Received: 19 August 2019 | Revised: 25 October 2019 | Accepted: 23 November 2019

DOI: 10.1111/aji.13213

ORIGINAL ARTICLE

AJRI American Journal of Reproductive Immunology WILEY

Establishment and characterization of immortalized human eutopic endometrial stromal cells

Zhi-Xiong Huang[1] | Xiao-Mei Mao[1] | Dian-Chao Lin[2] | Yi-Huang Hong[2] | Gui-Shuang Liang[2] | Qing-Xi Chen[1] | Qiong-Hua Chen[2]

[1]School of Life Sciences, Xiamen University, Xiamen, China

[2]Department of Obstetrics and Gynecology, the First Affiliated Hospital of Xiamen University, Xiamen, China

Correspondence
Qiong-Hua Chen, Department of Obstetrics and Gynecology, The First Affiliated Hospital of Xiamen University, Xiamen 361003, China.
Email: cqhua616@126.com

Funding information
the Natural Science Foundation of Fujian Province, Grant/Award Number: 2017J01362; The National Science Foundation of China, Grant/Award Number: 8187060862; the Science and Technology Foundation of Xiamen, Grant/Award Number: 2017S0060

Problem: The application of primary eutopic endometrial cells from endometriosis patients in research is restricted for short life span, dedifferentiation of hormone responsiveness.

Method of Study: Human telomerase reverse transcriptase (hTERT)-induced immortalized cells (iheESCs) were infected by lentivirus. mRNA level was examined by qRT-PCR, and protein expression was quantified by Western blot. CCK-8 and EdU assay were assigned to assess the proliferation. The migration and invasion of cells were assessed by transwell assay. Clone formation assay and nude mouse tumorigenicity assay were used to evaluate colony-formation and tumorigenesis abilities.

Results: hTERT mRNA and protein were significantly expressed higher in iheESCs compared to primary cells. iheESCs grew without morphological change for 42 passages which is much longer than 18 passages of primary cells. There was no obvious difference between primary cells and iheESCs in growth, mobility, and chromosome karyotype. Furthermore, the expression of epithelial-mesenchymal transition (EMT) markers and estrogen/progesterone receptors remained unchanged. The decidualization of iheESCs could be induced by progesterone and cAMP. Estrogen increased the proliferation and mobility of iheESCs, and lipopolysaccharides (LPS) induced the IL-1β and IL-6 promoting inflammatory response. The colony-forming ability of iheESCs, like primary cells, was lower than Ishikawa cells. In addition, tumorigenicity assay indicated that iheESCs were unable to trigger tumor formation in BALB/c nude mouse.

Conclusions: This study established and characterized iheESCs that kept the cellular physiology of primary cells and were not available with tumorigenic ability. Thus, iheESCs would be useful as in vitro cell model to investigate pathogenesis of endometriosis.

KEYWORDS

decidualization, estrogen and inflammatory response, eutopic endometrial stromal cells, Immortalized, tumorigenicity

1 | INTRODUCTION

Endometriosis is classically defined by the presence of endometrial tissues out of intrauterine locations. Endometriosis is an estrogen-dependent and inflammatory disorder that can result in chronic pelvic pain, dysmenorrhea, irregular uterine bleeding, and infertility.[1,2] The appearance of pelvic endometriosis approaches 10% in female of reproductive age.[3] Furthermore, the recurrence rate of endometriosis reaches 6%-67%, and some patients require three or more surgeries.[4] Thus, endometriosis impacts seriously the physical

and mental health, personal relationship, and financial status of patients.

The most widely accepted theory of endometriosis is that retrograded menstruation of endometrial tissues slough through patent fallopian tubes into the peritoneal cavity.[5,6] Therefore, the survival ability of desquamated endometrial lesions in peritoneal cavity is crucial for establishment of viable implants. It was reported that the heritable feature of endometriosis was certificated in clinical, and the occurrence of endometriosis in family with medical history was much higher than that of unaffected women.[7,8] Another study found that eutopic endometriums from patients with endometriosis were detected with several genomic alterations compared with normal female.[9,10] Furthermore, the aberrant progesterone receptor (PR) expression and progesterone resistance were found in eutopic endometrium and resulted in impaired endometrial receptivity.[11,12] These findings imply that genomic abnormality in eutopic endometriums from females with endometriosis may permit to result in endometriotic patient infertility and form endometriotic lesions. Thus, the study focused on abnormality in eutopic endometriums is vital to reveal the pathogenesis of endometriosis.

Primary endometrial cells are essential tools in research of endometriosis, but primary cells tend to undergo dedifferentiation during cell culture, including loss of hormone and cytokine responsiveness, short life span, and the change of migration and invasion. Thus, consistent supply of cells with a stable character and long life span is extraordinarily important for research application. This study established and characterized immortalized cells that maintained original morphology in continuous cell cultures, and the growth rate, mobility, and epithelial-mesenchymal proteins, estrogen/progesterone receptor expression remained unchanged. Estrogen stimulation and inflammatory response of iheESCs were observed. To sum up, iheESCs would be useful as in vitro cell model to investigate abnormality of eutopic endometriums and pathogenesis of endometriosis.

