Int J Clin Exp Med 2016;9(8):16450-16458
www.ijcem.com /ISSN:1940-5901/IJCEM0027741

Original Article

Establishment of human endometriosis-derived immortalized eutopic endometrium stromal and epithelial cell lines

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Received March 8, 2016; Accepted June 3, 2016; Epub August 15, 2016; Published August 30, 2016

Abstract: Endometriosis (EM) is commonly seen in women at reproductive age and can have a significant social and psychological impact. However, reliable, simple in vitro models of endometriosis are not common. To establish human EM-derived immortalized cell lines, we transfected cells from eutopic and ectopic endometria of endometriosis patients with the plasmid PCD2SV40T. The cells were then screened with G418, and monoclonal cells were isolated by the limited dilution method. Subclones were evaluated by karyotype analysis, immunocytochemistry, reverse transcription-polymerase chain reaction, cell doubling time, and nude mice implantation. The established immortalized eutopic stromal cell line was named hEM15A, and the ectopic epithelial cell line was named hEM5B2. hEM15A cells exhibited the characteristics of endometrium stromal cells, and no abnormalities in chromosome structure or number were observed. hEM5B2 cells exhibited the characteristics of epithelial cells, but had a polyploid karyotype. Both of these cell lines could be passaged continuously. No tumourigenicity was observed. hEM15A is the first immortalized EM-derived eutopic endometrium stromal cell line, and hEM5B2 is an immortalized ectopic endometrium epithelial cell line from Asian EM tissues. These cells could facilitate studies on the aetiology, molecular mechanisms, and therapeutic models of EM.

Keywords: Endometriosis, cell culture, immortalized cell line, SV40

Introduction

Endometriosis (EM) is a common gynaecological disease in women at reproductive age, in which the endometrium (gland and/or stroma) forms and grows outside the uterus. EM can cause pain, infertility, pelvic masses, and other symptoms through invasion, migration, and bleeding of the ectopic endometrium [1]. Although considered as benign disease, EM exhibits malignant biological behaviours such as invasion, metastasis, and recurrence [2].

Currently, rats, mice and rabbits are used in the most common *in vivo* models of EM. As rodents do not menstruate, they cannot develop spontaneous EM, and are therefore unsuitable for studies of EM pathogenesis or risk factors [3]. Macaques and baboons can mimic natural EM development [4], but these models are costly and present ethical limitations. Primary cells and cultured tissues are the main *in vitro* models for EM studies, but are limited by short lifespan, poor homogeneity, and need for advanced culture technology. The major limitations of recently developed EM tissue models [5], which enable direct and continuous observation of endometrium-peritoneum interactions, are the heterogeneity of specimen sources and the challenges associated with acquisition. Establishing immortalized EM cell lines that reflect the biological characteristics of EM and enable experimentation and in a homogenous culture are urgently needed.

Human cell lines can often be immortalized by treatment with Simian Virus 40 T antigen (SV40T), human papillomavirus E6/E7 (HPVE6/E7), human telomerase reverse transcriptase (hTERT), and other methods. SV40T is widely used in the immortalization of epithelial cells, and endometrial glandular epithelial cells are reportedly sensitive to SV40T [6]. However, the

process of inducing cell immortalization by SV40T is complicated and may involve the cell-cycle regulatory proteins P53, SNE6, and pRB, among others [7]. Numerous studies have shown that SV40T both increases growth rates of transfected cells and retains the original cells' differentiated phenotype, thus allowing cells to maintain the biological characteristics of the original cells [8].

Here, we report the establishment and identification of the first human EM-derived immortalized eutopic endometrium stromal cell line (hEM15A) and an ectopic endometrium epithelial cell line (hEM5B2) following transfection with SV40T.

