SPANDIDOS PUBLICATIONS

Establishment of an immortalized human extravillous trophoblast cell line by retroviral infection of E6/E7/hTERT and its transcriptional profile during hypoxia and reoxygenation

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Abstract. Investigation into the function of human trophoblasts has been largely restricted by a lack of suitable cell models. We aimed to produce normal human trophoblast cell lines with a long lifespan and to provide an ideal *in vitro* cell model. Primary human trophoblast cells were derived from a placenta that had undergone elective abortion at the 7th week of gestation. The cells were immortalized by infection with retroviral expression vectors containing the type 16 human papillomaviruses E6 and E7 in combination with human telomerase reverse transcriptase (hTERT). Characterization of the cell line was performed by immunocytochemistry using a panel of antibodies, Western blotting, real-time RT-PCR, an invasion assay, gelatin zymography, karyotype analysis and a nude mouse assay. Gene expression profiles under hypoxia (1% O_2, 1 h) and subsequent reoxygenation (20% O_2, 6 h) were analyzed using cDNA microarray. Immunocytochemistry revealed an extravillous trophoblastic phenotype by positive staining for hCGß, cytokeratin 7, HLA-G and CD9. A transwell insert invasion assay showed the invasiveness of this cell line and gelatin zymography detected the secretion of MMP-2 and MMP-9. Karyotype analysis exhibited an almost normal chromosomal number which ranged from 46 to 48 and the cells showed no tumorigenecity in a nude mouse assay. Forty-three genes showing reversible up- or down-regulation during hypoxia were detected using an oligonucleotide array. This newly immortalized cell line, HChEpC1b, is a useful model for the study of extravillous trophoblast function.

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Introduction

In humans, cells outside the morula form the trophoectoderm and differentiate into trophoblasts. These cells form the fetal compartment of the placenta during pregnancy. After the initial phase of implantation, human trophoblasts differentiate along either the villous or the extravillous trophoblast pathway. Multinucleate syncytiotrophoblasts, which form the epithelial layer of the villi by cell fusion, are involved in the exchange of gas and the nutrients between the mother and the fetus. Mononuclear extravillous cytotrophoblasts invade deep into the decidua, the myometrium and the uterine spiral arteries (1). Clinically, many pregnancy-associated conditions result from abnormal functioning of trophoblasts, such as abortion, intrauterine growth retardation and pre-eclampsia (2).

Investigation into the function of human trophoblasts has been largely restricted by the short life span of primary cultured trophoblasts *in vitro*. There are two major mechanisms that cause the limited life span of primary cultured cells. One is the telomere-based replication senescence (3,4) and the other is telomere-independent senescence, which is thought to be controlled by the Rb/p16 (5) and p53 pathways (6). Since HPV16 E6/and E7 are known to inhibit p53 and Rb function, respectively, we introduced E6/E7 and human telomerase reverse transcriptase (hTERT) into primary human trophoblast cells to immortalize them.

We obtained one cell line, HChEpC1b, that retained an extravillous phenotype and acquired immortality. This cell line expressed molecular markers for human extravillous trophoblasts and it demonstrated invasiveness and metalloproteinase production; nevertheless, it did not show tumorigenic potency. There have been a number of trophoblast cell lines that have gained a long life span spontaneously (7-12), or by transduction of the SV40 T antigen (7,13-17), HPV16 E6/E7 (18,19) and hTERT (20). However, this is the

first study of an immortalized trophoblast cell line produced by retroviral infection of HPV16 E6/E7 in combination with hTERT and this newly established cell line may provide a useful model for the study of extravillous trophoblast function as well as that of other cell lines.

Materials and methods

Preparation of human trophoblast primary cultures. A human placenta was obtained from a patient that had undergone a legal termination of a normal pregnancy at 7 weeks of gestation with informed consent. This study was approved by the Ethics Committee of the Jikei University. After removal of all attached decidual tissue and blood clots, chorionic villi were gently shaken in normal saline solution. The fallen cells and tissue fragments were collected and suspended in RPMI-1640 (Sigma-Aldrich, Tokyo, Japan) supplemented with 10% fetal bovine serum (Invitrogen Corp., Carlsbad, CA, USA), 50 IU/ml penicillin, 50 μg/ml streptomycin, and 100 mg/ml neomycin (Invitrogen Corp) and were cultured in a collagen I coated culture dish (Asahi Techno Glass, Tokyo, Japan). Two weeks later, colonies were collected using cloning rings with 0.05% trypsin and 0.53 mM EDTA (Invitrogen Corp) and were transferred to a 24-well culture dish. When the colonies became confluent, the cells were transferred to a 6-well culture dish. Three clones with epithelioid phenotypes [human chorionic epithelium cell-1 (HChEpC1) a-c] were selected for the next immortalization step.