2 | MATERIALS AND METHODS

2.1 | Reagents

Primary cells were separated by Collagenase IV (#A004186-0001) and DNase I (#B002004-0005) which were bought from Sangon Biotech. DMEM/F12 medium (#SH30023.01) was from Hyclone and fetal bovine serum (FBS; #04-001-1A) was acquired from Biological Industries (BI) 17β-Estradiol/estrogen/E2 (#E2758) and Lipopolysaccharides (LPS; #L4391) were from Sigma-Aldrich. GAPDH mouse monoclonal antibody (#40493) was procured from ABclonal. Cytokeratin 18 (#4548), E-cadherin (#14472), Vimentin (#5741), and N-cadherin (#13116) antibodies were from Cell Signaling Technology. Anti-TERT rabbit monoclonal antibody (#ab32020) was purchased from Abcam.

2.2 | Patients and primary cells culture

The tissues were collected in Department of Obstetrics and Gynecology in the First Affiliated Hospital of Xiamen University. The application of samples received permission from the ethics committee of the First Affiliated Hospital of Xiamen University, and all patients signed the informed consent. Normal endometrium tissues were obtained from a 32-year-old healthy female. Eutopic endometrium tissues were recruited from a 26-year-old female suffering from ovarian endometriosis who underwent surgery. All the tissue specimens were collected in the proliferative phase and histologically confirmed. All patients had regular menstrual cycles and were without hormone treatment for more than 3 months before the surgery.

The endometrium samples were minced and digested by collagenase IV and deoxyribonuclease I. After being filtered through nylon cell-strainers with a 100 mesh and then through a mesh size of 400, the cell supernatant was separated into endometrial epithelial gland cells which were retained by the strainer and the stromal cells which passed through the strainer in the filtrate. The filtered cells were cultured within DMEM/F12 medium containing 10% FBS in dishes at 10% CO_2 37℃ incubation.

2.3 | Establishment of iheESCs

hTERT, human telomerase reverse transcriptase, maintains telomere length and relieves a main barrier on cellular life span. hTERT coding regions were cloned and inserted into GV358 vector (GeneChem). hTERT-containing plasmid and lentivirus packing plasmids (VSVG/PMDL/REV) were mixed and transfected into 293T cells to generate lentivirus. The primary stromal cells were cocultured with viral supernatants for 24 h to generate infected cells.

2.4 | Drugs treatment

Cells were digested by trypsin and removed to 60 cm dishes in DMEM/F12 medium without phenol red. After 24 h culture to remove endogenous hormones, 200 nM estrogen and 100 μg/mL LPS were diluted in DMEM/F12 medium and exposed to cells for 48 h.

2.5 | RNA extraction and qRT-PCR

RNAiso Plus (#9108; Takala Biotechnology) was used to lyse cells. Total RNA was extracted by chloroform and isopropyl alcohol. cDNA was synthesized by PrimeScript RT reagent kit (#RR047A; Takara). PCR was performed by the SYBR Premix Ex Taq II (#RR820A, Takala) in Lightcycler 480 (Roche). The results were normalized based on GAPDH

expression, and the $2^{-\triangle\triangle CT}$ method was used to calculate the relative mRNA level. The primer sequences used in qPCR are listed in Table 1.

2.6 | Western blot assay

RIPA lysis buffer with protease inhibitor was used to harvest proteins. Proteins were separated by SDS-PAGE and then were transferred onto PVDF membranes (#IPVH00010; Merck). The membranes were incubated in primary antibodies for overnight at 4℃ and then incubated with HRP-labeled secondary antibody. The dilution rates of antibodies in the experiment were hTERT (1:1000), Cytokeratin 18 (1:1000), E-cadherin (1:500), Vimentin (1:1000), N-cadherin (1:1000), ERα (1:500), ERβ (1:1000), PR (1:1000), GAPDH (1:10 000), and secondary antibody (1:30 000). The signal was finally visualized by an enhanced chemiluminescence (ECL).

2.7 | Cell proliferation assay

1×10^3 cells were dispersed and seeded into 96-well plates. The drugs were added into medium after 24 h. Then, CCK8 was added into each well and reacted in 5% CO_2 37℃ incubator for 2 h every 24 h. The absorbance of supernatants was measured by an ELISA reader spectrophotometer (Dynatech Laboratories).

2.8 | Transwell assay

1×10^5 cells suspended in DMEM/F12 containing 0.5% BSA were planted into the 8 μm transwell chambers (#3422) which were incubated in complete medium. After 24 h, the chambers were stained by crystal violet and the cells were counted by microscope (Olympus Corporation). Invasion assay was similar but the chambers were coated with Matrigel (BD Bioscience).

TABLE 1 Primer sequences used in qRT-PCR analysis

Gene name	Sequences (5'-3')
GAPDH	Forward: GGAAGGTGAAGGTCGGAGTCA
	Reverse: GAGTCCTTCCACGATACCAA
hTERT	Forward: CGGCCTATTCCCCTGGT
	Reverse: ATGTTCCTCCCAGCCTTGA
ERα	Forward: CCACCAACCAGTGCACCATT
	Reverse: GGTCTTTTCGTATCCCACCTTTC
ERβ	Forward: AGAGTCCCTGGTGTGAAGCAAG
	Reverse: GACAGCGCAGAAGTGAG CATC
PR	Forward: AAGTGAGGTTAGGGCGAAATG
	Reverse: AAGGTAGTTGATTGCCAACGAA
IL-1β	Forward: ATGATGGCTTATTACAGTGGCAA
	Reverse: GTCGGAGATTCGTAGCTGGA
IL-6	Forward: ACTCACCTCTTCAGAACGAATTG
	Reverse: CCATCTTTGGAAGGTTCAGGTTG

2.9 | EdU labeling assay

After treatment with drugs for 48 h, the cells were incubated with 10 μM EdU solution for 3 h. Then as described in instructions of EdU assay kit (#C0071S; Beyotime Biotechnology), cells were fixed and permeabilized and cultured in click additive solution for 30 min. Finally, the cell nucleus was stained with Hoechst 33342 solution. The results were observed and captured using the fluorescence microscope (Olympus Corporation).

