Biochemical and Biophysical Research Communications 446 (2014) 335–340

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Reconstruction of functional endometrium-like tissue *in vitro* and *in vivo* using cell sheet engineering

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ARTICLE INFO

Article history:
Received 19 February 2014
Available online 3 March 2014

Keywords:
Regeneration
Endometrium
Glandular structure
Hormonal receptors
Cell sheet

ABSTRACT

Uterus is a female specific reproductive organ and plays critical roles in allowing embryo to grow. Therefore, the endometrial disorders lead to female infertility. Hence, the regeneration of endometrium allowing fertilized ovum to implant might be valuable in the field of fertility treatment. Recently, cell sheet engineering using a temperature-responsive culture dish has advanced in regenerative medicine. With this technology, endometrial cells were harvested as a contiguous cell sheet by reducing temperature. Firstly, mouse endometrial cell sheets were re-cultured for 3 days to evaluate the function. Histological analyses revealed that endometrial epithelial cell-specific cytokeratin 18 and female-specific hormone receptors, estrogen receptor β and progesterone receptor, were expressed. Furthermore, endometrial epithelial cells constructed epithelial layer at the apical side. Then, endometrial cell sheets from green-fluorescent-protein rat cells were transplanted onto the buttock muscle of nude rat for evaluating the function *in vivo*. Histological analyses showed that endometrial cell sheets reconstructed endometrium-like tissue, which was found to form uterus-specific endometrial glands having hormonal receptor to estrogen. In this study, endometrial cell sheets were speculated to contribute to the regeneration of functional endometrium as a new therapy.

1. Introduction

In the developed countries, there is a social issue regarding low birth rate with increasing women's social advancement. At the same time, female infertility caused by uterine diseases is also an important problem with respect to declining birth rate. As a treatment for infertility, assisted reproductive technology, e.g. *in vitro* fertilization and early embryo manipulation, has been progressed well. In contrast, infertility treatments except this technology are poorly advanced.

The uterus plays essential roles in maintaining embryo throughout its development. Because the endometrial disorder leads to severe infertility, surgical treatment is required for pregnancy and improvement in the quality of life for women. However, endometrial defect by surgical treatment induces complete or partial intrauterine adhesion and Asherman's syndrome [1,2]. Therefore, the regeneration of endometrium needs to be facilitated.

Uterine tissue reconstruction is investigated with cell transplantation [3] and tissue engineering [4,5]. Human endometrial-side-population cells transplanted under kidney capsule in super-immunodeficient mice led to the organization of human endometrium [3]. Whereas with rabbit endometrial cells, endometrium-like tissue is reconstructed *in vitro* using biodegradable scaffold, type I collagen/Matrigel mixture [4,5]. Recently, cell sheet engineering with a temperature-responsive culture dish of which surface is covalently grafted with a temperature-responsive polymer, poly(N-isopropylacrylamide) (PIPAAm) for obtaining an intelligent culture surface has been proposed [6]. PIPAAm is hydrophobic in culture medium at 37 °C and becomes hydrophilic a temperature below a lower critical solution temperature of 32 °C. Therefore, confluent cells can detach themselves as a contiguous cell sheet by reducing temperature at 20 °C. Using this technology, cell sheets have been clinically applied to a number of organs and tissues, e.g. cornea [7], heart [8], esophagus [9].

This study aimed to reconstruct endometrial tissues *in vitro* and ectopically regenerate endometrium-like tissue *in vivo* using cell sheet engineering. This study was then designed to determine endometrium-specific functions by analyzing histology and mRNA expressions in endometrium-like tissue.

