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Original Article

Role of Wnt5a in the differentiation of human embryonic stem cells into endometrium-like cells

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Abstract: Objective: To explore the effects of Wnt5a and Wint7a on the differentiation of human embryonic stem cells (hESCs) into endometrium-like cells, and provide a basis for establishing endometrium-like cell models and a cell source for carrying out further endometrium-related experiments. Methods: The hESCs established by our center were differentiated into endometrium-like cells in 4 different media including Wnt5a (Group A), Wnt7a (Group B), secreted frizzled related protein (sFRP, an inhibitor of Wnt signal pathway, Group C) and medium alone (Group D). In the differentiated terminal cells, the expressions of cytokeratin (CK) and vimentin were detected with immunofluorescence, and the mRNA levels of CK18, epithelial cell adhesion molecule (EPCAM), estrogen receptor (ER) and progesterone receptor (PR) were determined with RT-PCR. At the same time, the differentiated terminal cells were incubated in medium containing medroxyprogesterone followed by determination of prolactin (PRL). Results: RT-PCR indicated that mRNA levels of CK18, EPCAM, ER and PR were significantly higher in Group A (Wnt5a) than in other groups (all $P < 0.05$), but were significantly lower in Group C (sFRP2) than in other groups (all $P < 0.05$). The changing trend of PRL mRNA was consistent with that of above genes in the 4 groups. Immunofluorescence displayed that the expression of cytokeratin was the strongest in Group A (Wnt5a), and the weakest in Group C (sFRP2) among the 4 groups. Conclusion: Wnt5a has promotive effects on the differentiation of hESCs into endometrium-like cells, but Wnt7a has no marked effects.

Keywords: Wnt5a, Wnt7a, human embryonic stem cells, endometrium-like cells

Introduction

In assisted reproductive technology, endometrial thickness is strongly correlated with the rates of embryo implantation and clinical pregnancy [1]. The thin endometrium can significantly reduce the rates of embryo implantation and clinical pregnancy. It has not been completely clear that how the endometrium develops and which signal pathways are involved in the endometrial development.

Although endometrial stem cells have been isolated from human endometrial tissue, their differentiation direction is not easily controlled because self-differentiation readily occurs during *in vitro* culture of adult stem cells. Human embryonic stem cells (hESCs) with self-renewal capacity and totipotency are obtained by isolation from cell mass of blastocysts and in vitro differentiated culture [2]. Observing the differentiation of hESCs into endometrium-like cells is conducive to understanding the endometrial development and critical signal pathways associated with endometrial regeneration, providing a basis for application of stem cells in clinical practice.

At present, cytokines are commonly used in the differentiation of stem cells. Epidermal growth factor (EGF), tumor growth factor (TGF-α) and platelet-derived growth factor (PDGF-BB) can promote the growth of endometrial stem cell clones [3], and are present in some epithelial tissues such as the skin, gastrointestinal tract and endometrium [4-6]. The uterus develops from Mullerian ducts in which the cells with Wnt7a expression give rise to epithelial cells of the fallopian tubes and uterus, and the cells with Wnt5a expression give rise to stromal cells

Table 1. Sequences of specific primers

Primer	Sequence of forward and reverse primers 5'-3	GenBank accession no.	Annealing temperatuire (°C)
CK18	GGAAGATGGCGAGGACTTTA	NM_199187.	59
	AACTTTGGTGTCATTGGTCTC	1	
EPCAM	TGCTGTTATTGTGGTTGTGGTG	NM_002354.	61
	TACTTTGCCATTCTCTTCTTTCT	2	
ER-a	TGCCAAGGAGACTCGCTA	NM_001122742.1	60
	TCAACATTCTCCCTCCTC		
PR	ACACAAAACCTGACACCTCC	NM_001271161.2	60
	TACAGCATCTGCCCACTGAC		
PRL	GGTGGCGACGACTCCTGGAGCCC	NM_000948.5	61
	GACACCAGACCAACTGGTAATG		
GAPDH	AGAAGGCTGGGGCTCATTTG	NM_002046.	59
	AGGGGCCATCCACAGTCTTC	4	

Notes: CK18: cytokeratin-18; EPCAM: epithelial cell adhesion molecule; ER-a: estrogen receptor; PR: progesterone receptor; PRL: prolactin.

of the uterus, cervix and vagina [7, 8]. It is necessary to know whether Wnt5a and Wnt7a are involved in the differentiation of hESCs into endometrium-like cells and what roles they play.