Vector construction and retroviral transduction of E6/E7 and hTERT. Construction of retroviral vector plamids, pCMSCV puro-hTERT, pCLXSN-16E6E7, has been described previously (21). Production of recombinant retroviruses was carried out, as previously described (22). Briefly, a retroviral vector and packaging construct, pCL-10A1, was co-transduced into 293T cells using TransIT-293 (Mirus Bio Corp., Madison, WI, USA) according to the manufacturer's instructions. The culture fluid was harvested at 48 to 72 h post-transduction. The titer of the recombinant viruses was $>1x10^5$ drug-resistant colony-forming U/ml on HeLa cells. HChEpC1 cells seeded on 24-well dishes were inoculated with a 0.5 ml aliquot of the culture fluid in the presence of polybrene (4 μg/ml). Following inoculation with viruses, the cells were grown without drug selection as mock-infected cells and stopped growing within two weeks. To achieve different combinations of retroviral infections, the cells were serially infected with MSCVpuro-hTERT and LXSN-16E6E7 at passage 2.

Cell culture. The infected HChEpC1 cells were cultured in RPMI-1640 supplemented with 10% FBS, 50 U/ml penicillin G, 50 μg/ml streptomycin, and 100 mg/ml neomycin and were incubated in 5% CO_2 air at 37˚C. Serial passages were made in one to four splits. One clone, HChEpC1b, was selected for further characterization because it had the highest purity of trophoblast cells as proved by its uniformly positive staining for hCGß. The population doubling time (DT) was calculated as follows: $DT = T \times \log_2/\log (N_T/N_0)$, where N_0 is the initial number of cells and N_T is the number of cells harvested after T h culture.

The choriocarcinoma cell lines JAR and JEG-3 and the fibroblast cell line Hs795Pl were obtained from ATCC and propagated in RPMI-1640, MEM (Invitrogen Corp) and DMEM (Invitrogen Corp) respectively, supplemented with 10% FBS.

Karyotyping. Cells in the exponential phase were arrested by adding 0.03 μg/ml of colchicine for 6 h, before being treated with hypotonic solution (75 mM KCl, pH 8.0) and fixed twice in Carnoy's fixative (methanol/acetic acid). The G-band pattern was obtained using Giemsa staining and the chromosomes of 20 metaphases were analyzed.

Nude mouse assay. Single cell suspensions (10^7 cells) of HChEpC1b cells or JAR cells were subcutaneously inoculated into the right flank of 5 female mice (balb/c nu/nu) each. These mice were observed for up to 3 months and examined for tumor growth.

Immunocytochemistry. HChEpC1b cells were cultured on collagen I coated culture slides (BD Biosciences, Bedford, MA, USA) for two days, washed once with phosphate-buffered saline (PBS, pH 7.4) and fixed with 100% ethanol (room temperature) for 30 min. After three 5-min hydration periods in distilled water (room temperature) and two 10-min periods of washing in 0.1% Tween in PBS (4˚C), the cells were incubated overnight at 4˚C with the primary antibodies diluted in Antibody Diluent (Dako, Glostrup, Denmark) as described in Table I. The cells were washed three times with 0.1% Tween in PBS (4˚C) and incubated with EnVision Labelled Polymer, Peroxidase (Dako) for 1 h at room temperature. After washing three times with 0.1% Tween in PBS (4˚C), visualization was developed according to the manufacturer's instructions using diaminobenzidine (DAB) as the substrate. The cells were counterstained with hematoxylin, dehydrated and mounted for light microscopy.

Real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR). Total RNA were isolated from subconfluent cultured cells using the RNeasy mini kit (Qiagen, Gmbh Hilden, Germany). Reverse transcription was performed according to the manufacturer's protocol (Takara RNA PCR kit, Takara, Shiga, Japan) with 1 μg of total RNA. For real-time PCR, each target was amplified on the same plate with GAPDH as the reference using the Taq Man Universal PCR master mix and the ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, USA) and the relative mRNA amounts and range were determined. The primer/probe sets were purchased from Taq Man gene expression assays (Applied Biosystems, Assay IDs are listed in Table II). Relative mRNA levels were calculated by the comparative C_T method described in ABI User Bulletin 2.

