Regeneration of Uterine Horns in Rats Using Collagen Scaffolds Loaded with Human Embryonic Stem Cell-Derived Endometrium-Like Cells

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A variety of diseases may lead to hysterectomies or uterine injuries, which may form a scar and lead to infertility. Due to the limitation of native materials, there are a few effective methods to treat such damages. Tissue engineering combines cell and molecular biology with materials and mechanical engineering to replace or repair damaged organs and tissues. The use of human embryonic stem cells (hESCs) as a donor cell source for the replacement therapy will require the development of simple and reliable cell differentiation protocols. This study aimed at efficiently generating endometrium-like cells from the hESCs and at using these cells with collagen scaffold to repair uterine damage. The hESCs were induced by co-culturing with endometrial stromal cells, and simultaneously added cytokines: epidermal growth factor (EGF), platelet-derived growth factor-b (PDGF-b), and E2. Expression of cell specific markers was analyzed by immunofluorescence and reverse transcription-polymerase chain reaction to monitor the progression toward an endometrium-like cell fate. After differentiation, the majority of cells (>80%) were positive for cytokeratin-7, and the expression of key transcription factors related to endometrial development, such as *Wnt4*, *Wnt7a*, *Wnt5a*, *Hoxa11*, and factors associated with endometrial epithelial cell function: *Hoxa10*, *Intergrin β 3*, *LIF*, *ER*, and *PR* were also detected. Then, we established the uterine full-thickness-injury rat models to test cell function *in vivo*. hESC-derived cells were dropped onto collagen scaffolds and transplanted into the animal model. Twelve weeks after transplantation, we discovered that the hESC-derived cells could survive and recover the structure and function of uterine horns in a rat model of severe uterine damage. The experimental system presented here provides a reliable protocol to produce endometrium-like cells from hESCs. Our results encourage the use of hESCs in cell-replacement therapy for severe uterine damage in future.

Introduction

THE UTERUS IS A MAJOR FEMALE reproductive organ lined by the human endometrium. An excellent intra-uterine environment is essential for embryo implantation and pregnancy maintenance. The current advances in assisted reproductive technology have made it possible to overcome many causes of male and female infertility.¹ However, pregnancy is still notably difficult for patients with severe intrauterine adhesions (IUA), which is a possible complication of therapeutic procedures on the uterus.^{2,3} In fact, hysteroscopy identifies IUA in 11% of patients with repeated failure of assisted reproductive treatment.² Recovery of fecundity and repair of the endometrium are possible treatments for patients suffering from uterine factor infertility.

Severe IUA present the most difficult to treat. Even after hysteroscopic adhesiolysis, ~50% of these patients will become IUA again.^{4,5} In addition, in humans, no successful uterine transplantation longer than 18 months has been reported.^{6,7} New technologies to overcome these difficulties are in great demand. Our previous study demonstrated that the collagen scaffold extracted from bovine skin was appropriate for the rat uterine full-thickness-injury model and could improve the regenerative capability of the endometrium and myometrium⁸; however, due to a lack of seed cells, there were an insufficient number of glands and blood vessels, and it was difficult to maintain pregnancy. Stem cell therapy holds tremendous promise for repair and/or regeneration of damaged tissue.^{9,10} Human embryonic stem cells (hESCs) can proliferate indefinitely and are able to differentiate into cell types of all three germ layers both *in vivo*

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and *in vitro*; therefore, hESCs are a candidate for cell-replacement therapy.^{11,12}

In this study, we obtained the endometrium-like cells that were differentiated from hESC line, NJGLhES1, by co-culturing with endometrial stromal cells. Furthermore, we transplanted these hESC-derived cells along with collagen scaffolds into uterine full-thickness-injury rat models and performed an *in vivo* functional assessment.