2.10 | In vitro decidualization

iheESCs were planked in 60 cm dishes and cultured in phenol red-free DMEM/F12 medium with 10% charcoal-treated FBS (#04-201-1A) for 24 h to consume endogenous estrogen. Then, the cells were stimulated with 1 μM progesterone (#P0130, Sigma-Aldrich) and 0.1 mM cAMP (#A9501, Sigma-Aldrich) for 5-7 days. The treated cells were observed and captured by microscope and then lysed to harvested RNA.

2.11 | Karyotype analysis

1×10^5 cells were planted into in a 10 cm dish and cultured with DMEM/F12 medium with 10% FBS in cell incubator for 48 h. After treatment with 0.2 μg/mL colchicine (#HY-16569; MCE), the cells were treated with low osmotic pressure for 20 min and then fixed by fixative solution (methanol: acetic acid = 3:1). The fixed cells were added to the slide and stained by Giemsa. The chromosomes were observed and captured by microscope and photoshop image processing software was used for clipping and sorting.

2.12 | Immunocytochemistry (ICC)

Cells were cultured on polylysine coated glass slides for 48 h to stick to the slides and fixed by cold methanol. After permeating by 0.3% Triton-X, the slides were incubated with 5% bovine serum albumin (BSA) for 1 h and then covered in primary antibody overnight and followed by incubation of corresponding secondary antibody.

2.13 | Colony-formation assay

1×10^3 cells were counted and planked in 6 cm dish. After 7 days, the cells were washed with PBS and fixed by 4% paraformaldehyde for 10 min. Crystal violet was used to stain the cells for 15 min and the colonies were counted visually.

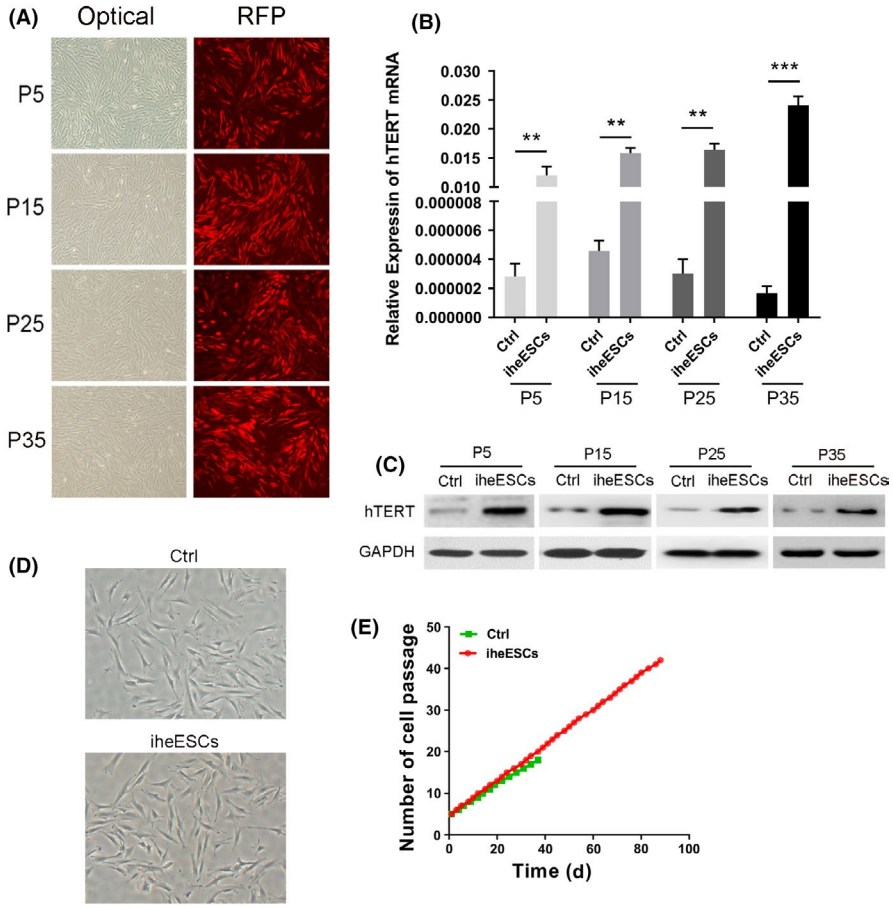

FIGURE 1 Establishment of immortalized human eutopic endometrial stromal cells (iheESCs) by infection of lentivirus. (A) Primary stromal cells were infected by lentivirus with red fluorescent protein tag. Expression of hTERT mRNA (B) and protein (C) after infection by lentivirus. (D) Cell morphology of primary cells and iheESCs. (E) Life span of primary cells and iheESCs processed by continuous culture. Data represent the mean ± SEM. $^{**}P < .001$, $^{***}P < .0001$

2.14 | Xenograft

Female BALB/c nude mice (5-8 weeks old and 15-20 g) were purchased and raised in Animal Research Laboratory of Xiamen University. After 1 week of acclimation, primary stromal cells, iheESCs, and Ishikawa endometrial cancer cells were subcutaneously injected into the right flank of the mice (1×10^7 cells/mouse). The mice were monitored for tumor growth for one month.

