Proliferation Profile of Uterine Endometrial Stromal Cells during *In Vitro* Culture with Gonadotropins: Recombinant versus Urinary Follicle Stimulating Hormone

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Abstract

BACKGROUND: Provision of optimal endometrial stromal cells is essential in uterine tissue engineering. Culture of these cells is significantly influenced by gonadotropin hormones. This investigation attempted to define the proliferation profiles of murine uterine endometrial stromal cells during *in vitro* culture with recombinant follicle stimulating hormone (rFSH), urinary follicle stimulating hormone (uFSH), and human chorionic gonadotropin (hCG).

METHODS: Murine uterine endometrial stromal cells were collected from 8-week-old mice and cultured *in vitro* up to 72 h, with rFSH, uFSH, or hCG. Cell cycles were analyzed by BrdU assay, and cyclin D1 expression was evaluated according to dose and duration of gonadotropin treatment.

RESULTS: BrdU assay showed a further inhibitory effect on murine uterine endometrial stromal cell proliferation when cultured with rFSH compared to uFSH, and a similar inhibitory proliferation profile when cultured with hCG at a specific range of concentrations. The expression of cyclin D1 of murine uterine endometrial stromal cells was down-regulated when cultured with rFSH, uFSH, or hCG, compared to control.

CONCLUSIONS: FSH may inhibit the proliferation of murine uterine endometrial stromal cells during *in vitro* culture. rFSH may have more significant inhibitory effects on the proliferation of endometrial stromal cells than uFSH. Establishing an optimal endocrine milieu is necessary using more advanced combination of female hormones for *in vitro* culture of this type of cells.

Keywords Uterus · Endometrial stromal cells · *In vitro* culture

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1 Introduction

Uterine endometrial stromal cells have a major role in embryonic implantation, fetal growth restriction, and pre-term birth [1]. *In vitro* culture of uterine endometrial stromal cells could afford a novel strategy of treatment for infertility of uterine or embryo receptivity cause, and be applied as an important platform for studies in gynecological field of tissue engineering and regenerative medicine [2, 3]. In particular, the effects of gonadotropins on endometrial stromal cells should be evaluated, because these medications are widely used in the procedures of assisted reproductive techniques such as *in vitro* fertilization (IVF) [4, 5].

Gonadotropins are the tropic glycoprotein hormones secreted from gonadotrope cells of anterior pituitary gland [5–7]. They act by binding to G-protein coupled receptors on gonads, ovaries in female [8]. Gonadotropins such as follicle stimulating hormone (FSH) and luteinizing hormone (LH) play critical roles in reproduction by regulating both gametogenesis and steroidogenesis. For inducing ovulation or recruiting oocytes used for assisted reproductive techniques, recombinant FSH (rFSH) and urinary FSH (uFSH) have widely been used to treat infertile female patients [9, 10]. Human chorionic gonadotropin (hCG) has also been clinically used to mimic endogenous LH surge [11, 12].

The presence of FSH receptors and hCG/LH receptors on uterine endometrial stromal cells has been demonstrated by previous reports [13–15]. A previous study implied that endometrial proliferation is affected by urinary gonadotropins [16, 17]. However, the effects of rFSH on endometrial stromal cell proliferation have not been fully evaluated, despite its wide use in clinical practice. Comparative analysis in regard to the effects of gonadotropins on the proliferation of endometrial stromal cells among rFSH, uFSH, and hCG treatment groups may provide useful knowledge for tissue engineering using *in vitro* culture of endometrial cells. This study attempted to define the proliferation profile of murine uterine endometrial stromal cells during *in vitro* culture with rFSH, uFSH, or hCG.

2 Materials and methods

2.1 Ethics

Entire experimental procedures were approved by Institutional Animal Care Unit Committee (IACUC) of Seoul National University Hospital [No. 16-0005-C1A0(1)]. Eight week-old C57BL/6 female mice were maintained in the animal facility and five mice per cage were housed in a room with a 12-h light and dark cycle and liberal access to food and water.

2.2 Evaluation of estrous cycle

To evaluate the estrous cycle, vaginal cells of each mouse were collected by gentle pipetting with PBS and replated onto slide glass and dried. Dried slides were then stained with 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO, USA). The stained images were observed under the inverted microscope (Tokyo, Japan) and the estrous cycle was determined based on cell type. Estrus cycle was determined by cornified epithelial cells.