Western blotting. Subconfluent HChEpC1b, JAR, JEG-3 and Hs795Pl cells were scraped from culture dishes, washed three times with cold PBS, and then incubated in the RIPA buffer [10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, complete mini (Roche, Basel, Switzerland)] for 30 min on ice. After centrifugation, the supernatants were subjected to Western blot

ntibodies used for immunocytochemistry and Western blotting.

Epitope	Clone name	Host	Isotype	Company	Cat. no.	Nation	Dilution
Cytokeratin 7	OVTL 12-30	Mouse	IgG1 κ	Acris Antibodies	DM057	Germany	1:50
HLA class I	W6/32HL	Mouse	IgG2a	Chemicon International	CBL139	USA	1:50
HLA-G	87G	Mouse	IgG2a	Exbio	10-437-C100	Czech	1:100
HLA-G	MEM-G/1	Mouse	IgG1	Serotec	MCA2043	UK	1:50
CD9	Polyclonal	Rabbit	IgG	Santa Cruz Biotechnology	sc-9148	USA	1:50
CD9	M-L13	Mouse	IgG1 κ	BD Biosciences	555370	USA	1:50
Vimentin	V9	Mouse	IgG1	Chemicon International	MAB3400	USA	1:200
E-cadherin	Polyclonal	Rabbit	IgG	Santa Cruz Biotechnology	sc-7870	USA	1:50
hCG ß	Polyclonal	Rabbit	IgG	Dako	A0231	USA	1:50
hPL	Polyclonal	Rabbit	IgG	Lab Vision	RB-9067-P	UK	1:50
PLAP	8B6	Mouse	IgG2a	Chemicon International	CBL207	USA	1:50

Table II. Details of assay ID for real-time PCR.

Gene	Assay ID
Cytokeratin 7	Hs00818825_m1
HLA-G	Hs00918802_m1
CD9	Hs00233521_m1
HPL	Hs01862611_g1
PLAP	Hs01654626_s1
Vimentin	Hs00185584_m1
Integrin α-1	Hs00235006_m1
Integrin α-6	Hs00173952_m1
Integrin ß-1	Hs00559595_m1
Integrin ß-4	Hs00236216_m1

analysis. Total proteins (20 μg) per sample was electrophoresed on a 4-20% SDS-polyacrylamide gel and transferred to a PDVF membrane. After washing in TBST (0.1% Tween in Tris-buffered saline), the membrane was incubated with primary antibodies (anti-cytokeratin 7, 1:500; anti-E-cadherin, 1:500; anti-vimentin, 1:1000) for 1 h at 37˚C, before being washed in TBST and incubated with secondary HRP-conjugated goat anti mouse or rabbit IgG goat antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at 37˚C. The antigen-antibody complexes were visualized using DAB.

Gelatin zymography. The gelatinolytic activities of matrix metalloproteinase-2 (MMP-2) and MMP-9 were assayed using gelatin zymography. Subconfluent cells were rinsed and cultured with serum-free medium for three days. The harvested medium was mixed 1:1 with 2X sample buffer [125 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol] and was then applied to gels for electrophoresis without boiling under nonreducing conditions in a 10% acrylamide gel containing 1 mg/ml gelatin (Invitrogen Corp). After electrophoresis, the gels were washed at room temperature for 1 h in a buffer consisting of 50 mM Tris-HCl (pH 7.5) and 2.5% Triton X-100 to remove SDS and were then incubated overnight in a buffer consisting of 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 10 mM $CaCl_2$ and 0.05% NaN_3 at 37˚C. The gels were stained with 0.25% Coomassie Brilliant Blue R-250 in 50% methanol and 5% acetic acid for 60 min and destained in 5% methanol and 7% acetic acid. MMP-2 and MMP-9 were visualized as clear bands against a dark background at 72 and 92 kDa, respectively.

In vitro invasion assay. Invasion assays were carried out using BD Matrigel Invasion Chambers, 24-well plate, 8-mm pore size (BD Biosciences). Cells were seeded at a density of 25,000 cells/well in 500 μl culture medium. Following incubation periods of 24 and 48 h, the filter inserts were removed and washed twice with PBS and the upper side of the filter was cleaned with a cotton tipped swab. For assessment of the number of invaded cells, the filters were fixed and stained with 0.2% crystal violet in 4% formaldehyde. The total number of cells that had invaded onto the underside of the filter was counted manually under a light microscope.