3 | RESULTS

3.1 | Establishment of iheESCs by infection of lentivirus

To generate immortalized cells with overexpression of hTERT, lentivirus was packaged and employed to infect eutopic endometrial stromal cells. As shown in Figure 1A, iheESCs stably expressed red fluorescence protein (RFP) in the passage 5, 15, 25, and 35 passages. The mRNA and protein of hTERT were significantly increased in iheESCs (Figure 1B,C). The morphology of iheESCs was unchanged compared with primary cells (Figure 1D). Furthermore, iheESCs could be passed down to 42 generations which was much longer than primary cells (Figure 1E).

3.2 | The cell physiology of iheESCs remains unchanged

To further monitor physiological changes of iheESCs, the growth and mobility of iheESCs were examined. The growth curve of primary cells and iheESCs were not significantly different (Figure 2A). Both migration and invasion of iheESCs were similar with that of primary cells (Figure 2B,C). Moreover, the protein expression of estrogen receptors, progesterone receptors, and epithelial-mesenchymal marker proteins between primary cells and iheESCs were consistent (Figure 2D). After long-term cultivation, the mRNA expression of ERs and PR in iheESCs remained unchanged compared to primary eutopic endometrium stromal cells which is different to normal endometrium stromal cells (Figure S1A-C). To distinguish epithelial and mesenchymal cells, immunocytochemistry was used to analyze the expression of Cytokeratin 18 and Vimentin. iheESCs and primary remained higher level of Vimentin while the expression of Cytokeratin 18 was lower (Figure 2E). To further study whether the cells were transformed, karyotype analysis was conducted to explore the chromosomal situation of iheESCs. As in Figure 2F, iheESCs maintained 23 pairs of chromatins, indicating that karyotype of iheESCs kept normal. These results demonstrated that iheESCs maintained the original proliferation and mobility capacity and the characteristics of primary

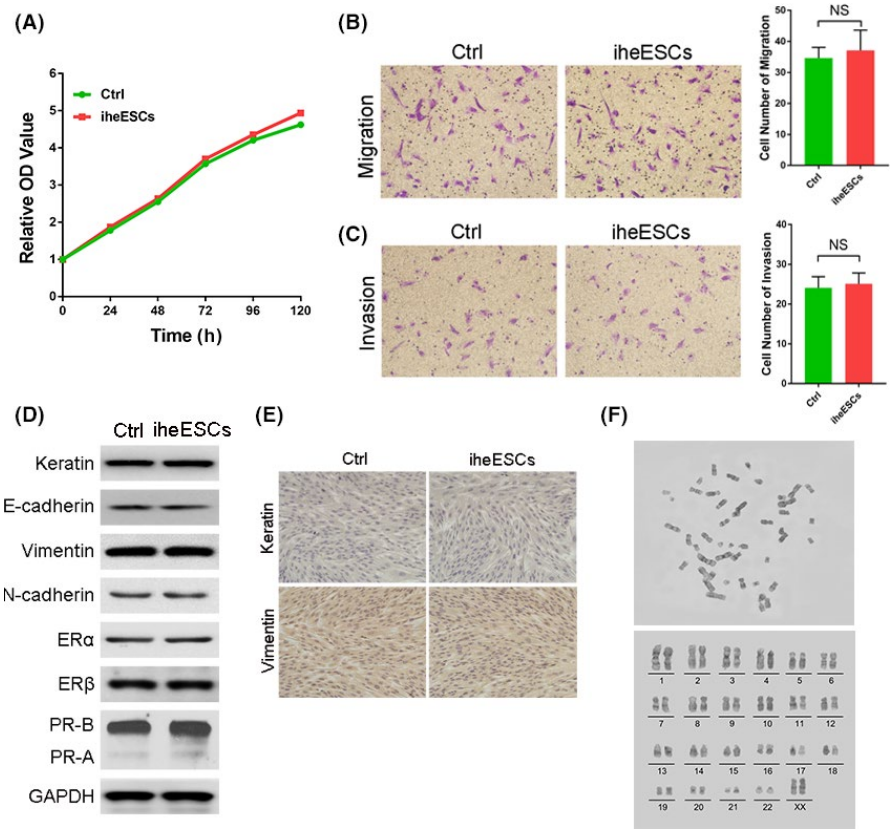

FIGURE 2 Cell physiology of iheESCs were unchanged. (A) Proliferation of primary cells and iheESCs assayed every 24 h by CCK8 assay. (B) Migration of primary ells and iheESCs assessed by transwell assay and quantified analysis (right). (C) Invasion assay of primary cells and iheESCs quantified analysis (right). (D) Expression of EMT markers, estrogen, and progesterone receptors in different cells. (E) Identification of epithelial and mesenchymal cells assessed by immunocytochemistry. (F) Chromosome karyotype analysis of iheESCs and sorting result (lower). Data represent the mean ± SEM. NS>0.05, $^{*}P < .01$

stromal cells and the expression of estrogen and progesterone receptors were unchanged.