2.3 Mouse endometrial cell preparation and culture (Fig. 1)

Uterine horns of female mice (C57BL/6) were excised using scissors after sacrificed. The excised horns were washed with DPBS (Invitrogen, Waltham, MA, USA) and chopped as $1 \times 1 \text{ cm}^2$ using surgical blades. The chopped uterine tissue pieces were collected and centrifuged. The pellets were digested with 5 mg/mL of type I collagenase (Invitrogen, Grand Island, NY, USA) for 1 h at 37 °C. Then, they were filtered through 70- μm cell strainer and the digested cells were blocked and centrifuged. The cells were re-suspended in culture medium and plated for incubation. After an initial culture for 30 min, the medium was changed to remove residual epithelial cells and further culture occurred in a fresh medium. The medium was consisted of DMEM/F12 phenol red-free (Invitrogen, Grand Island, NY, USA), supplemented with 10% FBS (Invitrogen, Grand Island, NY, USA), $1 \times$ Insulin-Transferrin-Selenium-G supplement (ITS, Invitrogen, Grand Island, NY, USA) and 50 IU/mL penicillin–streptomycin (Invitrogen, Grand Island, NY, USA).

2.4 Gonadotropin treatment

In vitro grown endometrial stromal cells were split at passage 2 using 0.25% trypsin–EDTA (Invitrogen, Grand Island, NY, USA) and re-plated as 1×10^4 density. Recombinant FSH (Merck-Serono, Darmstadt, Germany), urinary FSH (LG Chem, Ltd., Seoul, Korea) or hCG (IVF-C®, LG, Korea) at 15, 30, and 150 mIU/ml was treated to endometrial stromal cells for up to 72 h in 5% CO₂ environment. *in vitro* cultured cells were collected at 0-, 24-, 48- and 72-h after treatment for further analyses.

2.5 Immunocytochemical staining (BrdU)

Thirty mIU/ml of rFSH-, uFSH- and hCG-treated cells were fixed using 4% paraformaldehyde (PFA, Sigma-Aldrich) for 20 min at room temperature. After washing with PBS, samples were treated with 3% BSA (Bovine serum albumin, Sigma-Aldrich, St. Louis, MO, USA) solution for prevention of non-specific reaction for 12 h at 4 °C. And then, primary antibody, mouse anti-BrdU (Molecular Probes, Eugene, OR, USA) was treated as 1:100 concentration. After washing three times with PBST [PBS containing tween 20 (Sigma-Aldrich, St. Louis, MO, USA)], secondary antibody, goat anti-mouse IgG Alexa Fluor 488 (Invitrogen, Grand Island, NY, USA) was added to samples, and incubated for 1 h at room temperature. After washing three times with PBST, samples were mounted with anti-fade solution containing DAPI (Invitrogen, Grand Island, NY, USA) and images were captured

Fig. 1 Preparation of *in vitro* culture of murine uterine endometrial stromal cells.

A Uterine horn of 8-week-old mouse **B** isolation of murine endometrial stromal cells, *In vitro* culture of murine endometrial stromal cells [passage 1 (**C**) and 2 (**D**)]

using EVOS FL microscope (Thermo Fisher Scientific, Waltham, MA, USA)

2.6 Quantitative analysis using FACS (BrdU)

Cultured endometrial stromal cells were dissociated into single cells by treatment of 0.25% trypsin–EDTA for 45 min at 37 °C. Dissociated cells were washed and resuspended in 1x binding buffer. And then, samples were incubated with BrdU and propidium iodide for 30 min at room temperature. Cells were analyzed using FACS Calibur (BD Biosciences, San Jose, CA, USA).

2.7 Quantitative real-time polymerase chain reaction (qRT-PCR) for cyclin D1

Used primers for PCR reaction are listed as Table 1. Samples were collected and total RNAs were retrieved using TRIzol® (Invitrogen). For assessing cyclin D1, 50 ng of RNAs were reverse-transcribed for the synthesis of cDNA using an Accute RT PreMix (Bioneer, Daejeon, Korea). The amplification of specific genes was performed in RotorGene Q (Qiagen, Valencia, CA, USA) using QuantiTect SYBR green PCR kit (Qiagen, Germantown,

MD, USA). PCR reaction was performed under the following condition for 35 cycles: denatured at 95 °C and annealed at 60 °C. All the reactions were performed in triplicate and the Ct value was calculated based on GAPDH expression.

2.8 Statistical analysis

Data were expressed as means and standard deviations, and were compared using the Student's *t* test. A statistically significant difference was deemed with *P* < 0.05. Statistical Package for the Social Sciences for Windows (version 12.0, SPSS Inc., Chicago, IL, USA) was used for data analysis.