Materials and methods

Endometriotic tissue and primary cell culture

Ectopic and eutopic endometrium tissue samples were obtained from patients who had histologically confirmed EM and underwent laparoscopic ovarian cystectomies at Peking University People's Hospital (**Table 1**). All of the patients signed informed consent forms before participating in this study, which was approved by the Institutional Review Board of Peking University People's Hospital. None of the patients had received any steroid hormone treatment within 3 months before operation. Biopsy material was stored in sterile saline at 4°C and transported to the laboratory. We isolated and cultured the endometrial stromal cells and epithelial cells as described in the literature [9], with some modifications. Fresh endometrial biopsy specimens were washed three times with DMEM/F12 complete medium (Gibco, USA). Blood clots were then removed, and lesions rich in blood vessels were retained. The tissues were minced into small pieces (1 mm^3) and centrifuged in 50-mL centrifuge tubes for 5 min at 1000 rpm. After the supernatant was discarded, the tissues were incubated for 1 h at 37°C in a shaking water bath (150 rpm) in serum-free DMEM/F12 containing 1 mg/mL collagenase type IV (Gibco, USA). The resulting dispersed endometrial cells were filtered through a 150-μm cell strainer. After filtration again through a 50-μm cell strainer, the epithelial cells were collected by backwashing the sieve with complete DMEM/F12 medium, whereas dispersed stromal cells were retained in the filtrate. The epithelial and stromal cells were then centrifuged for 5 min at 1000 rpm and suspended in 1 mL of DMEM/F12 containing 15% foetal bovine serum (FBS; Gibco, USA). Cells were seeded in 25-mL culture flasks (Nunc, USA) at 1×10^6/mL and kept at 37°C in an incubator with 5% CO_2.

Plasmid extraction and transfection

The PCD2SV40T plasmid was constructed by Guangzhi Liu, Gynecology Oncology Center, Peking University People's Hospital. After expansion of PCD2SV40T/DH5a bacteria, the PCD-2SV40T plasmid was extracted using a Easy-Pure Plasmid MiniPrep Kit (Transgen Biotech, China) and the gene sequence was identified (BGI, China). The plasmid was transfected into the second generation of primary cells using FugeneHD reagent (Roche, Basel, Switzerland) when the cells reached 75% confluence. Cells were screened by adding 100 μg/mL G418 (Gibco, USA) for 14 days, beginning 24 h after transfection. Survival-resistant clones were passaged and amplified. Monoclonal cells were isolated by the limited dilution method.

Immunocytochemistry

The 15th, 30th and 50th passages of the epithelial and stromal cells were cultured on small glass slides in a 24-well dish for 24 h, fixed in 4% paraformaldehyde containing 0.015% Triton X-100 (ZSGB-Bio, China), and washed in phosphate-buffered saline (PBS) three times. Sections were quenched sequentially in 3% H_2O_2 (ZSGB-Bio), washed three times in PBS and blocked with 5% sheep serum (ZSGB-Bio) for 1 h at room temperature. Slides were then incubated at 4°C overnight with anti-cytokeratin C11 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:1000), anti-vimentin V9 (Santa Cruz Biotechnology, 1:1000), and anti-fibroblast anti-F (Abcam, Cambridge, UK; 1:100) antibodies. Slides were washed three times in PBS; primary antibodies were detected using a biotin-streptavidin HRP detection system (ZSGB-Bio, SP9000) according to the manufacturer's protocol. Control slides omitted the primary antiserum.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was prepared from hEM15A and hEM5B2 cells at passages 20 and 40 using TRIzol reagent (Invitrogen, USA), strictly accord-

Table 1. Characteristics of endometriosis specimens of enrolled patients

Tissue origin	Cycle phase	Number of patients	Age (years)
Single ovary	Proliferative phase	9	27-38
	Secretory phase	3	23-42
Both ovaries	Proliferative phase	6	28-42
	Secretory phase	1	33
Single ovary and eutopic endometrium	Secretory phase	3	30-39
Single ovary and abdominal wall	Secretory phase	1	47
Both ovaries and eutopic endometrium	Proliferative phase	1	28
Eutopic endometrium	Proliferative phase	1	28