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http://dx.doi.org/10.1016/j.bbrc.2014.02.107
0006-291X/

2. Materials and methods

2.1. Animals

A total of 26 female C57BL/6 mice aged 4 weeks old (Japan SLC, Shizuoka, Japan) for the re-culture of endometrial cell sheets and a total of 20 SD-Tg(CAG-EGFP) rats [green-fluorescent-protein (GFP) rat] aged 4 weeks old (Japan SLC) for preparing endometrial cell sheets were used. A total of 3 female nude rats (F344/NJcl-rnu/rnu) aged 3 weeks old (CLEA Japan, Tokyo, Japan) were used for the transplantation of endometrial cell sheets. All animals were caged in an environmentally controlled room with alternating 12 h dark/light cycles and ad libitum access to food and water. All animal experiments were performed in accordance with the guidelines outlined by the institutional animal committee located at Tokyo Women's Medical University.

2.2. Isolation of endometrial cells

Uterine horns were resected from immature mice and rats, and were minced. Uterine horn segments were treated with 0.2% collagenase type I (Life technologies, Carlsbad, CA, USA) in Dulbecco's Modified Eagle's Medium and Ham's F-12 at a volume ratio of 1:1 (DMEM/F12) (Life technologies) at 37 °C for 1 h with continuous shaking. And then, DMEM/F12 containing 10% charcoal-stripped fetal bovine serum (FBS) (Life technologies), 1% penicillin–streptomycin solution (Life technologies), 10 mmol/L HEPES (Sigma–Aldrich, St. Louis, MO, USA), 1 nmol/L β-estradiol (Sigma–Aldrich), and 100 nmol/L progesterone (Sigma–Aldrich) used as culture medium were added in the equal volume of cell suspension. After filtration through a 100 and 40 μm cell strainer (BD Biosciences, Bedford, MA, USA), the cells were centrifuged at 250×*g* for 5 min at 4 °C. Viable cells were counted by trypan blue exclusion test and plated on a 35-mm temperature-responsive cell culture dish (UpCell®) (CellSeed, Tokyo, Japan) coated with FBS for 4–6 h at a density of 2.5×10^6 cells per dish. Isolated cells were cultured at 37 °C for 1 h, and then the medium was replaced with fresh culture medium for removing non-adherent cells.

2.3. Harvesting and re-culture of endometrial cell sheets

At 3 days after the culture of mouse endometrial cells, culture temperature was reduced to 20 °C for 30–60 min for inducing the detachment of endometrial cells. After detaching itself from the dish, an endometrial cell sheet was allowed to shrink significantly by cytoskeletal reorganization, re-cultured for constructing a thick tissue for 3 days, and then harvested. Re-cultured cell sheets were used for histological analyses.

2.4. Transplantation of harvested endometrial cell sheets

At 2 days after the culture of endometrial cells of GFP rats, confluent cells detached themselves as a contiguous cell sheet by reducing temperature.

Female nude rats were anesthetized with 1.5–2% isoflurane (Escain) (Mylan, Canonsburg, PA, USA). Longitudinal incision was made in the median dorsal skin. Buttock muscle was exteriorized by removing fascia and connective tissues. Harvested and shrunk endometrial cell sheets were washed with saline. A total of 3 cell sheets were transplanted onto exteriorized muscle using a supporting plate made of polyethylene terephthalate. After the transplantation of sheets, the transplanted area was covered with a 0.5-mm-thickness silicon sheet, and the dorsal skin was closed with 5-0 nylon sutures (Johnson and Johnson, New Brunswick, NJ, USA).

2.5. Resection of transplanted site

At 6 weeks after the transplantation of endometrial cell sheets, transplant sites were resected with observing the site by a fluorescent stereomicroscope (Leica Microsystems, Wetzlar, Germany). The resected tissues were cut in half. One half specimen was fixed and embedded for histological analyses as described below, and another was frozen by liquid nitrogen for gene expression analyses, and the frozen specimens were kept at −80 °C until mRNA extraction.

2.6. Histological analysis

Endometrial cell sheets, resected endometrium-like tissues, and normal uteri were fixed with 4% paraformaldehyde (Muto Pure Chemicals, Tokyo, Japan) for 30–60 min and then immersed into gradient solutions of 10%, 20%, and 30% sucrose (Wako Pure Chemical Industries, Osaka, Japan) in phosphate buffered saline (PBS) (Sigma–Aldrich) one by one for overnight at 4 °C. After being frozen with Tissue-Tek OCT compound (Sakura Finetek, Tokyo, Japan) and sliced into 5-μm sections, the specimens were stained with hematoxylin and eosin (H&E) by conventional methods.

