Immortalization of normal human cytotrophoblast cells by reconstitution of telomeric reverse transcriptase activity

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Placental trophoblast cells are unique endocrine cells that play vital roles during the processes of embryonic implantation and placentation. However, research into the function of human trophoblast has been largely restrained mainly due to a lack of adequate cell models. A normal placenta-origin cytotrophoblast cell line (NPC) was previously established by our group, but these cells showed replicating senescence after 50 population doublings (PDs). In this study, the human telomerase catalytic subunit gene (hTERT) was transferred into B6 strain of NPC cells, and strains with reconstituted telomerase activity (B6Tert) were established. It was shown that B6Tert-1 cells produce various biomarkers of normal extravillous cytotrophoblasts during the early weeks of gestation. Meanwhile, the cell invasiveness was inhibited by transforming growth factor β (TGFβ). However, their ability to form syncytiotrophoblast was relatively low when stimulated with fetal calf serum (FCS). The cells maintained normal cell growth properties and failed to elicit tumours in nude mice. They proliferated continuously with no signs of senescence until the final count at 210 PDs. The growth rate of B6Tert-1 cells was increased when compared with the parental cells, which results, at least partly, from facilitating release of the G1/S checkpoint during the cell-cycle regulation. This is the first report of immortalizing human normal cytotrophoblast (CTB) cells by activation of telomerase activity. The cells will provide an ideal in vitro model for the study of human extravillous trophoblast (EVT) functions and consequently the mechanisms of embryonic implantation and placentation.

Key words: G1/S checkpoint/human cytotrophoblast/immortalization/telomerase

Introduction

Human placental villi consist primarily of an outer layer of multinuclear syncytiotrophoblast (STB) formed by fusion of mononuclear cytotrophoblast cells (CTB) that lie beneath the syncytiotum (Loke and King, 1995). Clinically, many pregnancy-associated diseases result from various malfunctions of trophoblast cells, such as abortion and pre-eclampsia because of incomplete invasion or hydatidiform moles and even chorioncarcinoma because of over-invasion of maternal tissues by trophoblasts (Norwitz et al., 2001). Therefore, studies on trophoblast function are vital for clarification of the physiological and pathological mechanisms involved in human pregnancy.

Research into the function of human trophoblasts has been largely restrained by a lack of pathological specimens and proper cell models, despite considerable research efforts. Replicating trophoblastic cell lines derived from chorioncarcinoma tissues (such as Jar, JEG, BeWo, NUC-1 and HCCM-5) may not represent normal trophoblastic functions in many aspects (Ringler and Strauss, 1990). For example, proliferation and invasion of normal trophoblast are strictly controlled processes and are inhibited by transforming growth factor β (TGFβ) derived from maternal decidua; however, those of chorioncarcinoma cells are not. For a long time, researchers have tried to establish several trophoblast cell lines derived from normal placenta tissues. A trophoblast-like cell line, TL, was established from a normal term placenta, but they were highly heterogeneous and triggered tumorigenesis in nude mice (Ho et al., 1987). Lei et al. (1992) transformed human CTB cells with temperature-sensitive mutant simian virus (SV)-40 large T antigen and obtained several cloned lines. The cells lost their malignant phenotype and grew as monolayers at the non-permissive temperature (40°C), while hCG secretion was high and could be stimulated by (Bu)2cAMP. This pattern of response differs from normal CTB cells. In 1993, Graham et al. reported a long lifespan human cytotrophoblast cell line (HTR-8/SVneo) derived from transfection of the first trimester CTB cells by SV40 large T antigen, whereas the reason for induction of hCG expression in the cells was unknown. We previously reported development of a normal placenta-origin cytotrophoblast (NPC) cell line from human placental villi at the first trimester (Li et al., 1996). The NPC cells were proven to maintain most endocrine functions of normal CTB and could proliferate actively in response to epithelial growth factor (EGF) and insulin (Li and Zhuang, 1997). However, replicating senescence was observed after 50 population doublings (PDs), which restricted the distribution of these cells among research groups.
It is well known that normal human somatic cells undergo limited rounds of cell division and ultimately enter replicative senescence stage that is probably because of sufficient shortening of the telomeres, the specialized nucleoprotein complexes located at the ends of eukaryotic chromosomes (Blackburn, 1991; Harley and V illepont eau, 1995). Telomerase compensates for the loss of telomeric repeats by adding telomeric DNA into chromosome ends (Harley and V illepont eau, 1995). More and more evidence has shown that forced expression of human telomeric reverse transcriptase (hTert) gene in human somatic cells is sufficient to produce telomerase activity that helps to circumvent the senescent stage. Furthermore, most cells immortalized with the hTert gene maintain the properties of their parental lines, which sheds new light on a possible method to immortalize human somatic cells (Bohn et al., 1998; Vaziri and Benchmark, 1998; Li et al., 1999; Morales et al., 1999; O'Hare et al., 2001; Kyo et al., 2003; Roy et al., 2004; Yin et al., 2004).