In this study, 4 different media including Wnt5a, Wnt7a, secreted frizzled related protein (sFRP, an inhibitor of Wnt signal pathway) and medium alone were used in the differentiation of hESCs into endometrium-like cells to compare the differentiation efficiencies between the 4 media and establish more efficient differentiation scheme. This study provides a basis for establishing endometrium-like cell models and a cell source for carrying out further endometrium-related experiments.

Materials and methods

All study methods were approved by Institutional Review Board and Ethics Committee of the First Affiliated Hospital of Zhengzhou University.

Materials

hESCs were established by our center. Recombinant human Wnt5a, Wnt7a and sFRP2 were purchased from R&D Company (Emeryville, CA, USA). Recombinant human EGF, TGF-α and PDGF-BB were purchased from GIBCO Company (Grand island, NY, USA). Recombinant human 17β-E2 was purchased from Sigma (St. Louis, MO, USA). Primary antibodies of mouse anti-cytokeratin and rabbit anti-vimentin were purchased from Santa Cruz (L.A, California, USA). ALLPrep DNA/RNA extraction kit was purchased from Qiangen (Munich, Germany).

Culture and inductive differentiation of hESCs

The hESC, ZZU-hESC-2, established by our center was used in this study. After feeder layer was prepared and clones were thawed, the thawed hESCs were incubated in the prepared feeder layer at 37°C in an atmosphere of 5% CO_2. Forty eight hours later, the adherent growth of clones was observed. hESC culture media mainly consisted of 80% KO-DMEM, 20% knockout serum replacement (KOSR), 1% non-essential amino acids (NEAA), 2mM L-glutamine, 0.1 mM β-mercaptoethanol and 8 ng/ml of basic fibroblast growth factor (bFGF). Medium was changed and the growth of clones was observed every day. One passage was performed with the mechanical method every 4-5 days.

The clones in good condition were used in inductive differentiation. The clones were respectively incubated in 4 different embryoid body-media containing 100 ng/mlof Wnt5a (Group A), 100 ng/mlof Wnt7a (Group B), 100 ng/mlof sFRP2 (Group C) and medium alone (Group D), respectively. Three days later, the embryoid bodies were placed in a dish covered by 0.1% of gelatin for inductive differentiation. Seven days later, these cells were continuously incubated in serum-free media instead of differentiated media for 14 days to obtain termi-