ing to manufacturer's instructions. First-strand cDNA was synthesized from 5 μg of total RNA by reverse transcription using SuperScript III Reverse Transcriptase (Invitrogen) with random primers. We used 2 μl of the product cDNA for the PCR reaction with 2×Taq PCR MasterMix (Tiangen Bio, China). Specific primers (Invitrogen) were used for each gene analysed: *SV40T* forward, 5'-CTCAGCCACAGGTCTGTACCA-3', reverse, 5'-TTGCCTGGAACGCAGTGAG-3'; oestrogen receptor (*ER*)-*α* forward, 5'-GGCCAGTACCAATGACAAGG-3', reverse, 5'-GGTTGGTGGCTGGACACATA-3'; *ER-β* forward, 5'-GGTCCATCGCCAGTTATCAC-3', reverse, 5'-ACAGCGCAGAAGTGAGCATC-3'; progesterone receptor (*PR*) forward, 5-GCATCAGGCTGTCATTATGG-3', reverse, 5'-ATGCCAGCCTGACAGCACT-3'; and *β-actin* forward, 5-CCACTGGCATCGTGATGG-3' and reverse, 5'-CGCTCGGTGAGGATCTTCAT-3'. Each cDNA sample was run in triplicate. Results were standardized to those for *β-actin*. PCR products were visualized on a 1% agarose gel.

Estimation of doubling time

HEM15A and hEM5B2 cells at passage 50 were initially seeded at 1 × 10^4 cells/mL in 6-well flat-bottomed plates and cultured in DMEM-F12 medium supplemented with 15% FBS. Cells were counted every 24 h. Cells in three wells were dissociated with trypsin/EDTA and counted. Average cell counts were used to draw cell growth curves.

Karyotyping

HEM15A and hEM5B2 cells at passages 12 and 40 were dissociated and centrifuged. The cells were then incubated for 30 min at 37°C in a hypotonic solution containing 0.075 M potassium chloride and fixed using three incubations with methanol: acetic acid (3:1) for 30 min. Chromosomes on air-dried slides were G-banded by trypsin using Giemsa staining.

Mouse xenograft experiments

The hEM15A, hEM5B2, and SKOV3 cells were resuspended in media at 10^7 cells/mL. We divided 15 6-week-old female BALB/c *nu/nu* mice (Vital River Laboratories, China) into three groups, which received subaxillary injections (200 μL) of hEM15A, hEM5B2, or SKOV3 cells. Tumour growth was monitored by weekly inspection over 90 days. All experiments were conducted with approval from the ethics committee.

