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The Isolation and Characterization of a Novel Telomerase Immortalized First Trimester Trophoblast Cell Line, Swan 71

Shawn L. Straszewski-Chavez^{1,4}, Vikki M. Abrahams², Ayesha B. Alvero², Paulomi B. Aldo², Yuehong Ma², Seth Guller², Roberto Romero³, and Gil Mor²

¹ Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, Connecticut

² Department of Obstetrics, Gynecology and Reproductive Sciences, Yale University School of Medicine, New Haven, Connecticut

³ Perinatology Research Branch, Eunice Kennedy Shriver National Institute of Child Health and Human Development, NIH, DHHS, Detroit, Michigan

Abstract

Studies using first trimester trophoblast cells may be limited by the inability to obtain patient samples and/or adequate cell numbers. First trimester trophoblast cell lines have been generated by SV40 transformation or similar methods, however, this approach is known to induce phenotypic and karyotypic abnormalities. The introduction of telomerase has been proposed to be a viable alternative for the immortalization of primary human cells. To investigate whether telomerase-induced immortalization might be a more feasible approach for the generation of first trimester trophoblast cell lines, we isolated primary trophoblast cells from a 7-week normal placenta and infected the cells with human telomerase reverse transcriptase (hTERT), the catalytic subunit of telomerase. Although this hTERT-infected first trimester trophoblast cell line, which we have named Swan 71, has been propagated for more than 100 passages, it still has attributes that are characteristic of primary first trimester trophoblast cells. The Swan 71 cells were positive for the expression of cytokeratin 7, vimentin and HLA-G, but do not express CD45, CD68 or the Fibroblast Specific Antigen (FSA), CD90/Thy-1. In addition, we also demonstrated that the Swan 71 cells secrete fetal fibronectin (FFN) as well as low levels of human Chorionic Gonadotrophin (hCG). Moreover, the Swan 71 cells exhibit a cytokine and growth factor profile that is similar to primary trophoblast cells and are resistant to Fas, but not TNF- α -induced apoptosis. This suggests that the Swan 71 cells may represent a valuable model for future *in vitro* trophoblast studies.

Keywords

apoptosis; hTERT; placenta; pregnancy; telomerase; trophoblast

Correspondence: Gil Mor, MD, PhD, Department of Obstetrics, Gynecology and Reproductive Sciences, Reproductive Immunology Unit, Yale University School of Medicine, 333 Cedar Street LSOG 305A, New Haven, CT 06520, Phone: 203-785-6294, Fax: 203-785-4883, Gil.Mor@yale.edu.

⁴Current Address: Institute for Stem Cell Biology and Regenerative Medicine, Stanford University, Palo Alto, California

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Introduction

Studies using first trimester trophoblast cells may be limited by the inability to obtain patient samples and/or adequate cell numbers to perform experiments. Moreover, isolated primary first trimester trophoblast cells are known to have a finite lifespan in culture (Manyonda, Whitley et al. 2001). Several Simian Virus 40 (SV40) or similarly transformed first trimester trophoblast cell lines have been generated, but this approach has been shown to induce karyotypic and/or phenotypic abnormalities (Aboagye-Mathiesen, Zdravkovic et al. 1996; Khoo, Bechberger et al. 1998; King, Thomas et al. 2000). As an alternative, trophoblast-derived choriocarcinoma cell lines have been used extensively, however, these cells are malignant and tumorigenic (Rachmilewitz, Elkin et al. 1995). Therefore, the results from these studies, although valuable, should be interpreted cautiously. In order to overcome these limitations, we have established a different technique, which utilizes the ribonucleoprotein enzyme, telomerase, for the immortalization of primary first trimester trophoblast cells.

Telomeres are repetitive DNA sequences at the end of chromosomes that are involved in DNA replication and stability. Telomere length is maintained by telomerase, a multi-component enzyme consisting of a RNA template and human telomerase reverse transcriptase (hTERT), the essential catalytic protein subunit of telomerase (Greider and Blackburn 1987). Using the RNA as a template, telomerase adds the DNA repeat sequence, TTAGGG in mammals, to the 3' end of DNA strands (Morin 1989; Wilkie, Lamb et al. 1990). Since adult somatic cells do not contain functional telomerase, their telomeres shorten with each mitotic division. This progressive shortening of telomere length is thought to be associated with the loss of replication potential and the aging process (Harley, Futcher et al. 1990; Hastie, Dempster et al. 1990).