3.3 | Induction of decidualization in iheESCs

To further confirm decidualization capacity of iheESCs, 1 μM progesterone and 0.1 mM cAMP were used to stimulate iheESCs to induce decidualization. The morphology of iheESCs changed into plump round from spindle shape (Figure 3A). Moreover, the mRNA expression of IGFBP-1 and PRL were remarkably increased (Figure 3B,C). Therefore, iheESCs showed an adequate decidualization phenotype with stimulation of progesterone and cAMP.

3.4 | Normal estrogen stress and inflammatory response are detected in iheESCs

To confirm the regulatory effect of estrogen in proliferation of iheESCs, CCK8 and EdU assay were performed to examine the

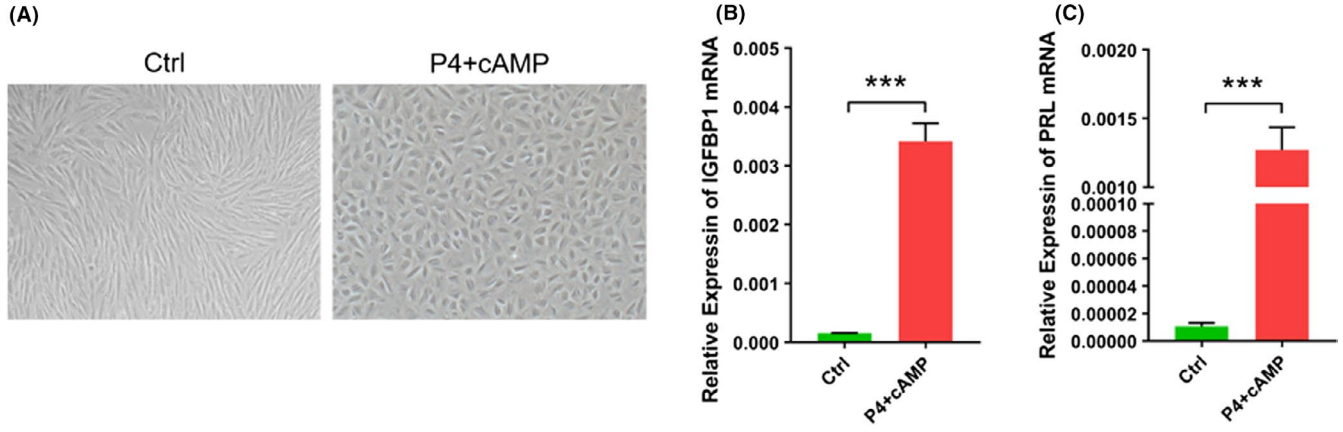

FIGURE 3 Induction of decidualization in iheESCs. (A) Observation of cell morphology with treatment of progesterone and cAMP. (B) Level of IGFBP1 mRNA after induction of decidualization. (C) Expression of PRL mRNA analyzed by qRT-PCR. Data represent the mean ± SEM. $^{***}P < .0001$