Materials and Methods

Cell culture and differentiation

Culture of hESCs. The hESC line, NJGLhES1 (a cell line derived from preimplantation human blastocyst at the Reproductive Medical Center, Drum Tower Hospital, Nanjing University, passages 15–29),¹³ was cultured on a feeder layer of mitotically inactivated mouse embryonic fibroblasts (MEF) at 37°C with a daily medium change containing 80% knock-out™ Dulbecco's modified Eagle's medium (DMEM; Gibco), 20% knock-out Serum Replacer (Gibco), 4 ng/mL bFGF (Peprotech), 0.1 mM β -mercaptoethanol (Gibco), 2 mM glutamine (Gibco), 1% nonessential amino-acid stock (Gibco), and 50 IU/mL penicillin and streptomycin (Sigma). To maintain undifferentiated hESCs, the cultures were passaged once per week by mechanically dissecting and transferring the hESC colonies onto a freshly prepared MEF feeder.

Isolation of human endometrial stromal cells. Human endometrial stromal cells were isolated from normal endometrium in the early proliferative phase of normal cycling women by endometrial biopsy at the time of diagnostic curettage before *in vitro* fertilization and embryo transfer. This study was approved by the Drum Tower Hospital Research and Ethics Committee, and patient consent was obtained before biopsy. As previously described,¹⁴ endometrial tissues were minced and enzymatically digested with 0.1% collagenase I (Gibco) in DMEM/F12 (Gibco), at 37°C for 1 h. Stromal cells were separated from intact glands by filtration of the digested tissue through a 40 μ m gauze. After centrifugation at 400 *g* for 5 min, the pellet was resuspended in DMEM/F12 and 10% heat-inactivated fetal bovine serum (FBS; Gibco). The endometrial stromal cells were then maintained in DMEM/F12 and supplemented with 10% FBS and 50 IU/mL–50 μ g/mL penicillin–streptomycin. The purity of the cultured stromal cells was greater than 95%, as determined by immunofluorescence staining with polyclonal antibodies against vimentin (Santa Cruz Biotechnology). The cells were used between passages 2 and 5.

Differentiation of endometrium-like cells. Endometrial differentiation of hESCs was induced by means of contact-independent co-culturing with human endometrial stromal cells. Undifferentiated hESC colonies were detached from MEF feeders by mechanical dissociation into small clusters. Then, in the co-culture system (cytokines/stromal cells group and stromal cells group), hESC clusters were grown on the bottom of a six-well plate in 2.5 mL of DMEM/F-12 with 10% FBS, and 1×10^5 human endometrial stromal cells were seeded on the 0.4- μ m polyester membrane of a transwell insert (Corning) in 1.5 mL of the same medium. The medium was changed every 2–3 days, and in the cytokines/stromal cells group the following factors were added to both

hESCs and stromal cells: 10 ng/mL of epidermal growth factor (EGF; Peprotech), 10 ng/mL of PDGE-BB (Peprotech), and 1×10^{-7} M of E2 (Sigma). In the cytokine group, cells were treated with cytokines alone. Every week, hESC-derived cells were dissociated with 0.25% trypsin and 0.27 mM EDTA (Gibco) in phosphate-buffered saline (PBS; Gibco) at 37°C for 5–10 min and plated onto a new six-well plate. The transwell insert with new human endometrial stromal cells was used. The cells were cultured in this manner for 4–5 weeks.

Reverse transcription and quantitative polymerase chain reaction analysis

Total RNA was extracted from cultured cells using Trizol (Invitrogen). First-stand cDNA was generated with a Superscript first-stand synthesis kit (Invitrogen). The primers, product lengths, and annealing temperatures are shown in Table 1. Quantitative polymerase chain reaction (qPCR) was performed using an Applied Biosystems 7500 Fast Real-Time PCR Detection System (Applied Biosystems). Triplicate wells were used for each gene. A total volume of 20 μ L per well containing 10 μ L of $2 \times$ Power SYBR Green PCR Master Mix (Applied Biosystems), 2 μ L of cDNA, and gene-specific primers was used. The cycling parameters for the qPCR were as follows: an initial denaturation of 95°C for 30 s followed by 40 cycles of 5 s at 95°C and 34 s at 60°C. To normalize template input, *18S* was used as an endogenous control and the transcript level was measured for each plate. The relative expression of the gene was then evaluated using 7500 Fast System Sequence Detection Software, Version 1.4. The value obtained for Ct represents the number of PCR cycles at which an increase in the fluorescence signal can be detected above background and the increase is exponential for the particular gene. Data were expressed as fold change relative to untreated controls after normalizing to *18S*.