Although the majority of normal human cells lack active telomerase, the introduction of ectopic hTERT was previously shown to restore telomerase activity in telomerase-negative cells (Bodnar, Ouellette et al. 1998; Vaziri and Benchimol 1998) and in certain cell types, the induction of telomerase activity was sufficient for immortalization (Counter, Meyerson et al. 1998). Indeed, our laboratory as well as others has successfully immortalized several cell lines by introducing exogenous hTERT without any effect on their karyotype or phenotype (Jiang, Jimenez et al. 1999; Morales, Holt et al. 1999; Alvero, Fishman et al. 2004; Krikun, Mor et al. 2004; Krikun, Mor et al. 2005). Previously, another group reported the successful immortalization of a telomerase-infected first trimester trophoblast cell line, B6Tert, but the primary parental cells used to generate the B6Tert cells, were infected with hTERT after several years and passages, which raises the question whether these cells had acquired phenotypic and genotypic traits not characteristic of primary trophoblasts before immortalization (Wang, Qiu et al. 2006). The aim of this study was to establish a new first trimester trophoblast cell line that retains the attributes of primary first trimester trophoblast cells following immortalization at an early passage number.

Materials and Methods

Tissue

A 7-week first trimester placenta was obtained from the fetal side of a normal pregnancy, voluntarily terminated for reasons unrelated to the present study. A signed, written consent form was obtained from the patient. The use of placental tissue, specimens and consent forms was approved by the Yale University Human Investigation Committee. The tissue specimen was collected in cold, sterile phosphate-buffered saline (PBS) and immediately transported to the laboratory for cell culture preparation.

Reagents and Antibodies

The agonistic anti-human Fas monoclonal antibody (mAb) (clone E0S9.1) and the vimentin mouse mAb (clone RV202; 1:50) were purchased from BD PharMingen (San Diego, CA). Recombinant human TNF- α (catalog #300-01A) was purchased from PeproTech, Inc. (Rocky Hill, NJ). The cytokeratin 7 (clone OV-TL 12/30; 1:150), CD45 (clone 2B11/DD7-26-16; 1:200) and CD68 (clone EMB11; 1:200) mouse anti-human mAb were obtained from DakoCytomation (Carpinteria, CA). While the HLA-G antibody (clone 5A6G7; 1:2000) was purchased from Exbio (Czech Republic), the Fibroblast Specific Antigen (FSA; CD90/Thy-1) mouse mAb (clone AS02; 1:2,000), which recognizes a 65 kDa band under both reducing and non-reducing conditions, was obtained from Calbiochem (San Diego, CA). The rabbit polyclonal antibody for B-actin (A2066; 1:10,000) was purchased from Sigma-Aldrich (St. Louis, MO). Primary antibody signals were detected using either a horseradish peroxidase (HRP)-conjugated horse anti-mouse, or HRP-conjugated goat anti-rabbit secondary antibody (1:10,000) from Vector Laboratories (Burlingame, CA).

Cell Lines

Both the first trimester human cytotrophoblast cell line, 3A, which was transformed by SV40 tsA255 (Chou 1978) and the Jurkat human T cell leukemia line were purchased from American Type Culture Collection (Manassas, VA). The SVneo transformed first trimester human extravillous trophoblast cell line, HTR8 (Graham, Hawley et al. 1993), was a gift from Dr. Charles Graham (Queen's University, Kingston, ON, Canada), while the monocytic cell line, THP-1, was a gift from Dr. Paul Guyre (Dartmouth Medical School, Lebanon, NH). Both trophoblast cell lines, the THP-1 cells and the Jurkat cells were cultured in RPMI 1640 (Gibco, Carlsbad, CA), whereas the third trimester placental fibroblasts, which were isolated by CD9 immuno-purification as previously described (Yui, Garcia-Lloret et al. 1994), were cultured in Basal Media, supplemented with 10% fetal bovine serum (Hyclone, South Logan, UT), 10mM Hepes, 0.1mM MEM non-essential amino acids, 1mM sodium pyruvate and 100 U/ml penicillin/streptomycin (Gibco) and maintained at 37 °C/5% CO₂.