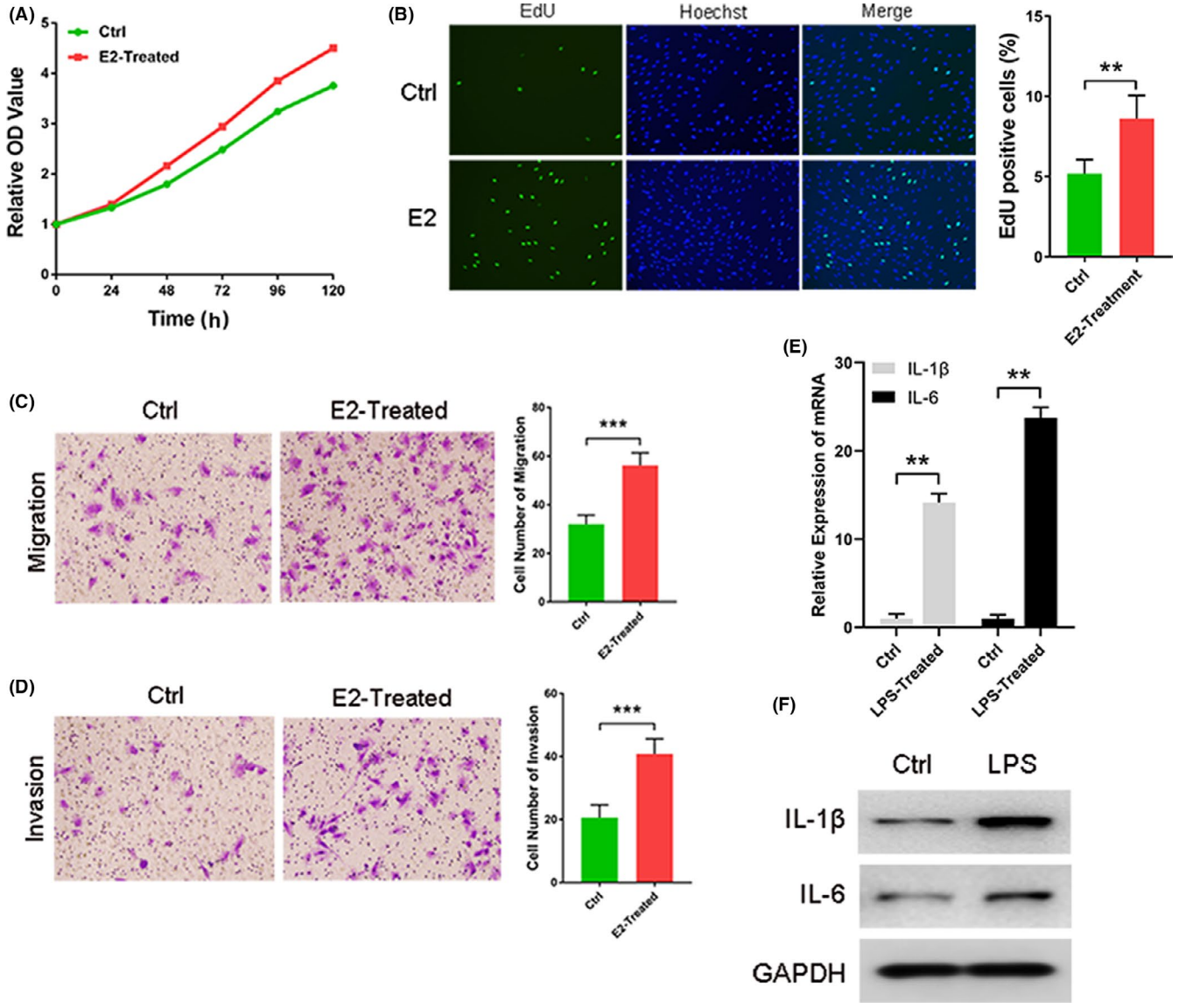

FIGURE 4 Normal estrogen stress and inflammatory response are found in iheESCs. (A) Proliferation of iheESCs with stimulation of estrogen. (B) EdU assay employed to monitor cell division and quantified analysis (right). (C) Ability of cell migration after treatment of estrogen detected by transwell assay and quantified analysis (right). (D) Evaluation of cell invasion exposed to estrogen and quantitative analysis (right). (E) Relative expression of IL-1β and IL-6 mRNA with treatment of lipopolysaccharide (LPS). (F) Protein level of IL-1β and IL-6 by Western Blot. Data represent the mean ± SEM. $^{**}P < .001$, $^{***}P < .0001$

proliferation and cell division. As in Figure 4A, the proliferation of iheESCs was significantly elevated with the stimulation of estrogen. The percentage of EdU-positive cells was remarkably increased in estrogen-treated cells (Figure 4B). Furthermore, estrogen-treated iheESCs were more capable of migration and invasion (Figure 4C,D). These results suggested that normal estrogen stress response was stabled presented in iheESCs.

To examine whether iheESCs remained normal inflammatory process, qRT-PCR and Western blot were employed to measure the expression of IL-1β and IL-6 in LPS-treated iheESCs. The mRNA and protein expression of IL-1β and IL-6 were both increased by LPS stimulation (Figure 4E,F). These results showed that iheESCs kept normal inflammatory responses.

3.5 | iheESCs are not available with tumorigenic ability

To further identify the tumorigenic ability of iheESCs, colony-formation assay and nude mouse tumorigenicity assay were employed to address the oncogenicity of iheESCs. The results of Figure 5A showed that colony formation of primary cells and iheESCs were similar and significantly less than Ishikawa cells. To examine whether iheESCs were worsened and acquired carcinogenic capacity, the cells were injected into BALB/c nude mouse. The primary cells and iheESCs were unable to trigger tumor formation, but positive control Ishikawa cells succeed forming tumors (Figure 5B). Thus, these results implied that iheESCs were not available with tumorigenic ability.

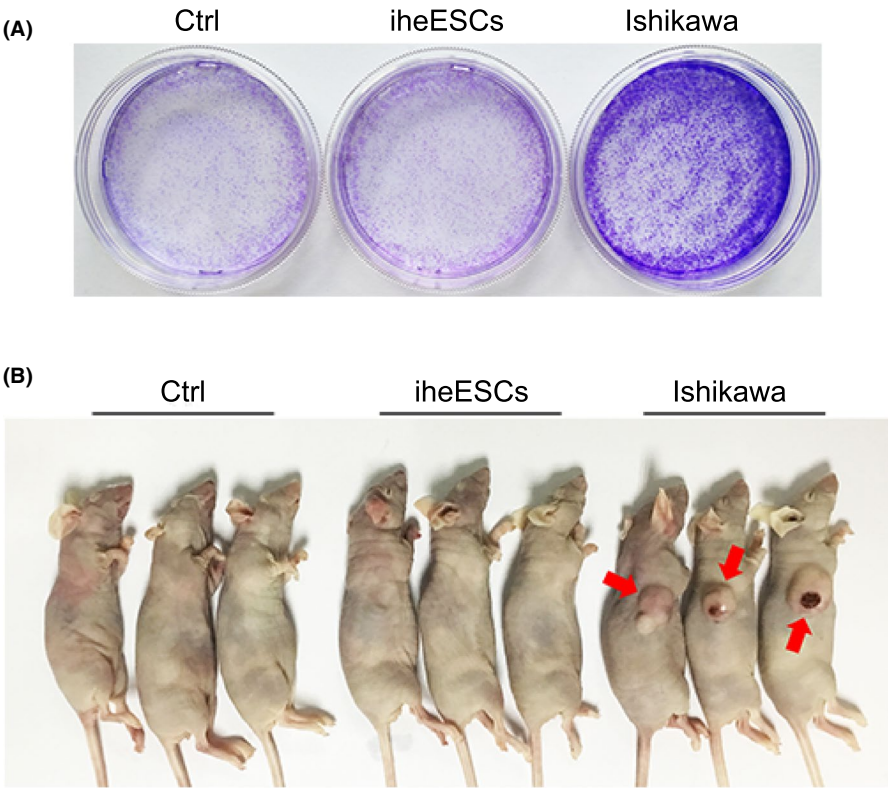

FIGURE 5 iheESCs are not available with tumorigenic ability. (A) Colony-formation assay used to assess the oncogenic potential of iheESCs. (B) Nude mouse tumorigenicity assay performed to evaluate tumorigenic capacity of iheESCs