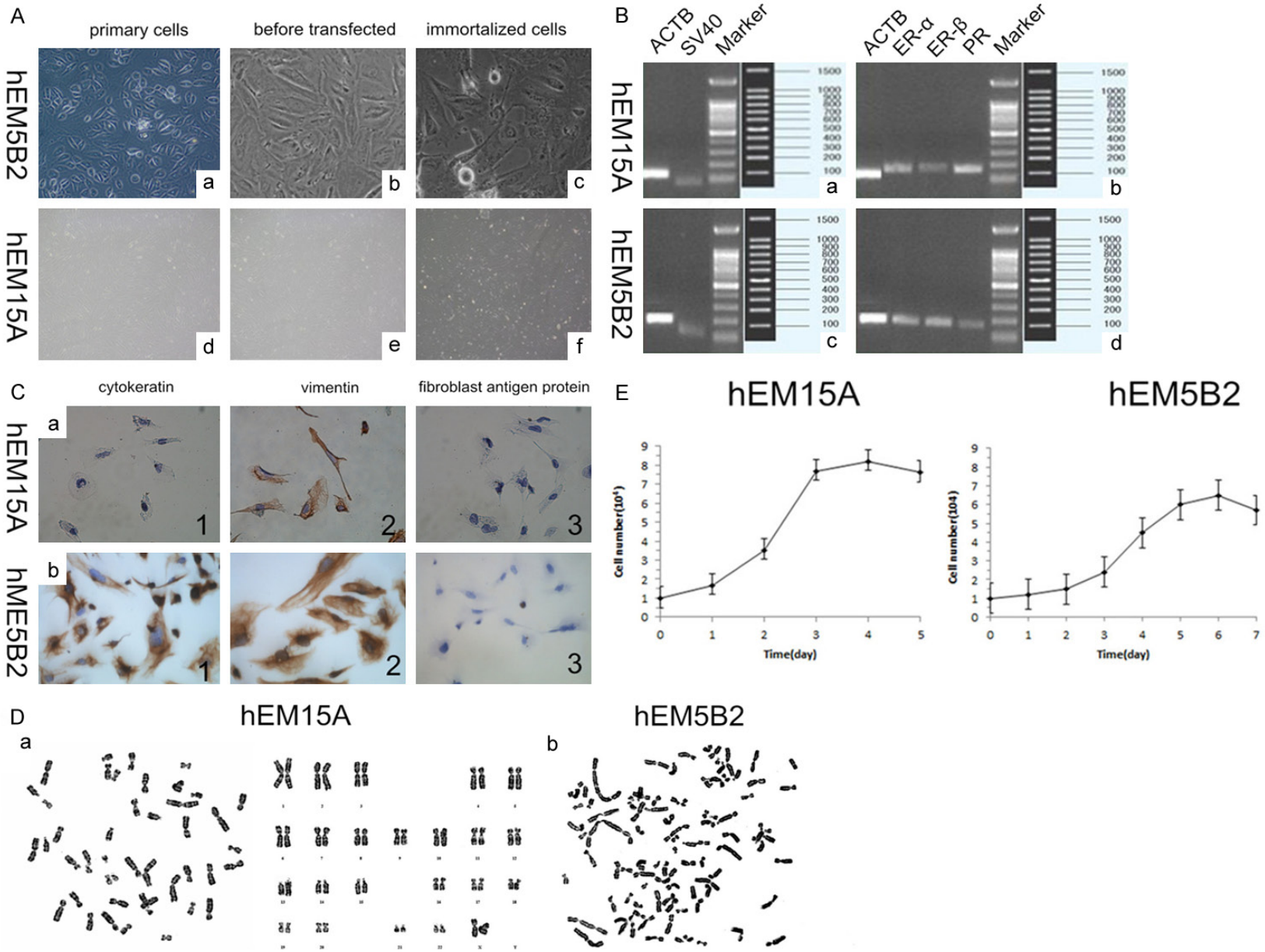
Results

In vitro growth and morphological properties of primary cells

We collected 38 tissue samples of peritoneal implants, ovarian endometriomas and eutopic endometrium from women with EM (**Table 1**), 32 (84.2%) of which were successfully cultured. The primary epithelial cells of the ectopic endometrium began to attach to the plates 24 h after being isolated from the tissues and grew in clusters or a spiral shape (**Figure 1Aa**). These cells survived *in vitro* for only approximately two weeks under natural conditions. Isolated stromal cells of the eutopic endometrium attached to the plate faster than the epithelial cells, within approximately 12 h. These cells exhibited spiral shapes (**Figure 1Ad**) and had better cell activity and condition; these cell survived approximately 1 month *in vitro* (about 10 passages).

Characterization of hEM15A and hEM5B2 cells

We successfully established two SV40T-immortalized endometriosis-derived cell lines: one from the eutopic endometrium stroma, designated hEM15A, and the other from the ectopic endometrium epithelium, named hEM5B2. The hEM15A cell line originated from the


A
primary cells
before transfected
immortalized cells
hEM5B2
hEM15A
a
b
c
d
e
f
B
ACTB
SV40
Marker
ACTB
ER-α
ER-β
PR
Marker
hEM15A
hEM5B2
1500
1000
900
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200
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a
b
c
d
C
cytokeratin
vimentin
fibroblast antigen protein
hEM15A
hME5B2
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b
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D
hEM15A
hEM5B2
a
b
E
hEM15A
hEM5B2
Cell number(10⁴)
Time(day)
Cell number(10⁴)
Time(day)