Transplantation and functional studies

The collagen membranes (Zhenghai Biotechnology) were freeze-dried collagen extracts from bovine skin that had been sterilized by 12 kGy Co60 irradiation. The samples were cut into 1.5 $\times$ 0.5 cm pieces. hESC-derived cells were isolated and resuspended in new media at a concentration of $\sim 10^5$ cells/ μ L. A total of 30 μ L of the cell suspensions were dropped onto one collagen membrane (1.5 $\times$ 0.5 cm) and incubated at 37°C for 15 min before use (Fig. 3A).

The Administrative Committee on Animal Research in Nanjing Drum Tower Hospital approved all animal experiment protocols. Animals were housed and treated according to the National Institutes of Health guidelines. A total of 132 uterine horns of 66 adult Sprague–Dawley female rats (200–250 g) were randomly assigned to four groups as follows: collagen ($n=35$ uterine horns), cells/collagen ($n=35$ uterine horns), natural regeneration ($n=35$ uterine horns), and sham-operated ($n=27$ uterine horns) groups (Table 2). After the rats were anesthetized with ketamine and diazepam (50 mg/kg, *i.p.*, respectively), the uterine horns were exposed using an abdominal midline incision. A segment of ~ 1.5 cm in length and 1/2–2/3 of the total circumference was excised from one horn of the uterus, and the mesometrium was retained. The collagen scaffolds (the collagen group) or scaffolds with cells (the cells/collagen group) were sutured in place with a 7/0

TABLE 1. PRIMER SEQUENCES AND PRODUCT LENGTHS FOR REVERSE TRANSCRIPTION-QUANTITATIVE POLYMERASE CHAIN REACTION

Gene	Product lengths (bp)	Primer sequence	Annealing temperature (°C)
<i>Wnt4</i>	360	5'-GCTGGAAGTCTCCACACTCG-3' 5'-CCCAGCATGTGTGTCAGGATGG-3'	60
<i>Wnt7a</i>	438	5'-GCCGTTACGTGGAGCCTGTGCGTGC-3' 5'-AGCACCTGCCAGGGAGCCCGCAGCT-3'	60
<i>Wnt5a</i>	273	5'-CTTCGCCCAGGTTGTAATTGAAGC-3' 5'-CTGCCAAAAACAGAGGTGTTATCC-3'	60
<i>Hoxa11</i>	304	5'-GATTTCTCCAGCCTCCCTTC-3' 5'-AGAAATTGGACGAGACTGCG-3'	60
<i>ER-α</i>	264	5'-TGCCAAGGAGACTCGCTA-3' 5'-TCAACATTCTCCCTCCTC-3'	60
<i>PR</i>	217	5'-ACACAAAACCTGACACCTCC-3' 5'-TACAGCATCTGCCCACTGAC-3'	60
<i>Hoxa10</i>	211	5'-GCCCTTCCGAGAGCAGCAAAG-3' 5'-AGGTGGACGCTGCGGCTAATCTCTA-3'	60
<i>Intergrinβ3</i>	231	5'-GACAAGGGCTCTGGAGACAG-3' 5'-ACTGGTGAGCTTTCGCATCT-3'	60
<i>LIF</i>	314	5'-GCAGATCATCGCCGTGTT-3' 5'-CAAGTAGAGGCAGAAAGTCCAG-3'	60
<i>18S</i>	174	5'-CGGCTACCACATCCAAGGAA-3' 5'-CTGGAATTACCGCGGCT-3'	60

prolene suture to replace the excised segment (Fig. 3B). In the natural regeneration group, after excision and complete hemostasis, marked defects were left open for natural healing. In the sham-operated group, after exposure by an abdominal midline incision, the uterine horns were left intact in the abdominal cavity without excision. All animals received an intramuscular injection of penicillin (80,000 U/100 mg) for 3 days after the surgery. In addition, to prevent immune rejection, injections of cyclosporine A (10 mg/kg i.p.) were given to the animals daily starting at 48 h before grafting and continuing for 5 days. Afterward, the animals drank a solution of the medicine (0.1 mg/mL) until death.