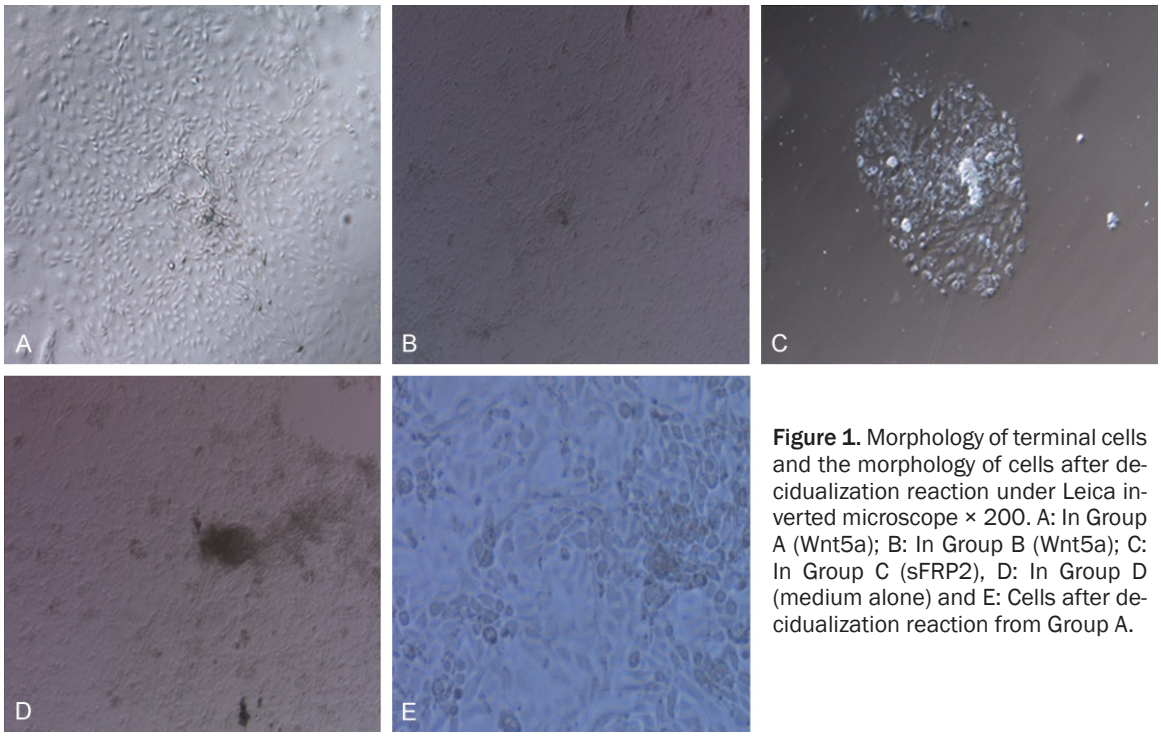

Figure 1. Morphology of terminal cells and the morphology of cells after decidualization reaction under Leica inverted microscope × 200. A: In Group A (Wnt5a); B: In Group B (Wnt5a); C: In Group C (sFRP2), D: In Group D (medium alone) and E: Cells after decidualization reaction from Group A.

nal cells. The differentiated media was prepared with DMEM containing 5% of serum, 1% NEAA, 10ng/ml PDGF-BB, 10ng/ml EGF, 10ng/ml transforming growth factor-α, transforming growth factor-α, 10^{-8}mol/L 17-β estradiol.

Decidualization reaction

Some cells in each group were incubated in the media containing 10^{-6}mol/L medroxyprogesterone for 10 days followed by determination of prolactin (PRL).

Immunofluorescence

The terminal cells were washed with PBS to get rid of residual media, fixed with 4% paraformaldehyde for 15 min, washed with PBS three times for 5 min of each time, punched with 0.1% Triton X-100 for 10 min, washed with PBS three times for 5 min of each time, blocked with 10% of pregnant mare serum for 30 min followed by addition of primary antibodies of mouse anti-cytokeratin (1:50) and rabbit anti-vimentin (1:50) at 4°C overnight. Samples were washed with PBS to get rid of residual media, and then secondary antibodies were added to incubate for one hour. After stained with DAPI and mounted, samples were observed.

RT-PCR

Total RNA was extracted using ALLPrep DNA/RNA extraction kit according to the instructions. cDNA was synthesized using reverse transcriptase kit. The reaction conditions were as follows: 50°C for 2 min, 95°C for 2 min; 95°C for 3s, 60°C for 30 s, 40 cycles. In this study, GAPDH was used as internal control and the levels of mRNA were calculated according to the following formula: ΔΔCt = (Ct of target gene-Ct of GAPDH) Sample A- (Ct of target gene-Ct of GAPDH) Sample B. Ct, a parameter without units, is the number of PCR cycles when the fluorescence reaches a threshold value. The design and synthesis of primers were performed by Takara Biological Engineering Co., Ltd (**Table 1**).

Statistical analysis

Statistical treatment was performed with SPSS13.0 software. Measurement data were expressed as mean ± SD. One-factor analysis of variance was used in the comparison among groups. The comparison between two groups was performed with the least significant difference. Statistical significance was established at $P < 0.05$.