In the present study, we attempted to circumvent cell senescence in NPC cells by reconstituting telomerase activity, and the phenotypic and biochemical characteristics of the cells were further determined. We aimed to obtain human normal CTB cell lines with a long lifespan and consequently provide an ideal in vitro cell model for the investigation of human trophoblast function and mechanism of embryonic implantation and placentation.

Materials and methods

Culture of human CTB cell line—NPC cells

B6 strain of NPC cell line was used in this study and maintained as previously described (Li et al., 1996). In brief, B6 cells were cultured in collagen I-coated flasks (collagen I, Cellmatrix Type I-A; Institute of Biochemistry, Osaka, Japan; flasks, Corning Inc.) with FD [F12 : Dulbecco's modified Eagle's medium (DMEM), 1 : 1; Gibco] medium supplemented with 10 ng/ml of EGF (Collaborative Research, Lexington, MA, USA) and 10 μg/ml of insulin (Sigma, St. Louis, MO, USA). The cells were incubated at 37°C in an atmosphere of 5% CO2; 95% air and were split at a ratio of 1 : 3 every 5 days during continuous culture.

Transfection of the B6 cells with hTert gene

The plasmid pGRN145 was received from Geron Corporation (Menlo Park, CA, USA), which contains the full coding region of hTert gene under the myeloproliferative sarcoma virus (MPSV) long terminal repeat (LTR) promoter. The pGRN145 plasmid was transfected into B6 cells at 25 PDs, according to the manufacturer’s instructions of FuGENE™ 6 Transfection Reagent (Boehringer Mannheim, Germany). The cells were subsequently treated with 10 μg/ml of hygromycin (Sigma) for 2 weeks, and surviving clones were expanded into stable lines in FD medium supplemented with EGF and insulin.

Telomeric repeat amplification protocol assay

Telomerase activity was determined by telomeric repeat amplification protocol (TRAP) assay (Kim et al., 1994). Briefly, assay tubes were prepared by sequestering 0.1 μg of CX primer (5′-CCCTAACCTTACCCTTACCCTAAA-3′) under a wax barrier (Ampliwax; Perkin Elmer Cetus, Foster City, CA, USA) until used. Three micrograms of cell extract was mixed with 0.1 μg of TS primer (5′-AATCTCCGTCCGAGCACTTG-3′) (Boehringer Mannheim) and 2 μCi [α-32P]dCTP at 3000 Ci/mmol (Amersham, Buckinghamshire, UK) and subjected to PCR amplification. The products were electrophoresed on a 15% polyacrylamide gel. Cell extract pretreated with RNaseA was included to check the specificity of the telomerase assay, and extract from Hela cells was used as positive control.

Terminal restriction fragment assay

Genomic DNA was extracted with phenol/chloroform as described (Sambrook and Russell, 2001). Five micrograms of DNA was digested with HindIII and RsaI, subsequently electrophoresed on a 0.8% agarose gel and then blotted to a nylon membrane. The membrane was hybridised to the 32P-labelled (TTAGGG)2 probe and then autoradiographed to X-film (Fuji Photo Film Co, Tokyo, Japan). The mean length of terminal restriction fragment (TRF) was estimated at the peak position of the hybridization signal using the Ultrascan XL (Pharmacia, Uppsala, Sweden).

Measurement of cell number—MTT assay

Cell number was evaluated using the (tetrazolium based colorimetric assay MTT) method as reported previously (Carmichael et al., 1987). Briefly, the cells were incubated with 0.5 mg/ml of thiazolyl blue followed by addition of 0.1 N HCl–isopropyl alcohol. Absorbance at 540 nm was recorded with a Microplate Reader (Molecular Devices Corporation, Sunnyvale, CA, USA), which corresponded to the equivalence of cell number.

Growth curve plotting and cell treatment

Cells were seeded at 5 × 104 per 35-mm dish, and MTT assay was performed in at least three dishes each day till day 11 after seeding. Growth curves were plotted accordingly.