2.7. Immunofluorescent staining

Mouse monoclonal antibodies against cytokeratin 18 (CK18) (1:100), vimentin (1:100), α-smooth muscle actin (α-SMA) (1:100), and progesterone receptor (PR) (1:100), and rabbit polyclonal antibody against estrogen receptor β (ERβ) (1:100) were obtained from Abcam (Cambridge, United Kingdom). Alexa Fluor 568 goat anti-mouse IgG antibody, Alexa Fluor 568 goat anti-rabbit IgG antibody, and Alexa Fluor 488 goat anti-mouse IgG antibody (Life technologies) were used as the secondary antibodies. Cell and section specimens were blocked with 10% normal goat serum in PBS containing 0.1% Tween 20 (Sigma–Aldrich) (PBST) (blocking solution) for 30 min at room temperature to prevent nonspecific binding, and then incubated overnight at 4 °C with the primary antibody diluted in blocking solution in a humidified chamber. After being washed with PBST, the specimens were incubated for 1 h at room temperature with the secondary antibody diluted in blocking solution in a humidified chamber. After being washed with PBST, the specimens were mounted with Prolong Gold antifade reagent with 4′, 6-diamidino-2-phenylindole (DAPI) (Life technologies) for nuclear stain.

2.8. Gene expression assay by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from each resected tissues using RNeasy Fibrous Tissue Mini Kit (QIAGEN, Hilden, Germany) in combination with DNase I for 15 min according to the instructions provided by the manufacturer. Synthesis of first strand cDNA was performed using SuperScript VILO Master Mix (Life technologies) as per the instructions provided by the manufacturer. The cDNA samples were subjected to real-time PCR using a StepOne Plus real-time PCR system (Life technologies) with TaqMan probes (Life technologies) as listed in Table 1. To determine the reference genes, 9 housekeeping genes (HKGs) were used as described previously [10]. The data of 9 HKGs were analyzed for evaluating and screening reference genes expression using RefFinder, a web-based comprehensive tool (http://www.leonxie.com/referencegene.php), including 4 statistical algorithms such as geNorm [11], NormFinder [12], BestKeeper [13] and comparative ΔCt method [14].

Table 1
Characteristics of target genes evaluated by the real-time reverse transcription-polymerase chain reaction.

Abbreviation	Gene name	Assay ID	Amplicon length (bp)
Esr2	Estrogen receptor 2 (ER beta)	Rn00562610_m1	89
Pgr	Progesterone receptor	Rn00575662_m1	75
Igf-1	Insulin-like growth factor 1	Rn00710306_m1	69
Wnt7a	Wingless-type mouse mammary tumor virus integration site family; member 7A	Rn01425352_m1	62
Wnt5a	Wingless-type mouse mammary tumor virus integration site family; member 5A	Rn01402000_m1	66
Foxa2	Forkhead box A2	Rn01415600_m1	65
Cdh1	Cadherin 1	Rn00580109_m1	105

2.9. Statistical analysis

All data were showed as mean ± SD. With the use of JMP software (Version 11.0, SAS Institute, Cary, NC, USA), an unpaired Student *t* test was performed to compare 2 groups.

3. Results

Uteri of immature female mice aged 4 weeks old are constituted of luminal epithelium, endometrial stroma, and myometrium as well as mature female uterus, and form a uterine cavity and endometrial glands (Fig. 1A). At 1 day after plating, endometrial cells were found to consist of CK18, vimentin, and α-SMA positive cells showing the characteristics of endometrial epithelial cells, endometrial stromal cells, and smooth muscle cells, respectively (Fig. 1B–D). The endometrial cells proliferated and became confluent at 3 days after culture (Fig. 1E). Confluent cells detached themselves as a contiguous cell sheet by reducing temperature (Fig. 1F). The endometrial cells in harvested and shrunk cell sheets showed a thick and cuboidal morphology (Fig. 1G). At 3 days after the re-culture, the surface area of the cell sheets became further smaller than before (Fig. 1H).