3 Results

3.1 *In vitro* culture of murine endometrial stromal cells

Isolated murine endometrial stromal cells were cultured for 2 passages (Fig. 1). To achieve comparable conditions using each gonadotropin, the cultured cells were plated on

Table 1 Primer sequences used for qRT-PCR

Gene	Sequences (5' → 3')	Sequences (3' → 5')
GAPDH	GAAGGTCGGTGTGAACGAAT	TTTGATGTTAGCGGGTCTC
CyclinD1	AGGCAGCGCGCGTCAGCAGCC	TCCATGGCGCGCCGCTCTGGG

Fig. 2 BrdU assay of murine uterine endometrial stromal cells cultured for 72 h with 30 mIU/ml of recombinant FSH, urinary FSH, or hCG treatment ($\times 200$, *rFSH* recombinant FSH, *uFSH* urinary FSH)

culture dishes at a 1×10^4 density. After 72-h-culture, BrdU assay of murine uterine endometrial stromal cells showed decreased expression of BrdU in rFSH-, uFSH- and hCG-treated cells compare to control (Fig. 2).

3.2 Proliferation of cultured murine endometrial stromal cell by analysis using FACS (BrdU)

After *in vitro* culture of murine endometrial stromal cells with rFSH treatment, the morphological comparison showed no significant differences (Fig. 3A). BrdU FACS analysis showed that the proliferation of murine endometrial stromal cells was decreased time-dependently both in control and all concentration of rFSH groups (Fig. 3B).

With uFSH treatment, cultured murine endometrial stromal cells showed no significantly different morphology. However, cells in control and all concentrations of uFSH groups showed decreased proliferation according to time (Fig. 4).

Culture with hCG treatment showed similar observational morphology in all concentrations compared to control. BrdU FACS analysis showed decreased cell proliferation at 72 h compared to basal, both in control and all concentrations of hCG groups (Fig. 5).

With increasing rFSH concentrations, the cell proliferation was decreased in a time- and dose-dependent manner (Fig. 6). The comparison of uFSH and hCG also showed time- and dose-dependently decreased proliferation (Figs. 7, 8). The pattern of decrease in proliferation was more significant in rFSH group compared to uFSH group.

3.3 Comparisons in expression of cyclin D1 according to concentration of gonadotropins

RT-PCR analysis showed decreased expression of cyclin D1 in murine endometrial stromal cells when cultured with rFSH compared to control (Fig. 9). The decreased expression of cyclin D1 in rFSH group showed a dose-dependent relationship. The expression of cyclin D1 in uFSH group was decreased, in a dose-dependent manner, compared to control (Fig. 10). The expression of cyclin D1 in 15 mIU/ml hCG group showed no significant differences up to 48 h compared to control (Fig. 11). However, the expression of cyclin D1 was decreased in cells treated with hCG at 72 h. The culture of 30 and 150 mIU/ml of hCG showed the decreased expression of cyclin D1 compared to control at all time points. In rFSH treatment groups, the relative expression of proliferation of cyclin D1 compared to control was lower than 0.5 after 24 h. In uFSH treatment groups, the relative expression of cyclin D1 was lower than 0.5 after 48 h. In 30 and 150 mIU/ml hCG treatment groups, the relative expression of proliferation of cyclin D1 was lower than 0.5 after 48 h.

4 Discussion

Uterus is an indispensable organ to support a pregnancy during reproductive period. For the treatment of intractable uterine factor infertility or subfertility, fine engineered uterine endometrial tissue should be offered to infertile couples [18–20]. A key component of uterus is endometrium whose stromal cells can be easily retrieved

Fig. 3 Proliferation of the murine endometrial stromal cells cultured *in vitro* with recombinant FSH treatment. **A** Morphological observation using inverted microscope ($\times 40$), **B** BrdU FACS analysis

and cultured *in vitro*. Thus, *in vitro* culture of uterine endometrial stromal cells may provide a novel treatment strategy for female infertility caused by uterine or embryo receptivity factor.

With the presence of their receptors on the endometrial cells, the effects of gonadotropins, used in assisted reproductive technique procedures, on endometrial stromal cells should be evaluated. rFSH, uFSH, and hCG are widely used gonadotropins for the treatment of female infertility [21–25]. This study aimed to evaluate the proliferation profile of murine uterine endometrial stromal cells when cultured *in vitro* with these gonadotropins.

