MK ( $r = 0.6362$ ,  $P = 0.0019$ ). No correlation was detected between the concentration of progesterone and MK ( $r = 0.00949$ ,  $P = 0.9676$ ).

#### Effect of MK on the Proliferation of LGC

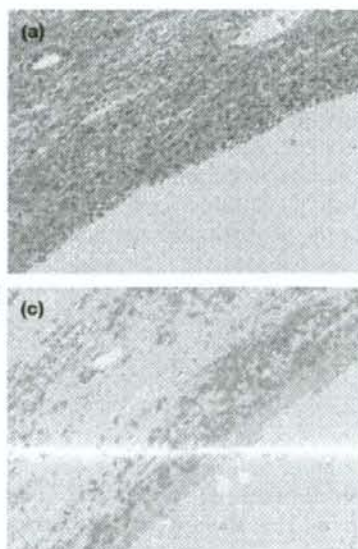
The effect of MK on DNA synthesis in LGC is shown in Fig. 5. MK at 100 ng/mL significantly increased BrdU incorporation to 140% of that of the control ( $P < 0.005$ ).

#### Discussion

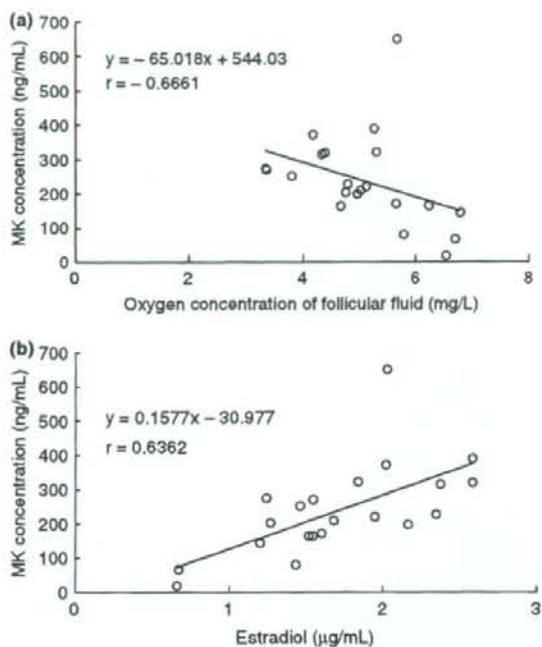
In the present study, we demonstrated the expression of MK in the human ovarian follicle. The concentrations of MK in FF were negatively correlated with those of oxygen and positively correlated with those of estradiol. *In vitro*, study showed the mitogenic activity of MK on human granulosa cells.

The present study verified that concentrations of MK in FF were relatively high as reported in our previous study.<sup>9</sup> A possible explanation of the high concentration is a local production of MK in the follicle. The notion is supported by our finding that MK mRNA was expressed in both granulosa and theca cell layers of the growing follicle. Interestingly, local gene expression of MK in the follicle has also been observed in the rat ovarian follicle although the expression is exclusively in granulosa cells.<sup>7,8</sup>

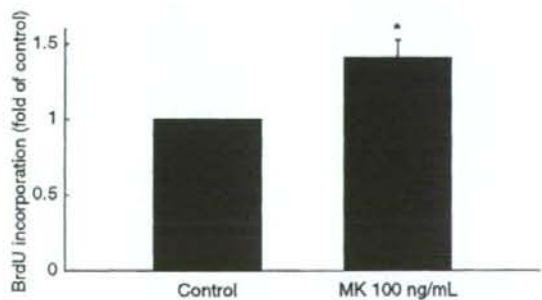
Vasculature of the follicle is limited to the thecal layer outside the basement membrane, and the oxygen tension tends to decline inside. Accordingly, angiogenesis is an essential event for a growing follicle to supply oxygen to cells comprising a follicle.<sup>15</sup> Indeed, several angiogenic factors have been suggested to play roles in the follicular development. It is generally known that hypoxic condition stimulate angiogenesis by upregulating various angiogenic substances. We previously reported that the volume of FF is negatively correlated with oxygen tension and positively correlated with the concentration of IL-8.<sup>4</sup>



**Fig. 3** *In situ* hybridization for midkine (MK) in the human ovarian follicle. Sections of ovarian tissue with large follicles were stained with hematoxylin and eosin (HE) (a). Slide sections were hybridized with digoxigenin (DIG)-labeled antisense (b) or sense (c) riboprobes. MK mRNA was strongly expressed in a human large follicle. Magnification,  $\times 100$ . The section represents four samples from different women stained with the same procedure.



**Fig. 4** Correlation between concentrations of midkine (MK) and those of oxygen and estradiol in follicular fluid (FF). (a) There was a significant negative correlation between the concentrations of oxygen and those of MK in FF ( $r = -0.6661$ ,  $P = 0.0013$ ). (b) There was a significant positive correlation between concentrations of estradiol and those of MK in FF ( $r = 0.6362$ ,  $P = 0.0019$ ).



**Fig. 5** Midkine (MK) stimulated the proliferation of luteinized granulosa cells (LGC). The effect of MK on the proliferation of LGC was examined by measuring 5-bromo-2'-deoxyuridine (BrdU) incorporation into DNA using the cell proliferation enzyme-linked immunosorbent assay (ELISA). LGC were treated with fetal bovine serum (FBS)-free media containing recombinant MK for 24 hr. As a control, FBS-free medium was used. Values are the means  $\pm$  S.E.M. of pentaplicate cultures. The data shown is representative of three experiments using different LGC preparations. Student's *t*-test was used for comparison of the BrdU incorporation. \* $P < 0.005$  (versus control).