The rats were anesthetized at 4 and 12 weeks after transplantation. The patency of uterine horns was tested with methylene blue perturbation via fimbrial opening. Then, the entire uterus was removed and subjected to hematoxylin-eosin (H&E) and immunostaining assay as described next. Uterine function was assessed by determining whether a fertilized embryo was able to implant into the regenerative uterine horn and whether the embryo was supported. Animals were mated with male Sprague-Dawley rats at 12 weeks postoperatively. Fertilization was confirmed by the presence of a vaginal plug. The rat gestation period is 19–22 days. The animals were euthanatized at 15 to 19 days after the presence of the vaginal plug, and the uterine horns were examined for the number and position of embryos.

Immunostaining on cultured cells and uterus slices

Cultured cells were fixed in dishes with 4% paraformaldehyde in PBS for 30 min at room temperature, while the entire uterus was fixed overnight. The rat uterine tissues were then routinely dehydrated in a series of alcohol gradients (70–100%), embedded in paraffin, and sectioned at 5 μ m. H&E staining was applied to observe the tissue structure. Specific primary antibodies were used as follows: mouse anti-cytokeratin-7 (CK7, 1:100; Santa Cruz Biotechnology), rabbit or mouse anti-vimentin (1:100), and mouse anti-human nuclei (HN, 1:100; Chemicon). For visualization, appropriate fluorescence-tagged secondary antibodies (1:100; Zhongshan Biotechnology Company) were used against the primary antibodies. Fluorescent-conjugated secondary antibodies were detected using fluorescence microscopy (AXIO Observer.A1). The immunostaining procedures were performed according to the manufacturer's instructions. Cell nuclei were stained with 5 ng/mL of 4', 6'-diamidino-2-phenylindole (DAPI; Sigma). Negative controls were set using PBS instead of the primary or secondary antibodies.

Statistical analysis

Statistical analyses were performed using the SPSS statistical package (SYSTAT Software, Inc.). The differences were

TABLE 2. GROUPING OF UTERINE HORNS^a

Variable	Sham-operated group (n = 27)	Natural regeneration group (n = 35)	Collagen group (n = 35)	Cells/Collagen group (n = 35)
4 week	8	10	10	10
12 week	10	10	10	10
Pregnancy test	9	15	15	15

^an indicates the number of uterine horns.

evaluated using the Chi-square test and Student's *t*-test. A *p*-value <0.05 was considered statistically significant.

Results

Morphology and cell markers of endometrium-like cells derived from hESCs

Differentiation of hESCs into endometrium-like cells. Till the time of this study, the hESC line NJGLHES1 had been maintained exclusively by manual microdissection of individual undifferentiated colonies with the morphological characteristics of hESC, a normal karyotype, positive expression of alkaline phosphatase and specific cell marker genes, and the potential of forming embryoids and teratomas (Fig. 1A). Monolayer stromal cells from the endometrium were got, and more than 95% of the cells in the cultures were stromal cells, which were characterized by a spindle shape, positive vimentin staining, and a negative cytokeratin-7 immunohistochemical reaction (Fig. 1B–C).

To prevent contamination of hESCs co-cultured with endometrial stromal cells, the stromal cells were cultured in hanging baskets. This system permitted soluble factors released from the stromal cells to influence the hESCs. Under differentiation conditions, the shape of the hESC colonies changed to multilayered clusters of an increasing number of small and flattened cells. After 12 days, the colonies were isolated and further differentiated with new stromal cells. Cell passaging resulted in considerable cell loss; less than 40% of the total cells were viable 1 day after the passage. However, surviving cells had high proliferation activity and

achieved 70–90% confluency after 4 days in culture. After 4–5 passages, the differentiated cells were uniform with a small and flat cell morphology and an endometrial cell appearance (Fig. 1D).