Our data showed that morphological findings did not significantly differ among the *in vitro*-cultured cells according to treated gonadotropins. However, we observed significant decreased proliferation of murine uterine endometrial cells treated with rFSH during *in vitro* culture. A previous study showed that, when treated with uFSH, *in vitro*-cultured human endometrial cells showed decreased proliferative patterns similar to our study [16]. Our results suggest that rFSH may have a negative effect on the proliferation of endometrial stromal cells, as in the case of uFSH.

Fig. 4 Proliferation of the murine endometrial stromal cells cultured *in vitro* with urinary FSH treatment (BrdU FACS analysis)

Fig. 5 Proliferation of the murine endometrial stromal cells cultured *in vitro* with hCG (BrdU FACS analysis)

Another report on the effects of rFSH showed similar proliferative pattern with our results when human endometrial cells were cultured [26]. Intriguingly, our results showed the more significant effects in rFSH group compared to uFSH and hCG groups. BrdU FACS analysis

and cyclin D1 expression suggested more suppressed proliferative pattern in rFSH group compared to uFSH and hCG groups. A recent study reported the presence of FSH receptor on the membrane of human endometrial glands cells throughout the glandular epithelium, and suggested

Fig. 6 Relative proliferation of murine endometrial stromal cells cultured *in vitro* with recombinant FSH (BrdU FACS analysis)

Fig. 8 Relative proliferation of murine endometrial stromal cells cultured *in vitro* with hCG (BrdU FACS analysis)

Fig. 7 Relative proliferation of murine endometrial stromal cells cultured *in vitro* with urinary FSH (BrdU FACS analysis)

Fig. 9 Relative cyclin D1 expression of murine uterine endometrial stromal cells cultured *in vitro* with recombinant FSH (RT-PCR)

that this receptor could enhance the intracellular cAMP in a dose–response and time-course manner [27].

From our data, the endometrial stromal cells *in vitro*-cultured with hCG showed decreased proliferation at specific concentrations. FACS analysis and cyclin D1 expression suggested less decrease in proliferation in hCG treatment group compared to rFSH and uFSH groups. This finding was consistent with a previous report of human endometrial stromal cells [16].

Consistent with this present study, previous studies suggested that precocious or prolonged hCG exposure may detrimentally affect endometrial receptivity by abrogating extracellular signal-regulated kinase phosphorylation

resulting in changes of tight junction integrity [28–32]. Although exogenous hCG is clinically used for ovulation triggering mimicking LH surge, endogenous hCG is one of the major hormones that have a role in the maintenance of endometrial receptivity to embryos [33]. In this context, the difference in effects on endometrial cell proliferation can be expected to occur between FSH and hCG was expected.

Findings of our present study may imply that gonadotropins could have negative effects on the proliferative competence of endometrial cells when transplanted into the uterus of women undergoing infertility treatment. The use of gonadotropins is almost mandatory in infertile women, particularly during assisted reproductive technique

Fig. 10 Relative cyclin D1 expression of murine uterine endometrial stromal cells cultured *in vitro* with urinary FSH (RT-PCR)

Fig. 11 Relative cyclin D1 expression of murine uterine endometrial stromal cells cultured *in vitro* with hCG (RT-PCR)

procedures. Therefore, consideration of this possible negative impact on endometrial cells should be taken when strategy of endometrial cell transplantation is prepared to treat infertile women with decreased endometrial receptivity.

Cautious interpretation of our data should be executed since this present study has a few limitations. Firstly, we only tested FSH and hCG, however, *in vivo* condition may be more complex due to orchestrated action of multiple steroid and glycoprotein hormones with common structural moieties, which may cause cross reactions at their receptor-ligand binding. Secondly, uterus has other tissue layer than endometrium, and furthermore, the endometrium has

glandular tissue in addition to stromal cells. The *in vivo* phenomena may differ from our observed findings with these variations considered. Thirdly, during the embryonic implantation period, many signals are released from embryo or fetus, which may overcome the observed gonadotropins' effects on the endometrial stromal cells. Lastly, human endometrial stromal cells may differ from those of rodents. Some data discrepancies may occur between previous reports and ours.

In conclusion, FSH may inhibit the proliferation of murine uterine endometrial stromal cells during *in vitro* culture. rFSH may exert more significant inhibitory effects on the proliferation of these cells compared to uFSH. hCG also has an inhibitory effect on this cell type with relatively lower significance compared to FSH. Establishing an optimal endocrine milieu is necessary using more advanced combination of female hormones for *in vitro* culture of this type of cells.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interests.

Ethical statement All of the animal studies were performed after receiving an approval of the Institutional Animal Care and Use Committee of the Biomedical Research Institute at the Seoul National University Hospital (SNUH-IACUC No. 15-0032).

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