To identify the dependency of cell proliferation on growth factors, we seeded cells at 5 × 104 per 35-mm dish and treated with various doses of EGF (0.1–100 ng/ml) and/or TGFβ (0.1–10 ng/ml) for 48 h. MTT assay was performed in at least three dishes at each dose.

To induce syncytium, we seeded cells in 35-mm dishes at 3 × 105 and maintained in FD medium containing 10% fetal calf serum (FCS) for up to 168 h. At 24-h intervals, at least three dishes were separately counted with haemacytometer, and the cells and media were harvested for hCGβ measurement.

RT–PCR analysis

Total RNA was extracted from the cultured cells with TRIzol reagent (Life Technologies), according to the manufacturer’s instructions. RNAs were subjected to DNase I digestion to avoid possible genomic DNA contamination and then reverse transcribed with oligo-dT primers and Superscript II reverse transcriptase (Gibco). The primer sequences and the reaction conditions used for PCR amplification are listed in Table I.

Gelatin zymography

The gelatinolytic activities of matrix metalloproteinase-2 (MMP2) and MMP9 were assayed using gelatin zymography as reported (Xu et al., 2000). MMP2 and MMP9 were visualized as clear bands against a dark background at 72 and 92 kDa, respectively.

Western blot analysis

The soluble cytoplasmic protein was extracted by incubating the cells with lysis buffer (20 mM Tris with Mg2+ and K+, 1 mM dithiothreitol (DTT), 0.2% NP-40, 100 μM PMSF, 5 μg/ml of aprotinin, chymostatin, leupeptin, pristane and trypsin inhibitor; Sigma), and the supernatant was harvested after centrifuging. Twenty-microgram protein extract was subjected to 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and electrotitransferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). Immunoblotting was performed with mouse monoclonal antibodies against human retinoblastoma (Rb, 1 : 250; Pharmigen), P53 (1 : 1000; Santa Cruz) and actin (1 : 100; Santa Cruz). Final visualization was achieved with a horse-radish peroxidase (HRP)-linked rabbit anti-mouse immunoglobulin G (IgG) (1 : 500; Pharmigen), P53 (1 : 1000; Santa Cruz) and actin (1 : 100; Santa Cruz). Final visualization was achieved with a horse-radish peroxidase (HRP)-linked rabbit anti-mouse immunoglobulin G (IgG) (1 : 500; Zymed) and a subsequent chemiluminescent reaction (ECL; Amersham).

Flow cytometry DNA content analysis

The cells were trypsinized at 70% confluence and then suspended in a phosphate-buffered saline (PBS)–ethanol (1 : 1) solution. The ethanol-fixed cells were treated with 0.02% RNase A (Sigma). After staining with 20 μg/ml of propidium iodide (PI; Sigma), the fluorescence of cells was analysed on a FACS Calibur flow cytometer (Becton-Dickinson Immunocytometry System, San Jose, CA, USA) through a 585/542-nm bandpass filter. Approximately 2 × 106 cells were measured each time. Data analyses were performed with CellQuest software (Becton-Dickinson).

Immunocytochemical analysis

Cells were fixed with 4% paraformaldehyde (PFA; Sigma) and incubated with 10% FCS, subsequently incubated with mouse anti-human integrin α1 (1 : 200; Chemicon), integrin β1 (1 : 100; Chemicon), cytokeratin-8 (CK8, 1 : 100;
**Table 1.** The primer sequences and reaction conditions used in the RT–PCR