Figure 1. Characteristics of immortalized cell lines. A. Phase-contrast microscopy of the primary cultured cells and their generated immortalized daughter cell lines. (a) Primary cultured epithelial cells (magnification: 200×). (b) Primary cultured epithelial cells before transfection with the plasmid (magnification: 200×). (c) hEM5B2 cells (magnification: 200×). (d) Primary cultured stromal cells (magnification: 200×). (e) Primary cultured stromal cells before transfection with the plasmid (magnification: 200×). (f) hEM15A cells (magnification: 200×). B. RT-PCR shows expression of SV40 and hormone receptor mRNA in EM cell lines. β-actin (145 bp; lane 1); and SV40 (97 bp; lane 2) of (a) hEM15A and (b) hEM5B2 cells; and β-actin (lane 1); ER-α (182 bp; lane 2); ER-β (83 bp; lane 3); and PR (176 bp; lane 4) of hEM15A (c) and hEM5B2 (d) cells. M: marker. C. Immunocytochemical staining for cytokeratin (CK), vimentin (VI), and fibroblast antigen protein (FAP) in EM cell lines. (a) hEM15A cells: CK^- (1), VI^+ (2) and FAP^- (3); (b) hEM5B2 cells: CK^+ (1), VI^+ (2), and FAP^- (3). Magnification: 400×. D. G-banded karyotypes of hEM15A and hEM5B2 cells. a. hEM15A cells showed a normal diploid karyotype of 46 chromosomes. b. hEM5B2 cells showed a hypertetraploid karyotype of 98 chromosomes. E. Mean cell doubling times were 24 h for hEM15A and 64.8 h for hEM5B2.

secretory-phase eutopic endometrium of a 33-year-old patient with ovarian endometrioma. The hEM5B2 cell line originated from a 23-year-old patient with ovarian endometrioma. The specimen was obtained from the ovarian ectopic foci during the secretory phase.

Morphology

When observed by phase-contrast microscopy, hEM5B2 cells grew by adhering to the wall in a swirling or cluster-like shape, with individual cells exhibiting polygonal shapes (**Figure 1Ac**), whereas hEM15A cells had a long fusiform shape (**Figure 1Af**), similar to fibroblasts. Neither hEM15A nor hEM5B2 cells showed any significant morphological changes after transfection with the plasmid (**Figure 1Ab**, **1Ac** and **1Ae**, **1Af**).

Expression of SV40T and sex steroid receptors

To confirm successful immortalization of these cell lines, we examined expression of *SV40T* by RT-PCR. We detected *SV40T* mRNA in both hEM15A and hEM5B2 cells (**Figure 1Ba**, **1Bb**). Both hEM15A and hEM5B2 cells also expressed mRNA transcripts of typical steroid receptors: *ER-α*, *ER-β*, and *PR* (**Figure 1Bc**, **1Bd**).

Immunocytochemistry

To confirm the origin of the established cell lines, we next examined the expression of epithelial and stromal cell markers using immunocytochemistry. In particular, cytokeratin (CK) is specifically expressed in epithelial cells, vimentin (VI) is expressed in mesenchymal-like cells but with little obvious tissue specificity, and fibroblast antigen protein (FAP) is specifically expressed in fibroblasts. Staining results of hEM15A cells revealed that the immortalized stromal cells were $CK^-/VI^+/FAP^-$ (**Figure 1Ca**); whereas the hEM5B2 cells consistently shown to be $CK^+/VI^+/FAP^-$ (**Figure 1Cb**).

Karyotype

Karyotype analysis revealed that hEM15A cells had a normal diploid karyotype with no clonal structural or numerical chromosome abnormalities (**Figure 1Da**). However, hEM5B2 cells contained between 78 and 123 chromosomes, exhibiting a hypertetraploid karyotype with chromosome breakage, deletion, and translocation in ~84% of the cells. The other cells (approximately 16%) exhibited hypodiploid, hyperheptaploid, and hyperoctoploid karyotypes (**Figure 1Db**).