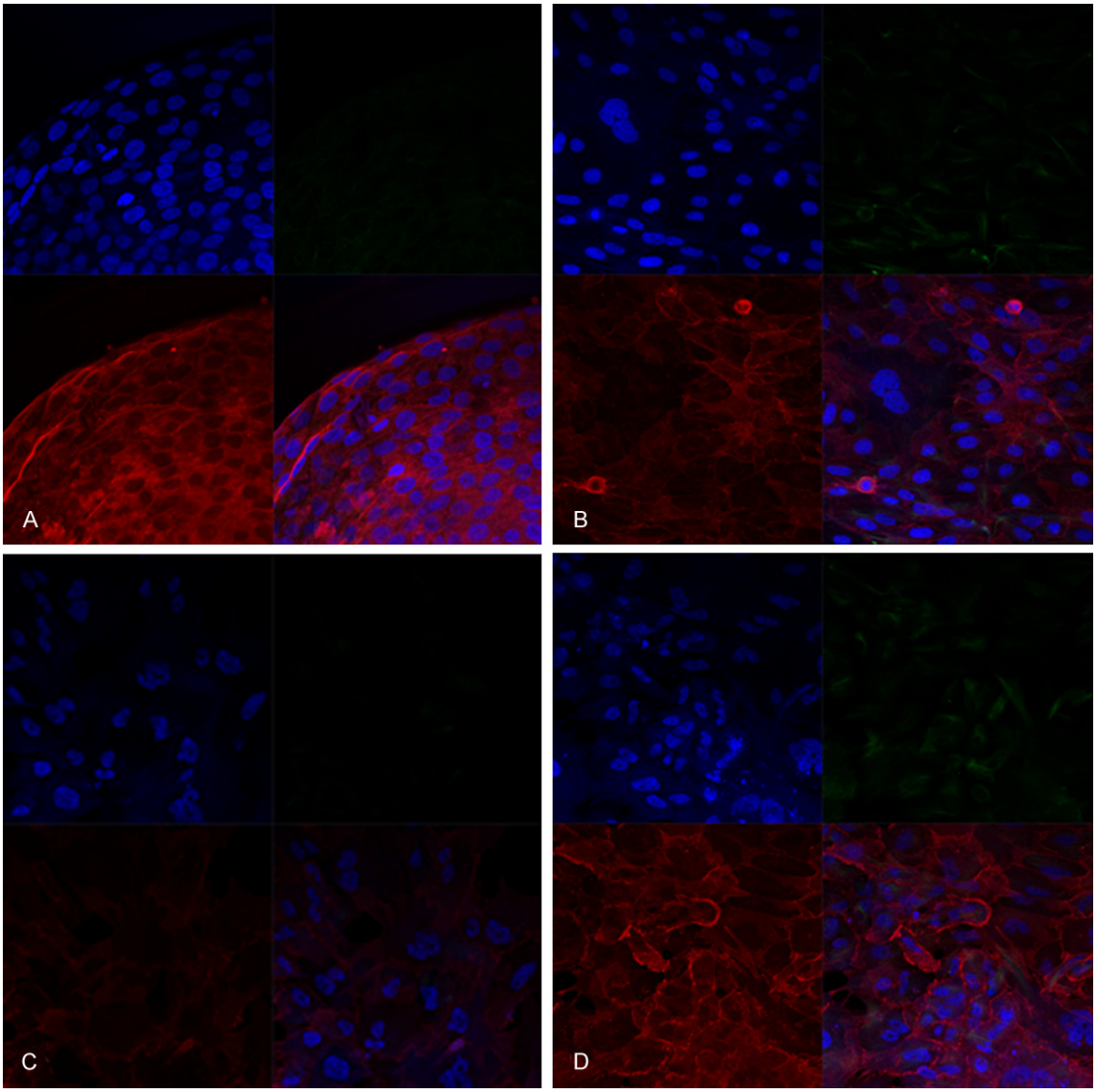

Figure 2. Immunofluorescence shows the expressions of cytokeratin and vimentin in terminal cells under Zeiss confocal microscope LSM710 × 400. Notes: cytokeratin is red, vimentin green and cell nucleus blue. A: In Group A (Wnt5a); B: In Group B (Wnt7a); C: In Group C (sFRP2) and D: In Group D (medium alone).