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequences (F: forward; R: reverse)</th>
<th>Annealing temperature (°C)</th>
<th>Cycle (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>htert</td>
<td>F: 5′-CGGAAGAGTGCTGCTGACAGAAA-3′&lt;br&gt;R: 5′-GGATGAGCGGGATCTTGA-3′</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>MMP2</td>
<td>F: 5′-ACCTGATACCCCGTGTTGAC-3′&lt;br&gt;R: 5′-TGTGGCAGCACCAGGCGC-3′</td>
<td>56</td>
<td>30</td>
</tr>
<tr>
<td>MMP9</td>
<td>F: 5′-GTCGCTGGCTCTGCTTTGCT-3′&lt;br&gt;R: 5′-GTCGCCTCAAAGGTTGAA-3′</td>
<td>67</td>
<td>30</td>
</tr>
<tr>
<td>MMP14</td>
<td>F: 5′-ATTGAAATTCCTAGGCCCTACCGA-3′&lt;br&gt;R: 5′-ATTTGACCTCGCCCTACCAAGA-3′</td>
<td>57</td>
<td>28</td>
</tr>
<tr>
<td>TIMP1</td>
<td>F: 5′-GCGTATTGAGATCGATGACC-3′&lt;br&gt;R: 5′-AGGCTCCAGCTTCACCTCC-3′</td>
<td>58</td>
<td>28</td>
</tr>
<tr>
<td>TIMP2</td>
<td>F: 5′-ATCCACCCTTGAGTTCTACTG-3′&lt;br&gt;R: 5′-ATTACAGCTGATTCTCAGGAAG-3′</td>
<td>58</td>
<td>28</td>
</tr>
<tr>
<td>TIMP3</td>
<td>F: 5′-TGC CAG AAA GAA TGA GGA ACC-3′&lt;br&gt;R: 5′-AGA GAG GGT GGT GCT TGT-3′</td>
<td>56</td>
<td>30</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: 5′-CTCAGACACATGGGGAAGGTGA-3′&lt;br&gt;R: 5′-ATGATCTTGAGGCTGTGCTAT-3′</td>
<td>55</td>
<td>20</td>
</tr>
</tbody>
</table>

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; htert, human telomerase catalytic subunit gene; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase.

Sigma), vimentin (1 : 200; Sigma) and HLA-G (1 : 200; Serotec, Kidlington, Oxford, UK). Binding of the primary antibody was visualized using either Dako Envision™ Kits (Dako) followed by counterstaining with haematoxylin or fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (Santa Cruz), followed by staining with PI (Sigma) and observation with a Leica TCS NT Confocal System (Leica, Wetzlar, Germany). Negative control was performed by replacing the primary antibodies with pre-immune mouse IgG.

**Karyotypic analysis**

Cells in the exponential phase were arrested by adding 0.03 μg/ml of colchicine for 6 h, treated with hypotonic solution (75 mM KCl, pH 8.0) and finally fixed twice in Carnoy’s fixative (methanol/acetic acid). The G-band pattern was obtained using Giemsa staining, and the chromosomes of 100 metaphases were analysed.

**Tumorigenicity test**

Female athymic nude BALB/c mice of 4–5 weeks (purchased from Animal Center, Chinese Academy of Sciences, Shanghai, China) were housed under pathogen-free conditions. Cells at 10^4/ml PBS were injected s.c. into the rear legs of the mice (10^7 cells/100 μl/injection; n = 5). The animals were observed for up to 3 months and examined for tumour growth.

**Northern blot analysis**

Northern blot analysis was performed as previously described (Xu et al., 2000). The pGEM-T plasmids containing cDNA fragments of α or β subunit of hCG were kind gifts from Dr X.Z. Shen at the Institute of Zoology, Chinese Academy of Sciences, Beijing, China. Specific cDNA probes for Northern blot analysis were labelled with [α-^32P]dCTP (Yahui, Beijing, China) using the Prime-a-gene System (Promega) and purified using nick column (Pharmacia). The hybridization signals were quantified with densitometric scanning, or fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (Santa Cruz), followed by staining with PI (Sigma) and observation with a Leica TCS NT Confocal System (Leica, Wetzlar, Germany). Negative control was performed by replacing the primary antibodies with pre-immune mouse IgG.

**ELISA for hCGβ**

Detection of hCGβ secretion was performed with a sandwich ELISA kit (Jiandian Pharm Co, Taiwan, China), according to the manufacturer’s instructions.

**Transwell invasion assay**

Transwell invasion assay was conducted in 24-well-fitted inserts with membranes (8-μm pore size, Millipore Corp, Bedford, MA, USA). Briefly, cells were plated in the transwell inserts pre-coated with collagen I (80 μg/ml; Cellmatrix Type I-A) at 2 × 10^4 cells in 200 μl of FD medium with or without 10 ng/ml of TGFβ1 (Sigma). Lower chambers were loaded with the same medium. After incubating for 24 h, cells on the upper surface of membranes were completely removed, and the migrated cells were fixed with 4% PFA and stained with haematoxylin. Cell-invasion indices were determined by counting the number of invaded cells in 10 randomly selected non-overlapping fields of the membranes under a light microscope.

**Statistics**

The experiments were repeated at least three times with different batches of the cells. Results are presented as the mean ± SD according to the repeated experiments. Statistical differences were evaluated by analysis with ANOVA, and values of P < 0.05 were considered significant.