Hypoxia-reoxygenation treatment. HChEpC1b cells were incubated under normoxic conditions (20% O_2) and then were subjected to hypoxia (1% O_2) for 1 h and subsequent reoxygenation under normoxic conditions (20% O_2) for 6 h. At the end of each culture period, cells were scraped from culture dishes and were centrifuged and snap-frozen. All samples were stored at -80˚C before analysis and total RNA were isolated using the RNeasy mini kit (Qiagen).

Microarray analysis. Human genome-wide gene expression was examined using the Human Genome U133 Plus 2.0 Array (HG-U133 Plus 2.0 Set: GeneChip, Affymetrix, Santa Clara, CA, USA), which contains almost 45,000 probe sets, representing >39,000 transcripts derived from ~33,000 well-substantiated human genes (http://www.affymetrix.com/products/arrays/specific/hgu133.affx). Double-stranded cDNA was synthesized and the cDNA was subjected to *in vitro* transcription in the presence of biotinylated nucleotide triphosphates. Ten micrograms of the biotinylated cRNA was hybridized with a probe array for 16 h at 45˚C and the

Table III. Expression of marker molecules for trophoblasts by immunocytochemistry.

	HChEpC1b	JAR	JEG-3	Hs795Pl
Cytokeratin 7[a]	+	+	+	-
HLA-G	+	-	+	-
CD9	+	+	+	+
Vimentin[a]	+	-	-	+
E-cadherin[a]	-	+	+	-
hCG	+	+	+	-
hPL	+	+	+	-
PLAP	-	+	+	-

[a]Same results were confirmed by Western blotting.

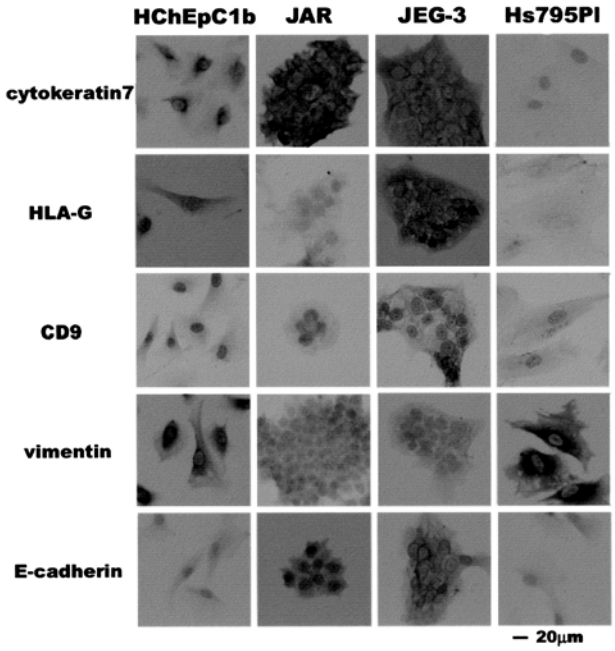

Figure 1. Immunocytochemistry of HChEpC1b, JAR, JEG-3 and Hs795Pl (each column, left to right), stained by cytokeratin 7, HLA-G, CD9, vimentin and E-cadherin (each row, top to bottom) at passage 75.

hybridized biotinylated cRNA was stained with streptavidin-phycoerythrin and was then scanned with a Gene Array Scanner. The fluorescence intensity of each probe was quantified using the GeneChip Analysis Suite 5.0 software (Affymetrix). The expression level of a single RNA strand was set as the average fluorescence intensity among the intensities obtained from 11-paired (perfect-matched and single nucleotide-mismatched) probes consisting of 25-mer oligonucleotides. If the intensities of mismatched probes were very high, gene expression was judged to be absent even if a high average fluorescence was obtained with the Microarray Analysis Suite 5.0 program (MAS5.0). The data was processed with Affimetrix's default parameters, except for scaling (Target Intensity 1000), without normalization procedures to calculate the level of gene expression as the signal.

Statistics. Assuming the changes are reversible, the detection p-value at normoxic conditions (P1) should equal to that at subsequent reoxygenation (P3). Therefore, we deleted genes for which the value P3/P1 was outside of the range 0.75-1.25. Then, we selected genes of which P2 x 2/ (P1 + P3) was >2, suggesting up-regulation by hypoxia (P2 was the detection p-value under hypoxic conditions). On the other hand, genes for which P2 x 2/(P1 + P3) was <0.5, were defined as down-regulated. The analysis was performed with Excel (Microsoft Office Excel 2007).