To evaluate the functions of endometrial cell sheets including endometrial reorganization and female specific hormonal response, the detached sheets were re-cultured onto a temperature-responsive cell culture dish. The thickness of re-cultured endometrial cell sheet increased from that of sheet before re-culture (Figs. 1G and 2A–C). In the normal uterus of nude rat, ERβ positive endometrial epithelial cells were formed along the lumen and the endometrial glands (Fig. 2D). At 3 days after re-culture, CK18 and ERβ positive endometrial epithelial cells were formed at the apical side of endometrial cell sheets, while PR positive cells were found in the cell sheet except the out-most surface, which had ERβ positive cells (Fig. 2B and C).

Endometrial cell sheets derived from immature GFP-rat uterus were transplanted onto exteriorized buttock muscle in female nude rats. At 6 weeks after transplantation, the endometrial cell sheets were confirmed to survive at the transplantation site (Fig. 3A). Uterus of recipient nude rat showed a normal uterine structure consisted of luminal epithelium, endometrial stroma and glands, myometrium, and uterine cavity (Fig. 3B). Histological examination of the resected tissues showed an endometrium-like structure, which consisted of the stroma, glands, and cyst surrounded with glandular epithelium (Fig. 3C). Immunohistochemical image of resected tissue

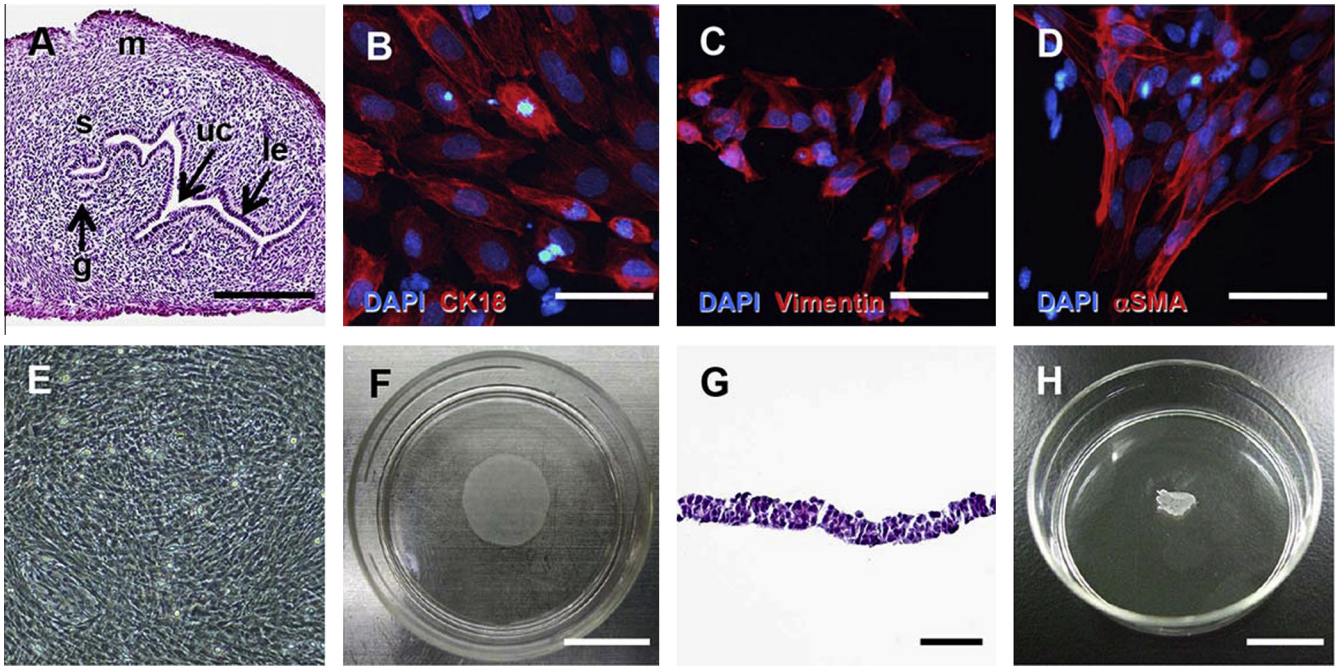