Results

Observation of terminal cells under light microscopy

In Group D (medium alone), hESCs grew into embryoid bodies in early phase. With the prolongation of culture time, the embryoid bodies formed marked cysts. After incubated in differentiated media, monostratal adherent cells grew out towards periphery with the embryoid bodies as the center on the bottom of culture dishes. The terminal cells in Group B (Wnt7a) were similar to that in Group D (medium alone). In Group A (Wnt5a), the growth of monostratal adherent cells was denser than that in Group D (media alone). In Group C (sFRP2), the growth of monostratal adherent cells was markedly inhibited (**Figure 1**).

Decidualization reaction

After the terminal cells were incubated in the media containing 10^{-6} mol/L medroxyprogesterone for 10 days, cells became large, round and transparent with rich cytoplasm (**Figure 1**).

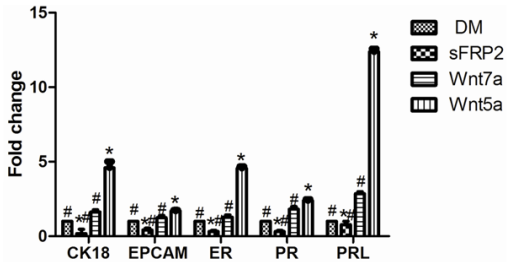

Figure 3. mRNA expressions of specific genes in terminal cells. Notes: CK18: cytokeratin-18; EPCAM: epithelial cell adhesion molecule; ER: estrogen receptor; PR: progesterone receptor; PRL: prolactin; DM: medium alone; sFRP2: secreted frizzled related protein2. Data are expressed as mean ± SD, n = 3; *Indicates $P < 0.05$ as compared with control group (medium alone). #Indicates $P < 0.05$ as compared with Group A (Wnt5a).

Expression of specific proteins in terminal cells of each group

LSM710 confocal microscope showed that cytokeratin (CK) expression was present in 60% of cells and vimentin expression in 10% of cells in Group A (Wnt5a), suggesting that although most cells were epithelial cells after differentiation, there also were stromal cells. In groups B (Wnt7a) and D (medium alone), CK expression was positive in nearly 40% of cells. In Group C (sFRP2), only 20% of cells exhibited positive CK expression (**Figure 2**).

Expression of specific genes in terminal cells of each group

CK18 and epithelial cell adhesion molecule (EPCAM) are the specific makers of epithelial cells, and the presence of estrogen receptor (ER) and progesterone receptor (PR) suggests that the endometrial epithelial cells can be regulated by estrin and progestogen. RT-PCR indicated that mRNA expressions of CK, EPCAM, ER, PR and PRL were significantly higher in Group A (Wnt5a) than in control group (medium alone) ($P < 0.05$), but were significantly lower in Group C (sFRP2) than in control group (medium alone) ($P < 0.05$), and were similar between Group B (Wnt7a) and control group (medium alone). mRNA expressions of CK, EPCAM, ER, PR and PRL were the highest in Group A (Wnt5a) among the 4 groups (all $P < 0.05$), suggesting that cell functions were the most closed to that of endometrial epithelial cells and cells were the most sensitive to decidualization reaction in Group A (Wnt5a) among the 4 groups (**Figure 3**).

Discussion

It has been an important issue to make hESCs differentiate into specific cell type. Exploring the differentiation of hESCs into endometrium-like cells can provide cell models and experimental basis for the researches about the early development of endometrium. It has been confirmed that the members of Wnt cytokine family play important roles in many developmental events [9-11], such as embryo development, cell proliferation, tissue regeneration and tumor formation.