In the present study, concentrations of MK were negatively correlated with those of oxygen in FF. Given that MK is upregulated by hypoxia<sup>16</sup>, our finding imply that MK is involved in angiogenesis of the developing follicle exposed to hypoxic environment.

Angiogenic factors that have hitherto been demonstrated in the FF include vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), angiogenin, and IL-8.<sup>1,3,4,17</sup> Interestingly, concentrations of VEGF, HGF, and angiogenin are all positively correlated with those of progesterone in FF, and not with those of estradiol. Thus, it seems quite unique that concentrations of MK are positively correlated with those of estradiol but not progesterone. It may be speculated that MK possesses specific feature in its effect on follicular steroid production, being different from other angiogenic factors in FF.

Midkine is known to have mitogenic activity in several cells.<sup>18–20</sup> We have previously shown that MK promoted the proliferation of endometriotic stromal cells.<sup>9</sup> In the present study, MK stimulated the proliferation of LGC. The expression of LRP1 in LGC suggests the presence of molecular complex of MK receptor that mediate the mitogenic function. Considering that granulosa cell proliferation promote follicular development and estradiol production, it is plausible that higher concentrations of MK are correlated with higher levels of estradiol in FF.

Pleiotrophin (PTN) has similar sequence and functions to those of MK, and the receptor and signaling systems of the two factors are closely related.<sup>5</sup> Recently, mice doubly deficient in genes of MK and PTN have been developed.<sup>21</sup> Interestingly, most of the female double deficient mice were infertile with reduced mature follicles. Given the possible importance of MK in human follicles, MK deficiency might be associated with human infertility.

In summary, the present study suggested that MK functions as a local regulator of follicular growth in the human ovary.

## Funding

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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## Human round spermatids from azoospermic men exhibit oocyte-activation and $\text{Ca}^{2+}$ oscillation-inducing activities

H. Yazawa<sup>1</sup>, K. Yanagida<sup>2</sup> and A. Sato<sup>3</sup>

Departments of Obstetrics and Gynecology, Fukushima Red Cross Hospital and Fukushima Medical University, Fukushima and International Health and Welfare, Nasuno-cyo Tochigi, Japan

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### Summary

During mammalian fertilization, intracellular  $\text{Ca}^{2+}$  oscillations are important for both oocyte activation and embryonic development. As the ability of round spermatids (ROS) to induce  $\text{Ca}^{2+}$  oscillations and oocyte activation is different between species, we examined  $\text{Ca}^{2+}$  oscillation- and oocyte activation-inducing abilities of human ROS originating from patients with non-obstructive azoospermia. Human ROS from 11 non-obstructive azoospermic patients were collected during their TESE-ICSI cycles. Following injection into mature unfertilized mouse oocytes, we examined the oocyte-activating and  $\text{Ca}^{2+}$  oscillation-inducing activities of ROS by using  $\text{Ca}^{2+}$  imaging and confocal laser scanning microscopy (mouse test). In these 11 cases, clinical TESE-ICSI using mature testicular spermatozoa was successful, with the exception of one case in which only one sperm-injected oocyte was not fertilized. The mean fertilization rate was 70.1% (40–100%); the mean cleavage rate was 97.9% (46/47). Two pregnancies were established from 10 transfer cycles (PR; 20%). When the ROS from these patients were injected into mouse oocytes, the ROS from all patients induced at least some intracellular  $\text{Ca}^{2+}$  oscillations (25–100%). In all patients, 40 out of 82 oocytes injected with ROS exhibited normal oscillation patterns of  $[\text{Ca}^{2+}]_i$ .

Human spermatogenic cells acquired oocyte-activating and  $\text{Ca}^{2+}$  oscillation-inducing abilities at the round spermatid stage, an earlier stage than found for rodent cells. These data indicate that human ROS might be useful for clinical treatments of non-obstructive azoospermic patients exhibiting mature spermatozoa in biopsied specimens.

Keywords: Calcium oscillation, Human, Non-obstructive azoospermia, Oocyte activation, Round spermatid

### Introduction

Spermatids are spermatogenic cells that have just completed meiosis and possess a haploid set of chromosomes, making them genetically equivalent to mature

spermatozoa. Recently, human round spermatids (ROS) have been used as a clinical treatment for non-obstructive azoospermic patients (Tesarik *et al.*, 1996; Vanderzwalmen *et al.*, 1997; Sousa *et al.*, 1999). As the pregnancy rates reported using ROS are poor in comparison with those using testicular or ejaculated spermatozoa, it is likely that functional immaturities of round spermatids, such as an insufficient activity of sperm-borne oocyte-activating factor (SOAF), result in inefficient fertilization.

In previous studies using experimental animal models, mouse ROS lack oocyte activation-inducing ability (Kimura & Yanagimachi, 1995; Yazawa *et al.*, 2000). At least in mice, SOAF becomes biologically active during spermiogenesis. In contrast, ROS from hamsters and rabbits exhibit oocyte-activating ability. These cells cannot, however, induce  $\text{Ca}^{2+}$  oscillations,

Correspondence should be addressed to: H. Yazawa,  
<sup>1</sup>Department of Obstetrics and Gynecology, Fukushima Red Cross Hospital, 11–31 Irie-cyo, Fukushima 960–8530, Japan.  
e-mail: ikyoku12@fukushima-med-jrc.jp

<sup>2</sup>Department of Obstetrics and Gynecology, Fukushima Red Cross Hospital, 11–31 Irie-cyo, Fukushima 960–8530, Japan.

<sup>3</sup>Department of Obstetrics and Gynecology, International Health and Welfare, 537–1 Iguchi, Nasuno-cyo Tochigi 329–2763, Japan.

<sup>4</sup>Department of Obstetrics and Gynecology, Fukushima Medical University, 1 Hikarigaoka, Fukushima 960–1295, Japan.