Fig. 1. Cultured endometrial cells, including endometrial epithelial and stromal cells, and myometrial cell. (A) The cross section of normal uterus of immature female mice was stained with hematoxylin and eosin (H&E). The endometrial cells cultured for 1 day were immunostained by antibodies against cytokeratin 18 (CK18) (B), vimentin (C), and α-smooth muscle actin (α-SMA) (D). Nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI). (E) The endometrial cells cultured for 3 days were observed to be in a confluent state by a phase-contrast microscope. (F) After the detachment of confluent cells by reducing culture temperature, shrinking endometrial cell sheet was harvested. (G) The cross section of single layer of endometrial cell was stained by H&E. (H) Single-layered sheet was detached at 3 days after re-culture. Mark "g" in the photographs indicates endometrial gland; le, luminal epithelium; m, myometrium; s, stroma; uc, uterine cavity. Scale bars indicate 200 μm in A, 50 μm in B–D, and G, and 10 mm in F and H.

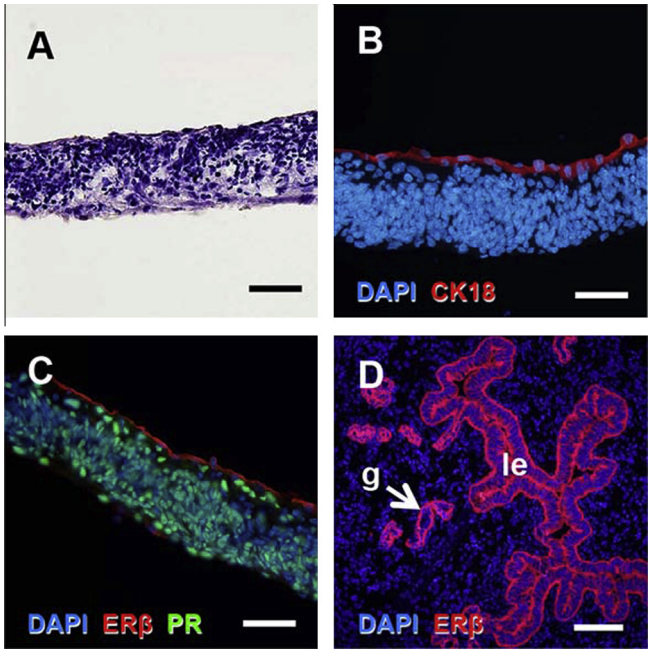

Fig. 2. Cross-sectional image of endometrial sheet at 3 days after the re-culture of single-layered sheet. (A) Endometrial cell sheet after re-culture was stained with hematoxylin and eosin. Typical immunofluorescent image of endometrial cell sheet after re-culture were stained with antibodies against cytokeratin 18 (CK18) (B) and the double staining of estrogen receptor β (ERβ) and progesterone receptor (PR) (C). Cross-sectional immunofluorescent image of normal uterus in mouse aged 8 weeks old was stained with ERβ (D). Nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI). Mark "g" in the photographs indicates endometrial gland; le, luminal epithelium. Scale bars indicate 50 μm in A–C, and 100 μm in D.

showed that the glandular epithelium and stroma were ERβ (Fig. 3D) and PR (Fig. 3E) positive respectively, similar to that of normal uterus (Fig. 3F).