So far, 20 kinds of secreted Wnt proteins have been found. They all can combine with the receptors of Frizzled family on the cell surface to activate three different signal pathways including Wnt/β-catenin classical pathway [12], and Wnt/cell polar [13] and Wnt/Ca^{2+} non-classical pathways [14]. At present, the mechanism about Wnt/β-catenin classical pathway is the clearest among the three signal pathways. The Wnt/β-catenin pathway is highly conserved during the evolution of species. It mainly regulates cell proliferation and differentiation, and plays a role in embryo development and tumor occurrence [15, 16]. When Wnt signal pathway is activated, Wnt molecule combines with specific receptors on cell surface to regulate transcription and expression of downstream genes. It is reported that sFRP may block this pathway through competitive binding with receptors on the cell surface [17]. Wnt signal pathways attract more and more attention because they play an important role in self-renewal potentiality and differentiation of hESCs.

There have been different reports regarding the effect of Wnt signal pathways on hESCs. On one hand, Wnt signal pathway, an important factor in cell division, plays a role in self renewal of stem cells. It was reported that the activation of Wnt/β-catenin pathway activated proliferative signals of murine hematopoietic stem/progenitor cells *in vitro* [18, 19]. On the other hand, there is no marked activation of Wnt signal pathways in quiescent stem cells. It was reported that the activation of Wnt/β-catenin pathway was found only in activated stem cells and progenitor cells derived from stem cells *in vivo* [20, 21], and Wnt signal pathways were

activated when primitive stem cells began differentiating [22-24]. However, in adult stem cells, self-renewal and differentiation are not mutually exclusive. During the regeneration and repair after tissue damage, only under the actions of activated Wnt signal pathways and proliferative signals, the quiescent stem/progenitor cells can produce offspring stem/progenitor cells, and finally form terminal cells [25, 26].

In embryo development, during the process that primitive neural crest cells migrate to the skin, high expression of Wnt5a leads to changes in the morphology of developed cells; and when cells reach the target region, Wnt5a gene expression decrease [27]. Similarly, in endometrial stroma, there is Wnt5a expression which is necessary for the development of endometrial epithelial glands [28]. The results above suggest that Wnt5a is a main regulatory factor and plays an important role in cell growth and differentiation.

In this study, immunofluorescence indicated that cytokeratin expression was the highest in Group A (Wnt5a), the weakest in Group C (sFRP2), and similar between Group B (Wnt7a) and Group D (medium alone). RT-PCR displayed that mRNA expression of CK18, EPCAM, ER and PR in terminal cells was significantly increased in Group A (Wnt5a) as compared with other three groups; while in Group C (sFRP2), mRNA expression of CK18, EPCAM, ER and PR was significantly decreased. Our results suggest that Wnt5a has marked promotive effects on the differentiation of hESCs into endometrium-like cells, sFRP2 can decrease differentiation efficiency, and Wnt7a has no marked effects. It is reported that there are expressions of Wnt5a, Wnt7a and Wnt11 and their receptors including Fzd6, Fzd2 and accessory receptor LRP6, but sFRP2, Wnt receptor antagonist, is not found in the endometrial epithelial cells of neonatal sheep; with the development of endometrial glands, the expressions of Wnt7a and Wnt11 decrease, but sFRP2 expression increases and the level of sFRP2 is consistent with the density of endometrial glands. It may be speculated from this result that the formation of endometrial epithelial cells requires Wnt5a, Wnt7a and Wnt11, sERP2 inhibits the formation of endometrial epithelial cells but promotes the development of endometrial glands.

In summary, this study explored the effects of Wnt5a and Wnt7a on the differentiation of hESCs into endometrium-like cells *in vitro* for the first time, and found that 100 ng/ml of Wnt5a could significantly promote the differentiation of hESCs into endometrium-like cells, but Wnt7a had no marked effects. However, the functions of terminal cells differentiated with the scheme used in this study remains to be further investigated.

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Disclosure of conflict of interest

None.

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