Results

Immortalization of HChEpC cells. After transduction of hTERT and HPV16 E6/E7, the clone HChEpC1b demonstrated continuous growth for >80 passages, which is equivalent to 160 population doublings at a split ratio of 1:4. The population doubling time was ~24 h. As no indication of cellular crisis throughout this period was observed, the cells were considered to be virtually immortalized.

Extravillous trophoblast phenotype of HChEpC1b. Immunocytochemistry was performed in JAR and JEG-3 cells, which are choriocarcinoma cell lines, and Hs795Pl, which is the fibroblast cell line that was derived from the term placenta. HChEpC1b showed positive staining for cytokeratin 7, HLA-G, CD9, hCG, hPL, and vimentin, but was negative for E-cadherin and PLAP (Fig. 1 and Table III). As discussed in the international workshop on trophoblast cell lines (23), the minimum characteristic requirements for an extravillous trophoblast cell line are the positive expression of cytokeratin 7, HLA-G and CD9. By immunocytochemistry, HChEpC1b showed expression of all of these extravillous markers. Some of these results were confirmed by Western blotting using the same antibodies.

Real-time RT-PCR demonstrated the same expression profiles as the immunological studies for CK7, HLA-G, CD7 and vimentin (Fig. 2.). The ability of these cells to produce hPL and PLAP was low compared to that of the chorio-carcinoma cells. The integrin expression profile revealed an extravillous trophoblastic phenotype.

The transwell invasion assay showed the invasiveness of HChEpC1b (Fig. 3A) and gelatin zymography detected the secretion of MMP-2 and MMP-9 (Fig. 3B). These results fulfill the biological criteria of extravillous trophoblasts suggested in the IFPA Workshop (24).

Growth potential, karyotyping and transformed properties. G-banding staining of HChEpC1b was conducted at passage 6, 14, 25 and 75. Karyotype analysis exhibited almost normal chromosomal numbers ranging from 46 to 48, but also contained minor structural alternations, probably associated with p53 inactivation (Table IV).

No tumors developed in nude mice after injection with HChEpC1b after a 3-month period. Therefore, immortalization of HChEpC1b was not accompanied by tumorigenic transformation.

Doubling time and karyotype of HChEpC1b.

Passage	Doubling time (h)	Chromosome no.	Karyotype
6	21.3	47	47,XY,+20[18]/ 47,idem,-14,+mar[2]
14	ND	46-47	47,XY,+20[2]/ 47,idem,-14,+mar[12]/ 46,XY[6]
25	24	46-48	47,XY,+20[7]/ 47,idem,-14,+mar[6]/ 46,idem,der(4;16)(p10;q10)[1]/ 48,idem,+6[1]/ 47,idem,i(14)(q10)[1]/ 47,XY,+14[1]/ 46,XY[3]
75	23.9	46-47	46,XY,add(11)(q13or21),-13,+20[14]/ 46,idem,+14,der(14;21)(q10;q10)[1]/ 47,idem,+der(1;2)(p10;q10),add(14)(p10),add(14)(q22)[1]/ 46,idem,t(13;16)(p10;p10)[1]/ 46,idem,add(18)(q12)[1]/ 47,idem,+mar1[1]/ 46,idem,-18,+mar2[1]