First trimester primary trophoblast cell isolation and culture

Primary trophoblast cells were isolated from the 7-week first trimester placenta according to Loke and King (Loke, Gardner et al. 1989) with a few modifications (Straszewski-Chavez, Abrahams et al. 2004). In brief, first trimester placental tissue was washed with cold Hanks' Balanced Salt Solution (HBSS) without calcium and magnesium (Gibco) to remove excess blood. Cells were removed from the membranes by scraping and transferred to trypsin-EDTA (Gibco) digestion buffer and incubated at 37°C for 10 minutes with shaking at 200 rpm. An equal volume of D-MEM media containing 10% FBS was added to inactivate the trypsin. This mixture was vortexed for 20 seconds, allowed to sediment and the supernatant was collected. The two previous steps were repeated twice and the collected supernatant was centrifuged at 1,500 rpm for 10 minutes. The pellet was resuspended in D-MEM media with 10% FBS and filtered through a 70 μ m cell strainer (BD Falcon, San Diego, CA) to remove tissue pieces and centrifuged at 1,500 rpm for 10 minutes. Contaminating red blood cells were removed from the filtrate by resuspending the cellular pellet in HBSS, layering this suspension over Lymphocyte Separation Media (ICN Biomedicals, Inc., Aurora, OH) and centrifuging the gradient at 2,000 rpm for 25 minutes. The interface, containing the trophoblast cells, was removed and incubated with an anti-CD45 mAb conjugated to magnetic beads (Dynabeads 450, Dynal, Oslo, Norway) at 4°C with rotation for 30 minutes (Kamsteeg, Rutherford et al. 2003). Following this incubation, the immune cells were magnetically separated from the negative cell fraction and the unbound cells were collected, washed and cultured at 37°C/5% in D-MEM media supplemented with 10% Human Serum (Gemini Bio-Products, Woodland, CA).

Telomerase (hTERT) infection

The isolated primary first trimester trophoblast cells were infected at passage 3 approximately 5 days later with a retroviral system consisting of the pA317-hTERT expressing cell line, which expresses human telomerase reverse transcriptase (hTERT), the essential catalytic protein subunit of telomerase, and the puromycin resistance gene as previously described (Krikun, Mor et al. 2004). In brief, trophoblast cells were infected with the supernatant of pA317-hTERT cells overnight (16 hours) in the presence of 10 µg/ml Polybrene (Sigma-Aldrich). The cells were allowed to recover in D-MEM media with 10% FBS for 48 hours before selecting with 800 ng/ml of puromycin (Sigma-Aldrich). Following 72 hours of selection, the remaining cell clones were serially propagated and cultured in D-MEM media plus 10% FBS with puromycin.

Detection of telomerase activity

Telomerase activity was assayed in triplicate using the TRAPeze ELISA Detection Kit (Chemicon International, Inc., Temecula, CA) per the manufacturer's instructions. Briefly, the infected trophoblast cells were lysed in 1X CHAPS lysis buffer and the cell extracts were quickly frozen on dry ice. Telomerase was allowed to add telomeric repeats (GGTTAG) onto the 3' end of a biotinylated Telomerase Substrate oligonucleotide (b-TS) at 30°C for 30 min. The extended products were amplified by polymerase chain reaction (PCR) using b-TS and RP (reverse) primers and a deoxynucleotide mix containing dinitrophenyl (DNP)-labeled dCTP. The labeled PCR products were immobilized onto streptavidin-coated microtiter plates by biotin-streptavidin interactions and detected by an anti-DNP antibody conjugated to HRP. The amount of product was determined by HRP activity using the HRP substrate 3,3',5,5'-tetramethylbenzidine (TMB) and subsequent color development. Using an automatic microplate reader (Model 550, Bio-Rad, Hercules, CA), the absorbance of the samples was measured at 450 nm and 690 nm and telomerase activity was determined using the equation: Absorbance = $A_{450} - A_{690}$. The lysis buffer alone, non-infected primary trophoblast cells and cell lysates heat-inactivated at 85 °C for 10 min served as negative controls.