Characteristics of endometrium-like cells differentiated from hESCs. Immunohistochemical analysis was performed at 5 weeks after differentiation. In the cytokines/stromal cells group, more than 80% of the cells expressed cytokeratin-7, and ~15% of the cells were positive for vimentin (Fig. 1E, F), suggesting that although most of the cells were epithelial cells, stromal cells were also present. In the stromal cells group, we obtained a similar proportion of cytokeratin-7 positive cells (~70%) (Fig. 1G–I). However, the cell proliferation ability was lower than that in the cytokines/stromal cells group. In the cytokine group, only about 35% of the cells were cytokeratin-7 positive cells (Fig. 1J–L).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed to further determine the cell characteristics. *Wnt4* prompts the early development of paramesonephric duct, and *Wnt7a* is involved in the further development of it. *Wnt5a* and *Hoxa11* expression suggest the genitalia and endometrial gland development.^{15,16} We also detected the gene associated with endometrial epithelial cell function. *PR* and *ER* suggest that the cells can be regulated by estrogen and progesterone hormone. *Hoxa10*, *Intergrinβ3*, and *LIF* are essential for embryo implantation and decidualization, and prompt endometrium receptivity.¹⁷ The analyses revealed that the expression of markers characteristic of endometrial development was induced during

FIG. 1. *In vitro* differentiation of human embryonic stem cells (hESCs) into endometrium-like cells. (A) Phase morphology of NJGLHES1 hESCs. (B) Morphology of human endometrial stromal cells. The cells were isolated from normal endometrial tissues. (C) More than 95% of the cells were stromal cells, which were characterized by a spindle shape, positive vimentin staining, and a negative cytokeratin-7 immunohistochemical reaction. (D–F) The hESC-derived cells in the cytokines/stromal cells group at the end of differentiation. (D) Morphology of hESC-derived endometrium-like cells. The differentiated cells were uniform with the small and flat cell morphology of an endometrial cell. (E) A high percentage (more than 80%) of the cells was positive for cytokeratin-7. (F) Some cells (~15%) were vimentin positive. (G, J) Representative images of the hESC-derived cells in the stromal cells group (G) and cells in the cytokine group (J). (H, K) CK7+ cells in the stromal cells group (H) and in the cytokine group (K). (I, L) Vimentin+ cells in the stromal cells group (I) and in the cytokine group (L). DAPI nuclear staining of the same field. Scale bars, 100 μm. DAPI, 4', 6'-diamidino-2-phenylindole. Color images available online at www.liebertpub.com/tea

FIG. 2. Expression profiles of hESC-derived cells after differentiation. Quantitative real-time-polymerase chain reaction was used to compare the mRNA expression pattern on stromal cell, stromal cell with cytokine, or cytokine treatment alone. Relative gene expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method. When the stromal cells existed (the cytokines/stromal cells group and the stromal cells group), the transcription factors *Wnt4*, *Wnt7a*, *Wnt5a*, *Hoxa11*, *Hoxa10*, *Intergrinβ3*, *LIF*, *ER*, and *PR* were expressed at higher levels. Color images available online at www.liebertpub.com/tea

in vitro differentiation. To look for evidence of endometrial stromal cell induction of endometrium-like cells, we compared the mRNA expression pattern on stromal cell, stromal cell with cytokine, or cytokine treatment alone using qPCR. The qPCR analysis showed that when stromal cells existed (the cytokines/stromal cells group and the stromal cells group), the transcription factors *Wnt4*, *Wnt7a*, *Wnt5a*, *Hoxa11*, *Hoxa10*, *Intergrinβ3*, *LIF*, and *ER* were expressed at higher levels, and in the cytokines/stromal cells group the gene expression levels for *PR* were increased by ~747.9-fold when compared with the untreated group (Fig. 2). These findings suggested that the endometrium-like cells were derived from hESCs, and they not only had the phenotype of endometrial cells but also had the endometrial function. The stromal cells play a key role in this process, which cannot be replaced by cytokines.

In vivo transplantation of endometrium-like cells derived from hESCs

Postoperative gross examination. Four weeks post-surgery, all rats had slight scar formation at the operative regions with the exception of the rats in the sham-operated group. However, in the natural regeneration group, the uteruses of a few animals exhibited extensive scar formation with the surrounding organs. In addition, some animals developed distal hydrometra (Fig. 3C–E). The patency rate of the cells/collagen group, collagen group, and sham-operated group were 90%, 80%, and 100%, respectively; these patency rates were much higher than in the natural regeneration group (50%). Twelve weeks postsurgery, the patency rate of each group had not changed much, and the rate of the natural regeneration group was still lower than in the other groups (Table 3).