**Results**

**Cloning and culture of htert-transfected cells**

The B6 cells were transfected with htert gene at 25 PDs, and four strains with positive htert mRNA expression were kept and designated B6Tert-1 to B6Tert-4.

In serum-free FD medium, both the parental B6 cells at earlier generations (Figure 1A) and the B6Tert cells (Figure 1D) grew as epithelial-like monolayers, with each cell containing one to four nucleoli. When seeded at low density, the cells elongated with extended fibres linking those over long distances (Figure 1E). Replicating senescence began to be observed in the B6 cells after 50 PDs. Cell growth began slowing down, and some of the cells appeared large and flattened, with centrally clustered multiple nuclei and highly vacuolated cytoplasm (Figure 1B and C). However, the population of B6Tert-1 cells continued active doubling, with no signs of senescence until the final count at 210 PDs when the manuscript was submitted in March, 2006.

By RT-PCR (Figure 2A) and TRAP assay (Figure 2B), it was shown that the B6Tert cells exhibited htert mRNA expression and telomerase activity, while the parental B6 cells appeared negative for both.

TRF assay revealed that the mean telomere length (MTL) in the B6Tert-1 cells was significantly elongated and continuously increased along with the generation when compared with that of the parental B6 cells at 25 PDs (Figure 2C).

**Biochemical characterization of B6Tert-1 cells**

Unless specially indicated, the B6 cells at 25 PDs and the B6Tert-1 cells at 80 PDs were used to determine their biochemical characteristics.

**Growth properties**

According to the growth curves, the PD time of the B6Tert-1 cells was ~36 h, which was 12 h less than that of the parental B6 cells. Cell
proliferation began to slow down and eventually ceased when the cells attained confluence 9 days after seeding (Figure 3A). Flow cytometry revealed a high percentage of the B6Tert-1 cells in S phase of the cell cycle (49.8%), which was almost 1-fold more than that of the parental B6 cells (24.6%) (Figure 3B).

It was revealed that growth of the B6 and B6Tert-1 cells retained stimulatory responses to EGF in dose-dependent manners, with the maximum stimulation at 10 ng/ml of EGF for the B6 cells and 100 ng/ml for the B6Tert-1 cells (Figure 3C). TGFβ alone did not influence cell growth but could partly abolish the effect of EGF, resulting in about 35% reduction of EGF-induced cell proliferation at 10 ng/ml in both cells (Figure 3D).

Tumorigenicity
The B6 cells at 25 PDs and B6Tert-1 cells at 80 and 210 PDs were non-tumorigenic when injected s.c. into nude mice separately at 10^7 cells per mouse (n = 5) and observed for up to 3 months (Figure 4A and B).

Chromosome analysis
Normal diploid karyotype was revealed in both the B6 and B6Tert-1 cells, which contained 46 chromosomes including the X and Y sex chromosomes (Figure 4C). However, in B6 cells over 70 PDs, abnormal karyotype could be observed with more than 60 chromosomes (Figure 4D).

In vitro differentiation towards syncytium
Spontaneous differentiation seldom occurred in both the B6 and B6Tert-1 cells. Northern blot analysis showed that hCGβ mRNA was expressed by both cells, while hCGα mRNA was scarcely detectable in these cells (Figure 5A). With RT-PCR, a much low level of hCGβ mRNA could be found in these cells after 35 cycles of amplification (Figure 5E).

When treated with FCS, growth rate of the B6 and B6Tert-1 cells were slowed down. From day 3 onwards, the number of FCS-treated B6Tert-1 cells was only 20–35% of control cells (Figure 5B). Meanwhile, morphological observation revealed that multinucleated cells began to appear from day 2 of FCS treatment in both the B6 and B6Tert-1 cells. The nuclei of the syncytium were centrally located as tight clusters, and the nuclear number in the syncytium was no more than six (Figure 5C). In the FCS-treated B6Tert-1 cells, the syncytial rate reached ~30% after the 4th day of treatment (Figure 5D). Result of semi-quantitative RT-PCR showed that hCGβ mRNA expression could be up-regulated by FCS in the B6 and B6Tert-1 cells, reaching a level of about 4-fold more than that of control (Figure 5E). However, hCGβ secretion in the FCS-treated cells was undetectable with ELISA.

Extravillous trophoblastic characteristics
More than 99% of the B6 and B6Tert-1 cells were positive for immunostaining of CK8, HLA-G, integrin α7 and integrin β1, while negative for vimentin (Figure 6).