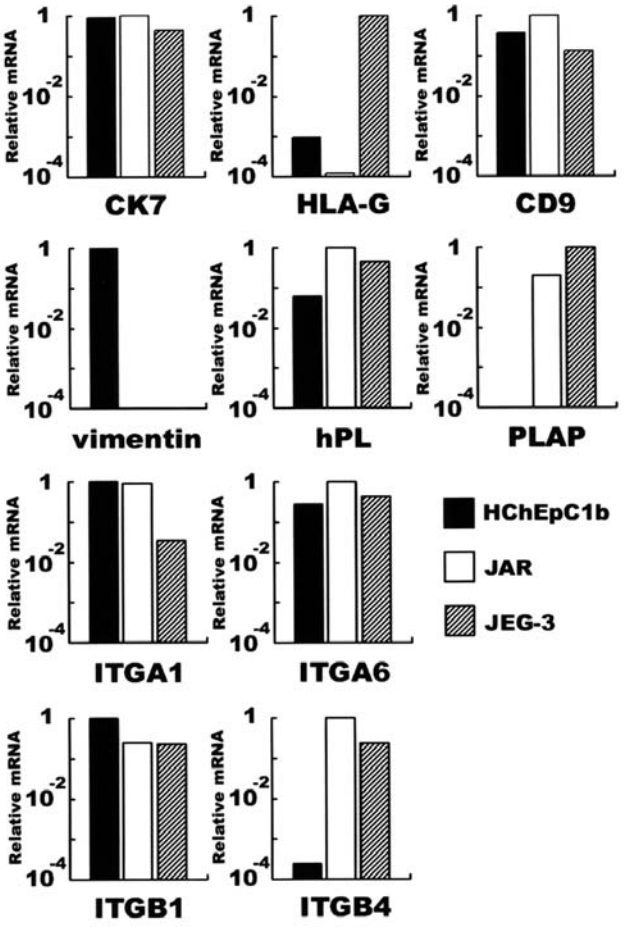

Figure 2. Real-time RT-PCR to detect the mRNA expression of molecular markers for trophoblast characterization.

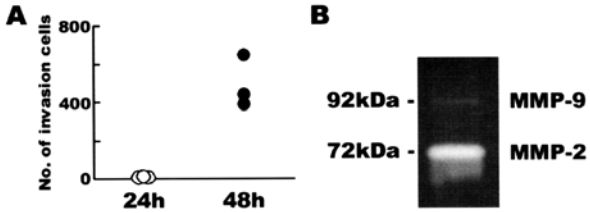

Figure 3. (A) A transwell insert invasion assay to show the invasiveness of HChEpC1b. The number of cells indicates the total count of cells that invaded through the insert membrane, which is coated with Matrigel Matrix, toward the underside of the membrane. (B) Gelatin zymography showing the secretion of MMP-9 and MMP-2.

Responsiveness to hypoxia reoxygenation treatment. The up-regulated and down-regulated genes that showed reversible expression in hypo-normo oxidative conditions are shown in Tables V and VI, respectively.

Discussion

In the present study, we established the cell line HChEpC1b, that acquired immortality after HPV16 E6/E7 and hTERT introduction. This cell line expressed molecular markers for human extravillous trophoblasts, including cytokeratin 7, HLA-G and CD9. Study of the biology of trophoblasts has been limited by the lack of suitable cell models that represent this unique cell population *in vitro*. In the past, choriocarcinoma cell lines, such as BeWo (25), JEG (26) and JAR (27) were the only immortal cell lines available and have been used extensively to study placental functions regardless of limitations related to their malignant origins. Recently, several cell lines have been established by transfection with viral

Table V. Up-regulated genes showing reversible expression in hypo-normo oxidative conditions.

UniGene ID_Affymetrix	Gene title
Hs.427236	MyoD family inhibitor domain containing
Hs.431081	Ubiquitin-specific peptidase 53
Hs.29725	Hypothetical protein FLJ13197
Hs.435991	Chromosome 4 open reading frame 16
Hs.469970	Splicing factor, arginine/serine-rich 4
Hs.522074	TSC22 domain family, member 3
Hs.631772	Solute carrier family 25 (mitochondrial carrier; ornithine transporter) member 15
---	Zinc finger protein 252
Hs.533499	Membrane-associated DNA binding protein
Hs.492348	SLAM family member 6
Hs.469840	Cytoplasmic linker-associated protein 1
Hs.369430	Peptidylglycine α-amidating monooxygenase
Hs.436121	Coiled-coil domain containing 112
Hs.459790	Vacuolar protein sorting 13 homolog A (*S. cerevisiae*)
Hs.302085	Prostaglandin I2 (prostacyclin) synthase /// prostaglandin I2 (prostacyclin) synthase
Hs.157818	Potassium voltage-gated channel, shaker-related subfamily, ß member 1
Hs.413801	Proteasome (prosome, macropain) activator subunit 4
Hs.512152	HLA-G histocompatibility antigen, class I, G
Hs.124565	Solute carrier family 23 (nucleobase transporters), member 3
Hs.441124	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3F

Table VI. Down-regulated genes showing reversible expression in hypo-normo oxidative conditions.