Immunohistochemical staining. Anti-human nucleus antigen was used to demonstrate the presence of human cells in the uterine horn. The immunostaining analysis revealed human cells in all uteruses transplanted with hESC-derived endometrium-like cells at 4 and 12 weeks after grafting. These cells were able to form glandular structures (Fig. 3F); however, the number of HN-positive cells was limited.

Histological analysis. The histological structures observed using H&E staining of the regenerative uteruses are shown in Figure 4. In the sections, we can observe the thickness of endometrium and myometrium, the collagen remains (if it exists), and vascular and glandular structures. Four weeks after surgery, the cells/collagen group exhibited faster degradation and better integration into adjacent tissue than did the collagen group. Sections of all experimental groups were lined by epithelial cells that were similar to the normal uterus. In addition, endometrial glands were also observed in these groups. However, the thickness of the regenerative uterine wall in the cells/collagen group was greater than in the other two groups. Twelve weeks after grafting, the collagen membrane of both scaffold groups was completely degraded. In comparison to the collagen and natural regeneration groups, the cells/collagen group had an obviously thicker wall and more endometrial glands in the scarred area; moreover, there was more apparent neo-vascularization and better organized tissue structure in this group. No teratoma formation was observed.

Uterine function after transplantation. The effect of the grafted cells on uterine function recovery was evaluated for 12 weeks after transplantation. As shown in Figure 3G–I, pregnancy can be maintained till a late stage (19 days after the presence of the vaginal plug) in all groups. However, the pregnancy rate of the cells/collagen group (80.0%) was much higher than the rate of the natural regeneration (26.7%) and collagen groups (33.3%) and similar to the rate in the sham-operated group (100%). The average number of gestational sacs in each uterine horn (the total number of gestational sacs/the number of pregnancy uterine) for each group was as follows: 5.4 (49/9) in the sham-operated group, 2 (8/4) in the natural regeneration group, 2.8 (14/5) in the collagen group, and 4.2 (50/12) in the cells/collagen group. Most embryos were implanted in normal areas rather than in grafted tissue; however, in the cells/collagen group, embryos could be found in both the normal and scarred areas, with a proportion of 8/15 uterine horns (Table 4). These findings suggest that uterine functional recovery of the cells/collagen group is better than that of the collagen group.

FIG. 3. Transplantation experiment. (A) hESC-derived cells were isolated, resuspended, and dropped on to the collagen scaffold. (B) Collagen scaffolds with or without hESC-derived cells were grafted to the remaining uterine horns, which were ~1.5 cm in length and 1/2–2/3 of the total circumference. (C) Gross view of intrauterine adhesions and distal hydrometra in natural regeneration group at 4 weeks after grafting. The *arrowheads* indicate hydrometra. (D, E) Gross view of reconstructed uterine horns in cells/collagen group at 4 weeks (D) and at 12 weeks (E) after grafting. The *arrowheads* indicate repair sites. (F) Immunostaining analyses on uterine slices of grafted rats killed at 12 weeks after transplantation in the cells/collagen group. Anti-human nucleus antigen (HN) was used to demonstrate the presence of human cells. HN staining is in red, and nuclei were stained with DAPI (blue). Scale bars, 100 mm. (G–I) Pregnancy in uterine horns at 12 weeks after surgery. Pregnancies in the natural regeneration (G) and collagen group (H) generally implanted in the normal tissue. In the cells/collagen group (I), embryos can be implanted in the grafted tissue. The *arrowheads* show the margins of the grafted tissue. Color images available online at www.liebertpub.com/tea

Discussion

A variety of diseases may lead to hysterectomies or uterine injuries, which may form a scar and lead to infertility or pregnancy loss. Re-establishment of fecundity by uterine transplantation has been previously attempted; however, the graft became necrotic after surgery and the attempt failed.¹⁸ Tissue engineering provides an alternative to organ and tissue transplants.^{19–21} Due to the physically and functionally special characteristics as well as hormonally complicated environment of the uterus, reports of uterine reconstruction are rare. Collagen has been a widely used biomaterial in many fields because of its excellent biodegradability and biocompatibility and weak immunogenic reactions.^{22,23} Our previous studies⁸ suggest that

collagen alone is not sufficient for uterine regeneration, especially in cases with larger and thicker defects. It is essential to promote angiogenesis and recruit cells to the injured site to accelerate natural healing and reduce scar formation.