Doubling time

The immortalized cell lines were routinely maintained in DMEM/F12 medium supplemented with 15% FBS. Direct viable cell counts indicated that their mean doubling times were ~24 h for hEM15A cells and ~64.8 h for hEM5B2 cells (**Figure 1E**).

Growth in nude mice

The tumourigenicity of the established cell lines was examined using nude mice. Control SKOV3 cells formed subcutaneous tumours 7 days after inoculation. Mice in the control group were killed 3 weeks later, when the mean volume of the tumours was approximately 22 mm^3. However, test group 1, which was inoculated with hEM15A cells, and test group 2, which was inoculated with hEM5B2 cells, did not exhibit tumour formation, even at 3 months after inoculation.

Discussion

EM is a common disease that affects up to 10% of reproductive-aged women. Although EM was first described more than 150 years ago, its pathogenesis remains unclear. EM is both

physically and psychologically debilitating, as it causes pain and infertility. Moreover, EM is associated with a high rate of relapse after treatment. Hence, better *in vitro* models of EM are urgently needed for both basic research and clinical investigations, and could benefit studies of EM physiology, pathology, and therapy. EM-derived immortalized cell lines have been established by several laboratories within the last 20 years and have provided practical and convenient research models (**Table 2**). Immortalization via SV40T or hTERT transfection results in greater homogeneity and less limitation in terms of lifespan; additionally, immortalized cells are easily accessible and can be cultured indefinitely [10-14]. An *in vitro* 3D spheroid model of endometriosis was reportedly established using the epithelial cell line EEC16 and the well-characterized endometriosis cell line EEC12Z [15].

Many laboratories in China have already successfully developed primary cultures of EM-derived cells; however, none of these cells have been immortalized. In this study, we established two human endometriosis-derived immortalized cell lines by introducing SV40T: the first, hEM15A, is the first eutopic endometrium stromal cell line derived from a patient with EM, and the second, hEM5B2, is an ectopic endometrium epithelial cell line. The hEM-15A cells were $CKCK^{-}/VI^{+}/FAP^{-}$ and also expressed typical sex steroid receptors, such as ER-α, ER-β, and PR. Cytokeratin is widely expressed in epithelial tissues and cells; VI has no significant tissue specificity, but is primarily expressed in the stroma and can be found in epithelial tissues; and FAP is specifically expressed in fibroblasts. Based on these results, we concluded that hEM15A cells were human endometriosis-derived eutopic endometrium stromal cells.

The hEM5B2 cell line was derived from the ovarian ectopic foci during the secretory phase. Consistent with previous reports [10-14], immunostaining showed the cells to be $CK^{+}/VI^{+}/FAP^{-}$. Because the cells also expressed ER-α, ER-β, and PR, we believed that hEM5B2 cells were human endometriosis-derived ectopic endometrium epithelium cells. As EM is a hormone-dependent disease, eutopic endometrium and ectopic endometrial lesions contain ER-α, ER-β, and PR [13]. Thus, the hEM15A and hEM5B2 cells maintain the original expression patterns for steroid receptors and may therefore be used to investigate EM-hormone interactions.