UniGene ID_Affymetrix	Gene title
Hs.368912	Dipeptidyl-peptidase 4 (CD26, adenosine deaminase complexing protein 2)
Hs.357128	Molybdenum cofactor synthesis 1 /// molybdenum cofactor synthesis 1
Hs.533499	Membrane associated DNA binding protein
Hs.617709	Chromosome 1 open reading frame 136
Hs.12929	Chromosome 17 open reading frame 80
Hs.470907	Adenylate kinase 2
Hs.471040	Hypothetical protein FLJ38973
Hs.529577	U2-associated SR140 protein
Hs.478000	Muscleblind-like (*Drosophila*)
Hs.502182	Brain-derived neurotrophic factor
Hs.443301	Centrosomal protein 63 kDa
Hs.321176	Transcribed locus, weakly similar to XP_850647.1 PREDICTED: similar to LINE-1 reverse transcriptase homolog [*Canis familiaris*]
Hs.28780	Zinc finger protein 449
Hs.554740	Fanconi anemia, complementation group B
Hs.340623	Hypothetical protein LOC644873
Hs.569809	Rho GTPase activating protein 27
Hs.461860	Nucleoporin 214 kDa
Hs.452398	MRNA; cDNA DKFZp564E143 (from clone DKFZp564E143)
Hs.16004	Chromosome 10 open reading frame 76
Hs.612891	Hypothetical protein FLJ21272
Hs.59159	Chromodomain helicase DNA binding protein 9
Hs.397001	CDNA clone IMAGE:4304686
Hs.512682	Carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)

's. The most commonly used gene was the early SV40 containing the small and large T-antigens that bind to and inactivate p53 and Rb. Although the expression of the T-antigen extends the life span of the cells, the onset of cellular senescence is inevitable. This is associated with a reduction of telomere length. Other commonly used viral genes include HPV16 E6 and E7, which inhibit the functions of p53 and Rb respectively. E6 has been reported to activate telomerase (28), which can stabilize the chromosome profile and avoid an immortalization-related crisis. Therefore, we infected human trophoblast cells with retroviral expression vectors containing type 16 human papillomaviruses E6 and E7 in combination with hTERT to obtain immortalized trophoblast cells showing approxinately diploid chromosomes.

Although cytokeratin-positive, vimentin-negative phenotypes are regarded as markers of trophoblast lineage (24), HChEpC1b expressed both cytokeratin and vimentin. Coexpression of cytokeratin and vimentin by extravillous trophoblasts was reported earlier (29), which is a characteristic similar to that of some cancer cells of epithelial origin (30-32), as part of an epithelial-mesenchymal transition (33,34). On the other hand, E6 is known to up-regulate vimentin (35) and another trophoblast cell line immortalized by E6/E7, TEV-1, was reported to express vimentin. From our results, it is difficult to distinguish whether the origin of HChEpC1b was the vimentin-positive extravillous trophoblasts or the vimentin negative trophoblasts that displayed the mesenchymal phenotype after E6 introduction. To answer this problem, further study is needed to compare the effects of E6 on each trophoblast population.

We obtained 43 genes that showed reversible up- or down-regulation during hypoxia. There are several interesting up-regulated genes including that for the MyoD family inhibitor domain containing protein isoform p40, which is involved in repair of muscle injury in ischemic micro-environments (36); prostaglandin I2 synthase, which is a potent vasodilator and inhibitor of platelet aggregation and plays an important role in the predisposition to lung cancer (37); human leukocyte antigen G (HLA-G) molecule, which plays an important role in immune response regulation and has been implicated in the inhibition of the cytolytic function of natural killer and cytotoxic T cells (38); and apolipoprotein B mRNA editing enzyme, a catalytic polypeptide-like 3C, which plays roles in growth or cell cycle control (39). Among the interesting down-regulated genes, there were adenylate kinase 2, which is localized in the mitochondrial intermembrane space and may play a role in apoptosis (40); BDNF brain-derived neurotrophic factor, which plays a role in the regulation of stress response and in the biology of mood disorders (41); and carcinoembryonic antigen-related cell adhesion molecule 1, which plays roles in the differentiation and arrangement of tissue three-dimensional structure, angiogenesis, apoptosis, tumor suppression, metastasis and the modulation of innate and adaptive immune responses (42). In future, we should confirm the expression of these genes by real-time RT-PCR and further examination should focus on a functional assay of these candidate genes to assess whether they play important roles during hypoxia.

In summary, we established a newly immortalized trophoblast cell line via infection with HPV16 E6/E7 in combination with hTERT and this cell line is a useful model for the study of extravillous trophoblast function.

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