Rapid epithelial repair is the key to preventing scar formation; therefore, we attempted to obtain more epithelial cells. Endometrium epithelial cell *in vitro* proliferation capacity is extremely limited, and not suitable for tissue engineering. hESCs can differentiate into multilineage cell types that are clinically relevant, but directly transplanted hESCs may cause tumors, so it is important to develop simple and reliable cell differentiation protocols.²⁴ As reported, during the *in vitro* spontaneous hESCs differentiation process, the gene expression associated with endometrial development,

TABLE 3. PATENCY RATE OF REGENERATIVE UTERINE HORNS^a

Time	Variable	Sham-operated group	Natural regeneration group	Collagen group	Cells/collagen group	p-Value
4 week	Patency	8 (8)	5 (10)	8 (10)	9 (10)	<0.05
	Percentage	100	50 ^b	80	90	
12 week	Patency	10 (10)	6 (10)	8 (10)	9 (10)	
	Percentage	100	60	80	90	

^an indicates the number of uterine horns. Chi-square test: critical level of significance $p < 0.05$.

^b $p < 0.05$, Sham-operated group versus natural regeneration group.

FIG. 4. Histological structures of the regenerative uterine horns at 4 weeks (A1–D1) and 12 weeks (A2–D2) in the sham-operated (A1, A2), natural regeneration (B1, B2), collagen (C1, C2), and cells/collagen (D1, D2) groups. The arrowheads indicate repair sites in (A1-1 to D1-1) and (A2-1 to D2-1). Em, endometrium; Mm, myometrium; SCEp, simple columnar epithelium; UG, uterine gland. The scale bar indicates 500 μ m. Color images available online at www.liebertpub.com/tea

the endometrial structure, and the endometrial cell can be detected²⁵; however, the directed differentiation of endometrial cells from hESCs has not yet been reported. Recently, it has been confirmed that both epithelial and stromal adult stem cells exist in the basal layer of the human endometrium; in particular, these cells can be found at the bottom of the endometrial gland and around vascularized stroma.^{26–31} In addition, cells derived from bone marrow transplants engraft in the uterus, where they can differentiate into endometrial cells.^{32–34} These results prompted the presence of an endo-

metrial stem cell microenvironment (niche). In recent years, researchers have hypothesized that a common feature of the mammalian stem cell niche is that, along with the extracellular matrix and various secreted molecules, they provide a microenvironment to regulate key adult stem cell functions.^{35–37} The “Niche” is more important than the stem cells themselves in determining cell destiny. Some researchers have already tried to obtain the endometrial cells that differentiated from bone marrow-derived mesenchymal stem cells (BMSCs) in a simulated uterus microenvironment. Zhang *et al.* found

TABLE 4. REPRODUCTIVE OUTCOME IN RATS WITH REGENERATIVE UTERINE HORNS^a

Variable	Sham-operated group (n=9)	Natural regeneration group (n=15)	Collagen group (n=15)	Cells/collagen group (n=15)	p-Value
Pregnancy (%)	9 (100) ^{c,d}	4 (26.7) ^e	5 (33.3) ^f	12 (80.0)	<0.05
Pregnancy at scar site (%)	—	1 (6.7) ^g	2 (13.3) ^h	8 (53.3)	
Average number of gestational sacs ^b	5.4 (49/9) ^{i,j,k}	2 (8/4) ^l	2.8 (14/5)	4.1 (49/12)	

^an indicates the number of uterine horns. Chi-square test and Student's *t*-test: critical level of significance $p < 0.05$.

^bThe average number of gestational sacs = the total number of gestational sacs/the number of pregnancy uterine.

^{c,i} $p < 0.05$, Sham-operated group versus natural regeneration group.

^{d,j} $p < 0.05$, Sham-operated group versus Collagen group.

^{e,g,l} $p < 0.05$, Natural regeneration group versus Cells/Collagen group.

^{f,h} $p < 0.05$, Collagen group versus Cells/Collagen group.