4 | DISCUSSIONS

In this study, we established and characterized immortalized human endometriosis eutopic stromal cells (iheESCs) by transfecting lentivirus vector encoding hTERT. iheESCs, sustaining high expression of hTERT, maintained original morphology in continuous cultures for 42 passages. The expression of estrogen/progesterone receptors and EMT markers in iheESCs kept stable consistent with primary cells. Moreover, iheESCs remained normal growth and ability of migration and invasion. iheESCs showed the ability of decidualization induced by progesterone and cAMP. Normal estrogen and inflammatory response were detected in iheESCs. Furthermore, iheESCs were not available with tumorigenic ability. Thus, iheESCs could be ideal tool cells in the study of abnormality of endometrium and pathology of endometriosis.

There are many great differences among endometriums from normal and endometriotic females. Eutopic endometriums display an increased proliferation compared with normal endometriums.[13-15] The higher migration and invasion capacity of eutopic endometriums compared to normal endometriums were reported in previous studies.[16,17] Our previous research found that EMT level was promoted markedly in eutopic cells.[18] Thus, the high proliferation, mobility, and EMT of eutopic endometriums may be closely related to the pathogenesis of endometriosis. iheESCs established by transfecting hTERT retained the original rate of growth, mobility, and EMT level. Moreover, iheESCs maintained the characteristics of mesenchymal cells after many generations. These results suggested the cellular physiology of iheESCs is not degraded. Thus, it is feasible to use iheESCs as model cells to replace primary cells in the study of endometriotic pathology.

Endometriums are dynamic steroid-responsive tissues that undergo repeated physiological cycles involving sequential proliferation, differentiation, breakdown, and repair, all of which are associated with steroid hormone.[19] Progesterone and its receptors regulate decidualization of the endometrium.[20] The decidualization of iheESCs was induced by progesterone and cAMP, which indicated that physiology of iheESCs was still deeply regulated by progesterone. Furthermore, the decidualization kept in iheESCs indicated that iheESCs was usable in research of endometrial receptivity and embryo implantation. For another, estrogen, interacting with inflammation, plays a paramount role in the maintenance of endometriosis.[21,22] Our previous study found that estrogen upregulates cell growth, mobility, and EMT level via p38 pathway.[18,23]Above assays indicated that the growth and mobility of the iheESCs were regulated by estrogen, thus cellular activity of iheESCs was regulated by the effect of estrogen. Moreover, the response to inflammation induced by LPS was detected in iheESCs. Accordingly, iheESCs can be used as a model cell line to investigate the effect and mechanism of estrogen and inflammatory process on endometriosis.

In addition, other groups had overcome the limitations of primary culture by immortalizing cells using various methods. Yuhki et al[24] established a human immortalized endometrial stromal cell line, KC02-44D, which kept normal decidualization capacity and inflammatory responses. Holdsworth et al[25] reported that immortalized human endometrial stromal cell lines maintained homozygous endometriosis-risk genotype and diverse responses to hormones (proliferation and decidualization changes) and inflammation. Same as hTERT immortalized endometrial cells which kept primary hormone response and inflammation, iheESCs remain a longer cell life span which is up to passages 40. The mobility and EMT level

of iheESCs were further observed unchanged. Moreover, iheESCs were incomplete transformed cells without malignant transformation. Therefore, iheESCs provide longer service life in more widely applied field and guarantee application security.

The process of establishing immortalized cells may lead to deterioration of cell, resulting acquisition of tumorigenic ability. However, iheESCs were not available with tumorigenic ability and kept growth characteristics of primary cells. Consequently, the results of our study suggested that iheESCs, keeping stable character and stress response, may serve as an ideal cell model to investigate the abnormality of eutopic endometrium and pathology of endometriosis.

ACKNOWLEDGMENTS

The authors would like to thank Dr Qian-Sheng Huang, Wei-Dong Zhou, and Rong-Feng Wu for the valuable comments and excellent technical assistance in this research. The author(s) disclosed receipt of the following financial support for the research: The National Science Foundation of China (No. 8187060862), the Natural Science Foundation of Fujian Province (No. 2017J01362), and the Science and Technology Foundation of Xiamen (No. 2017S0060).

CONFLICT OF INTEREST

All authors announced there were no potential conflicts of interest in this article.