The endometrium-specific gene expression levels of the endometrium-like tissues at 6 weeks after transplantation and normal recipient uterus were analyzed by quantitative RT-PCR. The most stable HKGs for this investigation were statistically determined by RefFinder. As a consequence of the analyses, ACTB was determined as the most appropriate reference gene (data not shown). The mRNA expression levels of Esr2, Pgr, Igf1, Wnt7a, Wnt5a, and Cdh1 normalized by ACTB showed no significant differences between the transplanted endometrium-like tissues and normal recipient uterus (Fig. 4).

4. Discussion

With the use of cell sheet engineering, this study established a preparing method of endometrial cell sheet that could maintain the function after re-culture *in vitro* and ectopically produce the endometrium. The endometrial cell sheet after re-culture was able to show their specific functions because of having the hormonal responses. The endometrial cell sheet was able to produce ectopically endometrium-like tissues forming uterus-specific endometrial glands. The functionalities were confirmed by observing endometrium-specific genes found in normal uterus.

Cell sheet engineering with PIPAAm-grafted temperature-responsive cell culture dishes allows the cells to be recovered as a contiguous cell sheet. The shape of cell in harvested cell sheet was cuboidal and comparable to that of cell *in vivo*. And the function of cuboidal cell is known to be higher than that of extended cell [15]. Furthermore, multilayered sheet cultured *in vitro* has showed that the function is higher than monolayer sheet [16]. In this study, re-cultured mouse endometrial cell sheets for 3 days

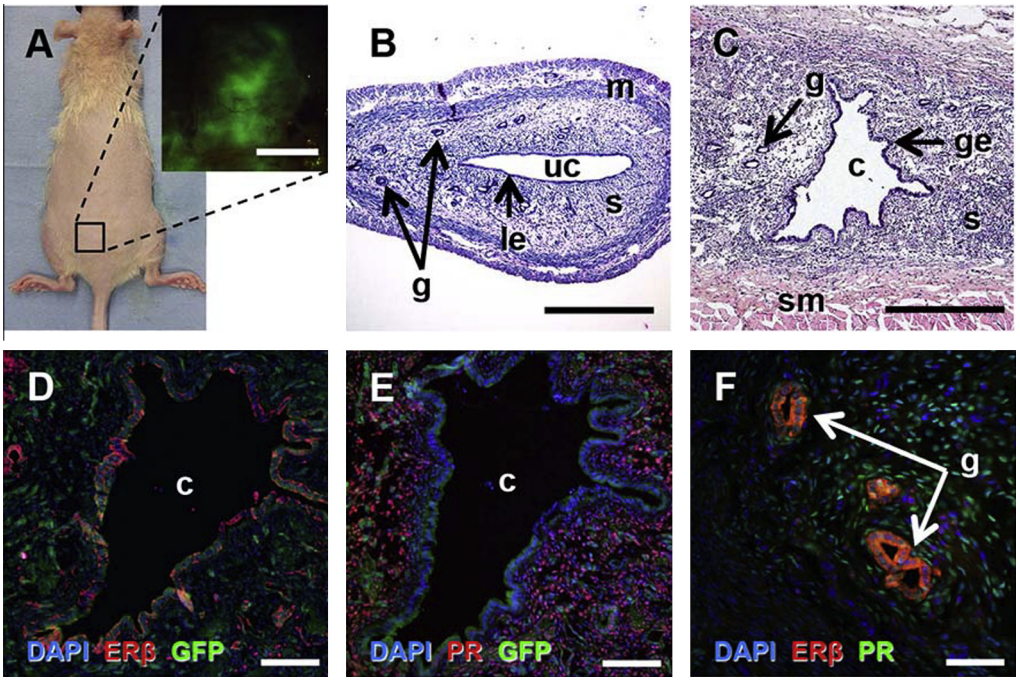

Fig. 3. Microscopic images of green-fluorescent-protein (GFP) rat-derived endometrial cell sheet transplanted onto the buttock muscle and normal uterus of recipient nude rat. (A) GFP positive endometrium-like tissue was reconstructed at the transplant site at 6 weeks after transplantation. Histological findings of normal uterus (B) and endometrium-like tissue reconstructed at the transplant site (C) by hematoxylin and eosin staining. Immunohistochemical images of horizontal section of endometrium-like tissue were stained with estrogen receptor β (ERβ) (D) and progesterone receptor (PR) (E). (F) Immunofluorescent images of cross section of endometrium-like tissue were stained with antibodies against ERβ and PR. Nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI). Mark "c" in the photographs indicates cyst; g, endometrial gland; ge, glandular epithelium; m, myometrium; s, stroma; sm, skeletal muscle; uc, uterine cavity. Scale bars indicate 5 mm in A, 500 μm in B and C, and 100 μm in D–F.