Immunocytochemistry

50–150 × 10³ cells (depending on the cell type) were plated per chamber in 4-well chamber slides (BD Falcon). The cells were grown to 70% confluence and fixed in 4% paraformaldehyde at room temperature (RT) for 30 min. Non-specific binding was inhibited by blocking the cells with 10% normal horse serum (Vector Laboratories) at RT for 1 hour. The cells were incubated with primary antibodies overnight at 4°C and secondary antibody (either peroxidase-conjugated or biotinylated horse anti-mouse, 1:1,000) at RT for 2 hours. If a biotinylated secondary antibody was used (cytokeratin 7), the cells were incubated with Vectastain ABC reagent (Vector Laboratories) at RT for 1 hour according to the manufacturer's instructions. Between each step, the cells were washed with PBS three times. Specific staining was detected by incubating the cells with DAB substrate (Vector Laboratories) at RT for 2–10 min. The cells were washed with distilled water three times and counterstained with hematoxylin (Sigma-Aldrich) before dehydration with ethanol and Histo-solve (Shandon, Inc., Pittsburgh, PA). Slides were mounted with permanent aqueous mounting medium (ScyTek, Logan, Utah) and visualized by light microscopy.

Western Blot analysis

5 × 10⁵ trophoblast cells were plated in 35 mm² petri dishes (BD Biosciences) and grown to 70% confluence for treatment. Following treatment, cells were lysed in 1% NP40 and 0.1% SDS in the presence of 0.2 mg/ml PMSF and a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Protein concentrations were calculated by BCA assay (Pierce Biotechnology, Rockford, IL). 20 µg of total cellular protein was loaded per lane and separated under reducing conditions by SDS-PAGE using 12% polyacrylamide gels and transferred to

PVDF membranes (NEN Life Sciences, Boston, MA) as previously described (Bechmann, Mor et al. 1999). The membranes were stained with Ponceau Red to ensure efficient transfer and equal loading of proteins. To inhibit nonspecific binding, membranes were blocked with 5% powdered milk in PBS/0.05% Tween-20 (PBS-T) prior to immunoblotting. The membranes were then incubated with primary antibody overnight at 4°C followed by the appropriate secondary antibody for 1 hour at room temperature in PBS-T/1% powdered milk. Following each step, the membranes were washed three times with PBS-T for 10 minutes. Finally, the blots were developed using the enhanced chemiluminescence (ECL) system (NEN Life Sciences) and the intensity of the signals was analyzed using a digital imaging analysis system and 1D Image Analysis Software (Kodak Scientific Imaging Systems, Rochester, NY). As a negative control, membranes were incubated with secondary antibody alone to validate the specificity of the signal.

human Chorionic Gonadotrophin (hCG) secretion

The secretion of hCG in 1 ml of cell-free supernatant from approximately 5×10^6 cells cultured for 48 hours was measured by an automated immunometric assay. A multi-well plate was coated with a capture antibody, which recognizes one site on hCG. The liquid form of a labeled tracer antibody, which recognizes a second site on hCG, was added. 200 μ l of trophoblast cell culture supernatant was added and if hCG was present in the sample, it linked the capture and tracer antibodies. The amount of tracer antibody captured was measured by radioactive detection in Counts Per Second (CPS) in The Endocrine Lab in the Department of Obstetrics, Gynecology and Reproductive Sciences at Yale University School of Medicine using a Diagnostic Products Corporation (DPC) Immulite 1 system number 4329 (Los Angeles, CA). The results were compared to the standard concentrations of hCG in mIU/ml and the amount of tracer antibody captured is, therefore, directly proportional to the amount of hCG present in the sample, which includes regular hCG, nicked hCG, hyperglycosylated hCG, hCG missing the β -subunit C-terminal peptide, free β -subunit, nicked free β -subunit, free β -subunit missing the CTP and urine β -core fragment.