^k $p < 0.05$, Sham-operated group versus Cells/Collagen group.

that mouse BMSCs have the potential to differentiate into endometrial epithelial cells when co-cultured with endometrial stromal cells *in vitro*³⁸; however, Jing Z showed that rat BMSCs could only differentiate into endometrial stromal cells.³⁹ In this study, we tried to simulate an *in vivo* endometrial stem cell niche and differentiate hESCs into endometrium-like cells.

The endometrium is believed to regrow from the basal layer, which is primarily driven by estrogen. Estrogen and progesterone interact with locally produced growth factors in an autocrine and/or paracrine manner.^{40,41} Endometrial epithelial cells synthesize and secrete several growth factors, including EGF, transforming growth factor- α (TGF α), and insulin-like growth factor-I (IGF-I).⁴² In addition, EGF, TGF α , IGF-I, and platelet-derived growth factor-b (PDGF-b) receptor expression are increased in epithelial cells during rapid growth associated with the proliferative stage.^{43,44} These growth factors may also modulate the effects of estrogen, progesterone, or each other by altering receptor expression.⁴⁵ To simulate an endometrial stem cell niche, we isolated stromal cells from normal endometrial tissues and added E2, PDGE, and EGF. We observed that the treatment under these conditions resulted in a high percentage of endometrium-like epithelial cells (~80%). The phenotypes of hESC-derived endometrium-like cells were also briefly confirmed by RT-PCR. We observed the transcription of genes linked to endometrial development, including *Wnt4*, *Wnt7a*, *Wnt5a*, and *Hoxa11*, after treatment with cytokines alone; however, the gene transcription was significantly increased by exposure to endometrial stromal cells. We also tested the genes that prompted endometrial function. *Hoxa10*, *Intergrin β 3*, and *LIF* are expressed in the glandular epithelial cells, and the increase in expression suggests good endometrial receptivity.^{15,16} These results suggested that stromal cells were very important in the development of the endometrium; however, the mechanism of induction remains unclear.

There are only a few reports using hESC-derived cells in uterine reconstruction. Moreover, these studies only examined endometrial repair rather than the entire uterine wall.⁴⁶ Here, we applied transplantation of uterine full-thickness-injury rat models to test the function of our hESC-derived endometrium-like cells *in vivo*. Twelve weeks after transplantation, the cells/collagen group showed a thicker wall, more endometrial glands, and better organized tissue structure in the scarred area. The recovered uterine function was also evaluated. The pregnancy rate and the number of gestational sacs of the cells/collagen group were much higher than in the natural regeneration and collagen groups. Furthermore, embryos were present within the grafted tissue in half of the uterine horns in the cells/collagen group. These results suggested a nearly complete recovery of uterine function in this group, which means that a fertilized ovum could implant into the endometrium of regenerated tissue. Furthermore, this endometrium could support the growth of embryos until near delivery. These results imply that hESC-derived endometrium-like cells could support uterine repair and function recovery. We also found that hESC-derived cells (HN-positive cells) were limited, and the cell quantity could not explain the restoration of function. We believe this may be because the transplanted cells themselves can constitute the new organization, and they (especially the hESC-derived endome-

trial stromal cells) may also promote the endogenous cell migration, proliferation, and differentiation through an autocrine and/or paracrine manner. These accelerated the scaffold degeneration and tissue reconstruction.

In conclusion, we developed a co-culture system to generate endometrium-like cells efficiently from hESC line, NJGLhES1, and we demonstrated that these cells along with collagen scaffolds could notably retrieve the structure and function of uterine horns in a rat model of severe uterine damage. Thus, the differentiation protocol presented in this study will be useful in further studies of human endometrial development, as well as for potential cell-replacement therapy of injured uteruses in the future.

Acknowledgments

The funding for this work was provided by the National Natural Sciences Foundation of China (81200410), the Chinese National Clinical Key Subject Construction Project, the Jiangsu Planned Projects for Postdoctoral Research Funds (1002036C), and the Fundamental Research Funds for the Central Universities (1127021452).

Disclosure Statement

No competing financial interests exist.

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Received: January 20, 2014

Accepted: August 1, 2014

Online Publication Date: September 17, 2014