Expression of MMP2, MMP9, MMP14 and tissue inhibitor of metalloproteinase-1 (TIMP1), TIMP2, TIMP3 mRNA was demonstrated in both B6 and B6Tert-1 cells by using RT-PCR (Figure 7A). Gelatin zymography revealed the same secretion pattern of MMP2 and MMP9 in these cells as that in the primary CTB cells at gestational weeks 6–7, with the level of pro-MMP2 much higher than that of pro-MMP9 (Figure 7B).

Transwell insert invasion assay showed that the B6 and B6Tert-1 cells exhibited similar invasion ability. Treatment with 10 ng/ml of TGFβ1 could evidently reduce cell invasion by about 70% in both cell types (Figure 7C).

Expression of P53 and phosphorylated Rb
To evaluate the influence of overexpressed HTERT on the cell proliferation cycle, we detected the expression of Rb and P53 proteins, which have been considered as the main regulatory proteins for G1/S checkpoint, by western blot analysis in the parental B6 cells and the B6Tert-1 cells. It was revealed that hyper-phosphorylated Rb protein expression in the B6Tert-1 cells increased to 5-fold of that in the B6 cells, while P53 level was only 25% of that in B6 cells (Figure 8).

Discussion
In this study, we successfully overcame proliferating senescence in NPC cells by inducing telomerase activity with overexpression of hTERT gene. The B6Tert-1 cells continued doubling until the final count reached to 210 PDs in March, 2006, and they have the potential to divide further. The lifespan has exceeded the limits of normal human somatic cells (~70 PDs), and therefore, the B6Tert-1 cells could be considered immortalized.

The aim of this study was to obtain a normal human cytotrophoblast cell line for the investigation of trophoblastic behaviour. This required immortalization of the cells without altering their phenotypic and genotypic characteristics. Morphological observation and immunostaining for CK8 and vimentin demonstrated an unaltered epithelial origin of the B6Tert-1 cells. Production of hCGβ is a common biomarker for identifying CTB cells, while hCGα is a marker for STB cells (Kliman et al., 1986). Similarly with the parental B6 cells, the immortalized B6Tert-1 cells expressed certain levels of hCGα mRNA, but little hCGβ, in parallel with the observation that there was little spontaneous syncytialization in these cells. In the presence of FCS, the B6 and B6Tert-1 cells differentiated into multinucleated cells. However, the rate of syncytium formation was relatively low (no more than 30%), and the nuclear number in the syncytium was less than six. This might lead to less production of hCGβ which was hardly detected using

Figure 1. Morphology of the human cytotrophoblast cell lines as observed by phase-contrast revert microscope. (A) B6 strain of normal placenta-origin cytotrophoblast cell line (NPC) cells at 25 PDs. (B) and (C) B6 strain of NPC cells at 45 PDs [Note the multinucleated cells (arrowhead) and replicating senescent cells with compact nuclear and much flattened cytoplasm (arrow)]. (D) B6Tert-1 cells at 80 PDs. (E) B6Tert-1 cells at 80 PDs seeding at low density [Note the long fibers extended by the cells (asterisk)]. Bar = 25 μm.
ELISA, even though mRNA expression was up-regulated. It has previously been demonstrated that syncytiotrophoblast differentiation is less extensive in the first trimester compared with that at term (Benirschke and Kaufmann, 1995). The B6 and B6Tert-1 cells were derived from placental villi from early pregnancy, and they retained low ability to form syncytiotrophoblasts. These results demonstrated the cytotrophoblastic nature of the B6 and B6Tert-1 cells and their potential ability to differentiate into STB.

One of the most important properties of extravillous trophoblast (EVT) cells is the ability of invasion/migration and production of various MMPs and TIMPs that are required for the controlled invasion of EVT cells into the uterine endometrium (Xu et al., 2000; Bai et al., 2005). RT-PCR showed expression of various MMPs and TIMPs mRNA in B6 and B6Tert-1 cells, and gelatin zymography revealed that the secretion pattern of MMP2 and MMP9 in these cells was similar to that of primary CTB cells at 6- to 7-week gestation as reported by Xu et al. (2000). Transwell invasion assay further demonstrated their in vitro invasion/migration abilities that could be negatively controlled by maternal decidua-derived factors such as TGFβ. Together with evidence that B6 and B6Tert-1 cells could produce several other biomarkers of EVT cells, including integrin α1, integrin β1 and HLA-G, we propose that B6 and B6Tert-1 cells are of extravillous cytotrophoblast lineage.