The consistent expression of SV40T in hEM-15A and hEM5B2 cells (as shown by RT-PCR) demonstrates stable transfection with the PCD2SV40T plasmid. Both hEM15A and hEM-5B2 cell lines have now been maintained *in vitro* for over 60 passages, indicating that they were successfully immortalized by SV40T. Notably, neither of the two patients has been diagnosed with EM-associated ovarian cancer or other cancers. Chromosome analysis revealed that hEM5B2 cells had an aneuploid karyotype. The distribution of chromosome numbers showed a peak in the hypertetraploid region of 78-123 chromosomes. This karyotypic alteration was more remarkable than that reported in other studies [10-14]. For example, an endometriotic epithelial cell line (Clo03) immortalized by SV40 also had a hypertriploid karyotype, a spontaneously immortalized endometriotic epithelial cell line (FbEM-1) had a hypotetraploid karyotype, and epithelial and stromal endometriotic cell lines immortalized by hTERT both had no clonal structural or numerical chromosome abnormalities. The normal diploid karyotype of the hEM15A cells was unexpected. Indeed, several reports have shown that some cells present an altered karyotype after transfection with SV40T and immortalization [16, 17], and some do not [18, 19]; the underlying mechanism of this phenomenon remains unclear. It is known that *SV-40T*, as a viral oncogene, integrates into the host cell DNA and regulates the cell cycle via a variety of pathways; SV40T can even transform normal cells into malignant cells. Transformation to tumour cells is a complex process, typically requiring at least four steps. First, the length of the telomere must be protected. Then, SV40T is introduced, followed by inactivation of the p53 and pRB pathways. The last step is the activation of *Ras* and other oncogenes. The cells remain at the immortalization stage before oncogene activation, which means the immortalized cells are incompletely transformed cells at a stage between normal cells and malignant cells, with a capacity for unlimited proliferation [20]. Interestingly, despite the karyotypic alteration of hEM5B2 cells, neither hEM15A nor hEM5B2 cell lines

Table 2. Comparison of existing endometriosis-derived cell lines

	Immortalization method	Cell type	CK	Vim	Anti-F	ER-α	ER-β	PR	Karyotype	Growth in nude mice	Doubling time (h)	Growth pattern	Passages
Bouquet et al. [10]	Spontaneous	Epithelial	+	+	/	-	-	+	Hypotetraploid	-	38	Suspended	> 50
Akoum et al. [11]	SV40T	Epithelial	+	+	/	+	+	+	Hypertriploid	/	32	Adherent	80
Zeitvogel et al. [12]	SV40T	Epithelial	+	+	/	+	+	+	/	/	31	Adherent	165
		Stromal	-	+	/	/	/	/	/	/	/	Adherent	/
Boccellino et al. [13]	hTERT	Epithelial	+	/	/	+	+	+	Normal Diploid	/	/	Adherent	> 80
		Stromal	-	/	/	+	+	+	Normal Diploid	/	/	Adherent	> 80
Bono et al. [14]	cyclinD1, cdk4 and hTERT	Epithelial	+	/	/	+	/	+	/	-	/	Adherent	/
Yanhong et al.	SV40	Epithelial	+	+	-	+	+	+	Hypertetraploid	-	64.8	Adherent	> 50
		Eutopic Stromal	-	+	-	+	+	+	Normal Diploid	-	24	Adherent	> 50

showed tumourigenicity in xenograft experiments in nude mice, which is consistent with other studies [10, 14]. A possible explanation is that abnormal chromosome patterns are not always associated with malignant tumours [21].

These cell lines might be ideal *in vitro* models to explore the aetiology, molecular mechanism, and therapeutic aspects of EM. The cell line established by Akoum [11] reportedly secreted migration inhibitory factor, which promotes the mitosis of endothelial cells *in vivo*, indicating the importance of angiogenesis in ectopic endometrial cell growth [22]. Banu tested cell lines established by Zeitvogel [12] and found that the cells overexpressed prostaglandin E2 and had higher matrix metalloproteinase-2 and -9 activities [23]. These cell lines were used in many mechanistic studies, which suggested that romidepsin [24], trichostatin A [25], prostaglandin EP2/EP4 receptor inhibitors [26] in addition to tumour necrosis factor-soluble high-affinity receptor complex [27] could be potential treatments for EM.

In conclusion, we established hEM15A and hEM5B2 as novel endometriosis-derived immortalized eutopic endometrium stromal and ectopic endometrium epithelial cell lines. These two cell lines have been preserved in the China Center for Type Culture Collection and Cell Resource Center of Peking Union Medical College. These cell lines may be ideal *in vitro* models to explore the aetiology, molecular mechanisms, and therapeutic aspects of EM.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (No. 81170544).

Disclosure of conflict of interest

None.

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