Cytokine array studies

Cytokine production was determined using a Human Cytokine Array 3.1 (for cell lysates) and III (for cell culture supernatants) (RayBiotech, Atlanta, GA) as previously described (Abrahams, Bole-Aldo et al. 2004; Abrahams, Visintin et al. 2005). Briefly, 100 μ g of protein from whole cell lysates or 1 ml of cell-free supernatant from approximately 5×10^6 cells cultured for 48 hours was incubated with the array membrane. Following incubation with primary biotin-conjugated Abs and HRP-conjugated streptavidin, detection of signals was performed by ECL (PerkinElmer). The intensity of the signals was quantified by densitometry using a digital imaging analysis system and 1D Image Analysis Software (Kodak Scientific Imaging, Melville, NY). The signal intensities were adjusted for the internal negative controls and normalized against the internal positive controls on each array membrane. An expression level below 0.2 U was considered below the detection limit of the assay as determined by the software.

Fetal fibronectin (FFN) secretion

The release of fetal fibronectin (FFN) from the infected cell line was measured as previously described (Rosen, Krikun et al. 1998). In brief, the cells were maintained in serum-free medium or in medium containing 10% charcoal-stripped fetal bovine serum with and without 100 nM dexamethasone for 48 hours. FFN levels in the culture media were measured by an ELISA using the FDC-6 monoclonal antibody according to information provided by the manufacturer (Adeza Biomedical, Sunnyvale, CA). The concentration of FFN in the culture media was

determined in triplicate and normalized to cell protein using the DC Protein Assay from Bio-Rad Laboratories (Hercules, CA).

Cell Viability Assay

Cell viability was evaluated using the CellTiter 96 assay according to the manufacturers instructions (Promega, Madison, WI). Briefly, 5×10^3 cells were plated in triplicate wells in a 100 μ l volume per well in a 96-well microtiter plate (BD Biosciences). The cells were grown to 70% confluence, at which stage the medium was replaced with reduced serum phenol depleted Opti-MEM (Gibco) and the cells were cultured for an additional 6 hours prior to treatment. Following treatment, 20 μ l of the Cell Titer 96 Aqueous One Solution was added to each well and the plate was incubated at 37°C for 1–4 hours. Optical densities of the samples were measured at 490 nm using an automatic microplate reader (Model 550, Bio-Rad). The values of the treated cells were compared with the values generated from the untreated control and reported as percent viability.

Caspase-3 activity assay

Caspase-3 activity was measured using the Caspase-3 Glo assay according to the manufacturers' instructions (Promega). Briefly, 10 μ g of total cellular protein from cell lysates were incubated at RT for 1 hour in the dark with the proluminescent caspase-3 substrate. Following incubation, luminescence was measured using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). All samples were assayed in triplicate. Luminescence was expressed as Relative Light Units (RLU) and is proportional to the amount of caspase-3 activity present in the sample.

Treatment with anti-Fas mAb and TNF- α

To evaluate the sensitivity of the first trimester trophoblast cell line to anti-Fas and TNF- α , cells were treated with 500 ng/ml of the agonistic anti-Fas monoclonal antibody (mAb) for 24 hours and 25 ng/ml of TNF- α for 48 hours. As positive controls, Jurkat cells and the trophoblast cell lines, 3A and HTR-8, were treated with similar concentrations of the anti-Fas mAb and TNF- α , respectively. Cell viability was determined using the CellTiter 96 assay, while caspase-3 activity was measured using the Caspase-3 Glo assay.

Statistical Analysis

The data is represented as the average $\pm$ the standard deviation and analyzed for statistical significance ($p < 0.05$) or less using one-way ANOVA with the Bonferonni correction. All experiments were repeated three times with similar results.

Results

The hTERT-infected trophoblast cells are morphologically similar to primary trophoblast cells

Primary trophoblast cells were isolated from a 7-week normal placenta and infected with hTERT, the essential catalytic protein subunit of telomerase. Following 72 hours of selection with puromycin, the remaining cell clones were serially propagated and cultured in the presence of puromycin. One surviving clone, which we have named Swan 71, was selected and further characterized. While the parental trophoblast cells, PL129, reached senescence after 4 passages (Figure 1A.1), the hTERT-transfected Swan 71 cells continued to proliferate and have been maintained in culture for over 100 passages without exhibiting any signs of senescence (Figure 1A.2). The Swan 71 cells were routinely frozen and thawed and after each thawing, approximately 85% of the cells attached. With each pass, the Swan 71 cells reached 70% confluence within 24–48 hours and no morphological changes could be detected between

passages. Analogous to the primary trophoblast cells, the Swan 71 cells retained the ability to fuse spontaneously, as evidenced by the formation of occasional multi-nucleated cell clusters (data not shown).