Normal cell growth properties were observed in the parental B6 and immortalized B6Tert-1 cells, including contact inhibition of cell growth and ‘growth plateau’ in the growth curve. We previously demonstrated that EGF was one of the vital factors supporting human CTB cell survival and growth in vitro (Li et al., 1996; Li and Zhuang, 1997). Here, proliferation of B6 and B6Tert-1 cells responded to EGF stimulation in a dose-dependent manner. Cell growth became slower, and cell senescence appeared after 10–15 PDs when EGF was removed from the culture system. Filla and Kaul (1997) demonstrated that growth of tumour trophoblasts, such as choriocarcinoma BeWo and JAR cells, failed to respond to exogenous EGF because overexpression of the EGF receptor led to cell proliferation at a maximal and uncontrolled rate. In the B6 and B6Tert-1 cells, the stimulation of proliferation by EGF could be partially abolished by low doses of TGFβ, consistent with the data of Li and Zhuang (1997) for normal human CTB. In choriocarcinoma cells, neither repression of cell growth by TGFβ nor contact inhibition could be observed. Furthermore, the B6 and B6Tert-1 cells could not initiate tumorigenesis when injected into nude mice. All these data indicate that normal cell properties are not altered in human CTB cells after transfection with hTERT gene. Several trophoblast cell lines have been reported in the literature. Most of them were derived from transforming CTB cells with SV40 large T antigen. For instance, HTR-8/SVneo cells reported by Graham et al. (1993) have an unlimited lifespan and possess many properties of EVT cells. However, there is little MMP9 production, and invasion/migration is not affected by TGFβ in these cells. Meanwhile, the
hCG expression is induced after SV40 transfection for unknown reasons. Other well-characterized EVT cell lines are MC4/SGHL4 and L10 developed by Choy and Manyonda (1998). The cells maintained many characteristics of EVT lineages, while their epithelial origin could not be determined because they exhibited negative immunostaining for CK and positive immunostaining for vimentin. The B6Tert-1 cell line reported here maintains normal cell growth properties and most of the phenotypic and biochemical characteristics of normal CTB cells, which indicates that transfection of the hTERT gene does not alter the biochemical characteristics of CTB cells. Meanwhile, their EVT cell properties indicate that the cell line will be an ideal in vitro cell model to investigate regulation of EVT cell behaviour as well as the interaction between trophoblast and endometrium in human. What’s more, it may be a cell model to discover the molecular mechanism underlying the pregnancy-associated diseases originating from malfunction of trophoblast cells.

In 1998, Bodnar et al. (1998) and Vaziri and Benchimol (1998) first reported the immortalization of human fibroblast and retinal epithelial cells by exogenous expression of the hTERT gene. Subsequent reports indicated that normal cell-cycle regulation existed in these cells (Jiang et al., 1999; Morales et al., 1999). Since then, stimulation of telomerase activity has been widely used to induce immortalization in various kinds of cells (O’Hare et al., 2001; Carney et al., 2002; Condon et al., 2002; Kyo et al., 2003; Lee et al., 2003; Wege et al., 2003; Krikun et al., 2004; Roy et al., 2004; Yin et al., 2004). Some cell types could apparently be immortalized by introduction of the hTERT gene alone (Bodnar et al., 1998; Vaziri and Benchimol, 1998; Carney et al., 2002; Condon et al., 2002; Lee et al., 2003; Wege et al., 2003; Roy et al., 2004; Yin et al., 2004), while co-transfection of either HPV E6E7 or SV40 large T antigen gene was required for other cells to overcome proliferating senescence (O’Hare et al., 2001; Kyo et al., 2003). The underlying mechanisms for these differing cellular responses need further investigation, but it probably depends, at least
Figure 5. Detection of syncytialization properties of human cytotrophoblast cell lines. (A) Northern blot analysis showing the expression of hCGα and hCGβ mRNA in B6 cells at 25 PDs (NPCB6/PD25) and B6Tert-1 cells at 80 PDs (B6Tert-1/PD80). Hybridization with RNA derived from normal placental villi tissue at gestational week 7 [villi (week 7)] was used as positive control. (B) Cell growth pattern of B6Tert-1 cells cultured in serum-free (control) or fetal calf serum (FCS)-containing medium. *, Compared with corresponding control, P < 0.05. (C) Morphology of the B6Tert-1 cells cultured in serum-free medium (a) and those treated with FCS for 3 days (b) and 7 days (c). The multinucleated cells were indicated by arrow. Bar = 25 μm. (D) Syncytial rate of B6Tert-1 cells cultured in serum-free (control) or FCS-containing medium. *, compared with the corresponding control, P < 0.01. (E) Detection of hCGβ mRNA expression by semi-quantitative RT-PCR in B6Tert-1 cells cultured in serum-free medium (B6tert-1) or in serum-containing medium (B6Tert-1/FCS) for 7 days. RNA derived from normal placental villi tissue at week 7 [villi (week 7)] was used as positive control.
in part, on the physiological telomerase activity and the regeneration ability of the tissues from whence the cells were derived. Previous reports have shown the presence of relatively strong telomerase activity in placental tissues during the very early pregnant stage, but not at term, and telomerase activity was found to be present in CTB but not in STB (Kyo et al., 1997; Cheung et al., 1999; Nishi et al., 1999). It is suggested that telomerase contributes to the active proliferation of CTB cells during the very early stages of pregnancy. This may be the physiological basis for elongating the lifespan of human CTBs in vitro by exogenous activation of telomerase.