ORCID

Qiong-Hua Chen https://orcid.org/0000-0003-4637-9813

REFERENCES

1. Giudice LC, Kao LC. Endometriosis. *Lancet*. 2004;364(9447):1789-1799.
2. Burney RO, Giudice LC. Pathogenesis and pathophysiology of endometriosis. *Fertil Steril*. 2012;98(3):511-519.
3. Eisenberg VH, Weil C, Chodick G, Shalev V. Epidemiology of endometriosis: a large population-based database study from a healthcare provider with 2 million members. *BJOG*. 2018;125(1):55-62.
4. Bozdag G. Recurrence of endometriosis: risk factors, mechanisms and biomarkers. *Womens Health*. 2015;11(5):693-699.
5. Sampson J. Intestinal adenomas of the endometrial type: their importance and their relation to ovarian hematomas of endometrial type (perforating hemorrhagic cysts of the ovary). *Arch Surg*. 1922;5(2):217-280.
6. Sampson J. Peritoneal endometriosis due to menstrual dissemination of endometrial tissue into the peritoneal cavity. *Am J Obstet Gynecol*. 1927;14(4):93-94.
7. Borghese B, Zondervan KT, Abrao MS, Chapron C, Vaiman D. Recent insights on the genetics and epigenetics of endometriosis. *Clin Genet*. 2017;91(2):254-264.
8. Saha R, Pettersson HJ, Svedberg P, et al. Heritability of endometriosis. *Fertil Steril*. 2015;104(4):947-952.
9. Wang Y, Guo SW. Statistical methods for detecting genomic alterations through array-based comparative genomic hybridization (CGH). *Front Biosci*. 2004;9:540-549.
10. Bischoff FZ, Simpson JL. Heritability and molecular genetic studies of endometriosis. *Hum Reprod Update*. 2000;6(1):37-44.
11. Pei T, Liu C, Liu T, et al. miR-194-3p represses the progesterone receptor and decidualization in eutopic endometrium from women with endometriosis. *Endocrinology*. 2018;159(7):2554-2562.
12. Zhou M, Fu J, Xiao LI, et al. miR-196a overexpression activates the MEK/ERK signal and represses the progesterone receptor and decidualization in eutopic endometrium from women with endometriosis. *Hum Reprod*. 2016;31(11):2598-2608.
13. Park JS, Lee JH, Kim M, Chang HJ, Hwang KJ, Chang KH. Endometrium from women with endometriosis shows increased proliferation activity. *Fertil Steril*. 2009;92(4):1246-1249.
14. Klemmt PA, Carver JG, Koninckx P, McVeigh EJ, Mardon HJ. Endometrial cells from women with endometriosis have increased adhesion and proliferative capacity in response to extracellular matrix components: towards a mechanistic model for endometriosis progression. *Hum Reprod*. 2007;22(12):3139-3147.
15. Lin A, Yin J, Cheng C, Yang Z, Yang H. Decreased expression of FOXA2 promotes eutopic endometrial cell proliferation and migration in patients with endometriosis. *Reprod Biomed Online*. 2018;36(2):181-187.
16. Mulayim N, Savlu A, Guzeloglu-Kayisli O, Kayisli UA, Arici A. Regulation of endometrial stromal cell matrix metalloproteinase activity and invasiveness by interleukin-8. *Fertil Steril*. 2004;81(Suppl 1):904-911.
17. Li M-Q, Hou X-F, Lv S-J, et al. CD82 gene suppression in endometrial stromal cells leads to increase of the cell invasiveness in the endometriotic milieu. *J Mol Endocrinol*. 2011;47(2):195-208.
18. Wu RF, Huang ZX, Ran J, et al. Lipoxin A4 suppresses estrogen-induced epithelial-mesenchymal transition via ALXR-dependent manner in endometriosis. *Reprod Sci*. 2018;25(4):566-578.
19. Young SL. Oestrogen and progesterone action on endometrium: a translational approach to understanding endometrial receptivity. *Reprod Biomed Online*. 2013;27(5):497-505.
20. Mulac-Jericevic B, Mullinax RA, DeMayo FJ, Lydon JP, Conneely OM. Subgroup of reproductive functions of progesterone mediated by progesterone receptor-B isoform. *Science*. 2000;289(5485):1751-1754.
21. Ferrero S, Remorgida V, Maganza C, et al. Aromatase and endometriosis: estrogens play a role. *Ann N Y Acad Sci*. 2014;1317:17-23.
22. Laux-Biehlmann A, d'Hooghe T, Zollner TM. Menstruation pulls the trigger for inflammation and pain in endometriosis. *Trends Pharmacol Sci*. 2015;36(5):270-276.
23. Chen S, Wu RF, Su L, Zhou WD, Zhu MB, Chen QH. Lipoxin A4 regulates expression of the estrogen receptor and inhibits 17beta-estradiol induced p38 mitogen-activated protein kinase phosphorylation in human endometriotic stromal cells. *Fertil Steril*. 2014;102(1):264-271.
24. Yuhki M, Kajitani T, Mizuno T, Aoki Y, Maruyama T. Establishment of an immortalized human endometrial stromal cell line with functional responses to ovarian stimuli. *Reprod Biol Endocrinol*. 2011;9:104.
25. Holdsworth-Carson SJ, Colgrave EM, Donoghue JF, et al. Generation of immortalized human endometrial stromal cell lines with different endometriosis risk genotypes. *Mol Hum Reprod*. 2019;25(4):194-205.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Huang Z-X, Mao X-M, Lin D-C, et al. Establishment and characterization of immortalized human eutopic endometrial stromal cells. *Am J Reprod Immunol*. 2019;00:e13213. https://doi.org/10.1111/aji.13213