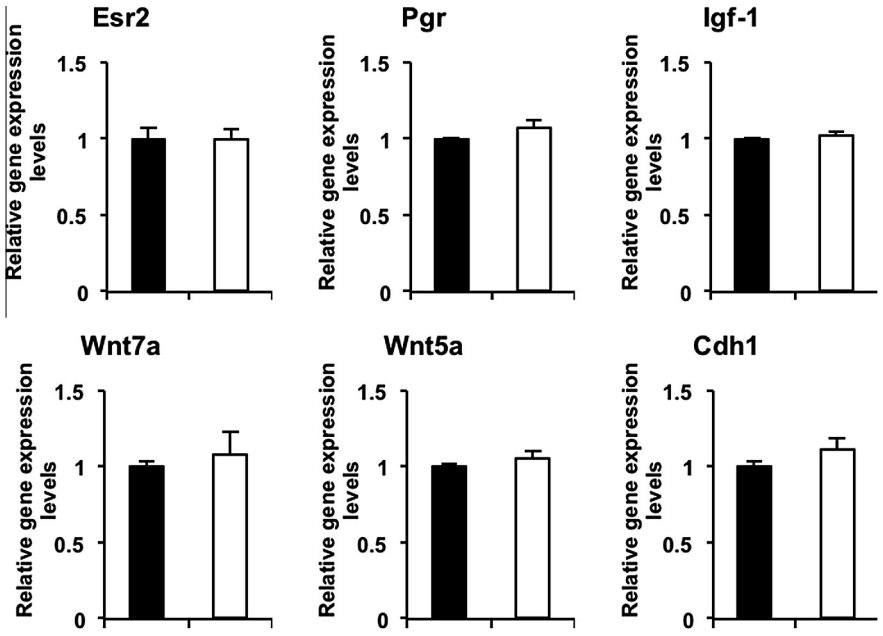

Fig. 4. Expression levels of the target genes of the normal uteri from recipient nude rat and endometrium-like tissues reconstructed at the transplant site. The abbreviations of target genes are given in Table 1. The mRNA expression levels of target genes were determined by quantitative real-time reverse transcription-polymerase chain reaction. The data were given as relative ratios to those of the normal uterus. The closed and the open bars show the expression levels of normal uteri and endometrium-like tissues, respectively. The numbers of specimens for both groups were 3.

in vitro formed a three-dimensional (3D) structure by longitudinal proliferation and consequently became a thick endometrium-like tissue formed CK18 and ERβ positive epithelial layer at the apical surface. Because re-cultured endometrial cell sheets showed an endometrium-specific hormonal response to estrogen and progesterone, the endometrial cell sheets were found to maintain uterine functions in culture.

Uterine regeneration plays an important role in treating endometrial defect, e.g. intrauterine adhesion and Asherman's syndrome, resulted in infertility. The human endometrial mesenchymal stem-like cells are investigated to evaluate the stem cell properties and the endometrial reconstruction *in vivo*. After the transplantation of the cells under the kidney capsule of super-immunodeficient mice, endometrium-like tissues including endometrial glands are established [3]. Although regenerative therapy using cell transplantation is clinically performed, cell transplantation has serious issues for lower level of survival rate because injected cells almost die and only a few cells survive. Whereas endometrial epithelial and stromal cells isolated from rabbit uterus mixed with collagen/Matrigel scaffold are cultured after stack in layers, and consequently endometrium-like tissue was reconstructed *in vitro* [4,5]. Although scaffold-based tissue engineering has used biodegradable scaffolds, these scaffolds sometimes lead to an inflammatory response *in vivo*. In recent cell sheet engineering, the transplantation of multilayered myoblast sheet is found to be more effective in treating the infarcted myocardium [17]. Moreover, thyroid cell sheets are found to recover their function as an endocrine gland, because thyroid cell sheet can make a 3D structure *in vivo* [18]. In this study, the transplanted cell sheets became endometrium-like tissues having a 3D structure, which was found in native endometrium, and a cytokine secreting function, because transplanted endometrial cell sheets gave a cell interacting and crowding environment. As a result, endometrium-like tissue showed a hormonal sensitivity and formed endometrial glands.