Telomerase is usually thought to elongate telomeres and thus prohibit cells from entering ‘proliferating senescence’ (Harley and Villeponteau, 1995). The comparison of telomere length between the B6 and B6Tert-1 cells during long-term cultures lends support to this classical function of telomerase in human CTB cells. Recent studies have shown that human epithelial cells immortalized by telomerase commonly harbour additional genetic or epigenetic aberrations of genes involved in cell-cycle regulatory pathways (Farwell et al., 2000; Jin et al., 2004). Some of these genetic changes, such as deletion of chromosomes 9p and 13q and amplification of 20q, are commonly present in immortalized cell lines and cancer cells (Fountain et al., 1992; Hodgson et al., 2003). The involvement of genes located on these chromosomes, such as the p16 gene on chromosome 9p and the Rb gene on chromosome 13q, may play roles in the regulation of the G1/S cell-cycle checkpoint. Whether such chromosomal changes exist in the B6Tert cells has yet to be studied. Here, Western blot analyses demonstrated increased level of hyper-phosphorylated Rb protein and decreased expression of P53 in B6Tert-1 cells compared with those in the parental B6 cells at earlier generations, suggesting that hTERT transfection facilitated release of the G1/S checkpoint during cell-cycle regulation in human CTB. This may lead to the increased growth rate exhibited by the B6Tert-1 cells, as shown by the cell-growth curve and DNA content analysis with flow cytometry. These data are consistent with that of Xiang et al. (2002), who demonstrated accelerated

Figure 6. Immunocytochemistry for cytokeratin-8 (CK8), vimentin, integrin α1, integrin β1 and HLA-G in B6 cells at 25 PDs (NPCB6/PD25) and B6Tert-1 cells at 80 PDs (B6Tert-1/PD80). Negative control was performed by replacing the primary antibodies with normal mouse IgG. Bar = 25 μm.
growth of immortalized lens epithelial cells through the Rb pathway after hTERT transfection, but are in contrast with that of Morales et al. (1999) and Jiang et al. (1999), who reported unaltered cell-cycle regulation in immortalized human fibroblast and retinal epithelial cells after exogenous expression of the hTERT gene. This discrepancy indicates that different regulatory mechanisms may be involved in the immortalization of various kinds of cells.

In summary, an immortalized human placental CTB cell line was established by the forced expression of hTERT gene. The cells maintained normal phenotypic and trophoblastic functions, and acceleration of cell growth through the Rb pathway was indicated in cell-cycle regulation.

Acknowledgements

The authors greatly appreciate the editorial assistance of Robert G. Newcomer at the Florida State University and the help of Dr. Yong Zhao in IOZ, CAS in tumorigenicity test with athymic nude BALB/c mice, as well as the technical assistance of Ms Li-hong Jiao, Shi-wen Li in SKLRB, IOZ, CAS in electronic photograph preparation. The plasmids containing cDNA fragment of HCGβ and HCGβ are kind gifts of Dr. Xiao-zhou Shen in SKLRB, IOZ, CAS, and the antibodies against HLA-G are kindly provided by Dr. Yuan-qing Yao from the Fourth Military Medical University, China.

This work was supported by grants to Y.L.W. from National Nature Science Foundation (30070373, 30530760 and 30370542), and the Fellowship from Sun Yat-sen Foundation.

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Submitted on March 29, 2006; resubmitted on May 8, 2006; accepted on May 16, 2006