The uterine development is facilitated by various growth factors and their receptors through the use of autocrine and paracrine pathways. Interaction between epithelium and stroma plays critical roles for uterine development [19]. Insulin-like growth factor I (IGF-I) is related to the development of rat uterus, and has a critical role in developing uterus in perinatal period [20]. Whereas, wingless-type mouse mammary tumor virus integration site family member (Wnt) 7a, Wnt5a, and cadherin 1 (Cdh1) included in the Wnt signaling pathway are related to the development of endometrial glands [21–24]. The endometrial glands produce two essential proteins, (1) leukemia inhibitory factor, which significantly affects the implantation and invasion of blastocyst and stromal decidualization [25], and (2) calcitonin, which greatly influences the implantation during early pregnancy [26]. These results indicate that the construction of functional endometrium is essential to form not only intrauterine cavity but also endometrial glands. In this study, analyses of mRNA expression levels showed that the reconstructed endometrium-like tissue retained uterus-specific functions comparable with normal uterus of recipient nude rat. Therefore, the endometrium-like tissues were ectopically constructed, and numerous endometrial glands were formed in the tissues. Moreover, this study revealed that endometrial glands had a secretory function by cyst formation, because secreted fluid was found to accumulate in a glandular structure. Hence, the regenerated endometrium was speculated to partially retain an important function for successful pregnancy.

Intrauterine adhesion and Asherman's syndrome showing complete or partial obliteration is caused by curettage due to abortion and miscarriage, or endometrial ablation, and results in amenorrhea and infertility [1,2]. Treatment for preventing the recurrence of intrauterine adhesion after hysteroscopic adhesiolysis uses an intrauterine contraceptive device or intrauterine catheters to maintain the uterine cavity, while hormonal therapy using the high doses of estrogen is performed to promote the endometrial regeneration [2]. As a measure to enhance the regeneration of functional endometrium, cell-sheet transplantation could be a promising candidate. For obtaining the measure, the confluent cells can easily harvest as a contiguous cell sheet, and then conveniently transplant to the target region. Hence, the endometrial cell sheet might completely be able to transplant at the defect site. To regenerate

endometrial cell sheet quickly *in vivo*, estradiol administration is effective approach. Therefore, the endometrial cell sheet might serve as a valuable therapy for preventing intrauterine adhesion and Asherman's syndrome.

In conclusion, this study demonstrated that endometrial cell sheets were able to maintain the function after re-culture. Furthermore, endometrium-like tissues after transplantation were found to form endometrial glands functioning as a secretory gland. Hence, the regeneration of functional endometrium using cell sheet engineering speculates to serve as a new therapy for endometrial defect.

Conflict of interest

Teruo Okano is a director of the board of CellSeed Inc. Masayuki Yamato and Tatsuya Shimizu are scientific consultants for CellSeed Inc. These 3 authors are the stakeholders of the company and are inventors of cell-sheet-related patents. Tokyo Women's Medical University is receiving research fund from CellSeed Inc.

Acknowledgments

The authors thank Dr. Norio Ueno for English editing of the manuscript. This study was supported by Creation of innovation centers for advanced interdisciplinary research areas Program in the Project for Developing Innovation Systems "Cell Sheet Tissue Engineering Center (CSTEC)" from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

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