The Swan 71 cells exhibit high levels of telomerase activity

In order to confirm that the isolated Swan 71 cell clone had been successfully infected with hTERT, the Swan 71 cells and the parental trophoblast cells were assayed for telomerase activity. The average change in absorbance, which correlates with telomerase activity, of the primary trophoblast cells was 0.09 ± 0.003 , whereas the average change in absorbance of the telomerase-infected Swan 71 cells was 3.57 ± 0.169 ($p < 0.001$; Figure 1B). As a control, telomerase activity was also evaluated in the corresponding heat-inactivated samples, which exhibited values similar to the background ($p < 0.0001$). Since the average change in absorbance of the hTERT-infected trophoblast cells was greater than 0.150, the Swan 71 cells are, therefore, considered positive for telomerase activity.

Cytokeratin 7 expression in the Swan 71 cells

To determine whether the isolated telomerase-infected Swan 71 clone expressed trophoblast specific markers, the expression of the intermediate filament protein, cytokeratin 7, was assessed by immunocytochemistry using the HTR-8 trophoblast cell line as a positive control. Analogous to the HTR-8 cells (Graham, Hawley et al. 1993) (Figure 2A.3), 100% of the Swan 71 cells expressed cytokeratin 7 (Figure 2A.2), whereas no cytokeratin 7 immunostaining was observed in the negative control (Figure 2A.1). These findings were confirmed by Western Blot analysis, with the expression of a band corresponding to cytokeratin 7 (54 kDa) detected in the Swan 71 cells as well as in the HTR-8 and 3A trophoblast cell lines (Figure 2B).

The Swan 71 cells do not express CD45 or CD68

A potential contaminant in trophoblast cell preparations is immune cells, particularly macrophages. Therefore, we evaluated the expression of immune cell markers in the Swan 71 cells by immunocytochemistry. Using PMA-differentiated monocytic THP-1 cells as a positive control, the Swan 71 cells were assessed for the expression of CD45 and CD68, which are pan leukocyte and monocyte/macrophage markers, respectively. In contrast to the THP-1 monocytic cell line (Figure 3A.3 and Figure 3B.3), the Swan 71 cells did not express CD45 (Figure 3A.2) or CD68 (Figure 3B.2), nor was any immunostaining detected in the negative controls (Figure 3A.1 and Figure 3B.1). This suggests that the telomerase-infected Swan 71 cells are devoid of immune cell contamination.

The Swan 71 cells express vimentin, but not the Fibroblast Specific Antigen (FSA)

Since fibroblasts are one of the most common cellular contaminants of primary trophoblast cell preparations (Yui, Garcia-Lloret et al. 1994), the Swan 71 cells were evaluated for the presence of fibroblast cells. To accomplish this, the expression of vimentin, an intermediate filament protein that has been shown to be expressed in mesenchymal cells such as fibroblasts (Puts, Vooijs et al. 1986), was assessed in the Swan 71 cells by Western Blot analysis. Analogous to HeLa cells, both the Swan 71 cells and the HTR-8 trophoblast cell line expressed vimentin (57 kDa) (Figure 3C), which is in accordance with previous studies demonstrating that certain extravillous trophoblast populations might express vimentin (Aboagye-Mathiesen, Laugesen et al. 1996; Loke and Butterworth 1997). This suggests that the Swan 71 cells may exhibit characteristics of extravillous trophoblast.

In order to confirm the absence of fibroblasts in the Swan 71 cell culture, we examined the expression of the Fibroblast Specific Antigen (FSA), CD90/Thy-1, which has been shown to be confined only to fibroblast cells (Saalbach, Aneregg et al. 1996) by Western Blot analysis

using third trimester placental fibroblasts as a positive control (Saalbach, Aneregg et al. 1996). While the third trimester placental fibroblasts expressed high levels of FSA (65 kDa), no band corresponding to FSA could be detected in the Swan 71 cells (Figure 3D), confirming the purity of the Swan 71 cell clone.

HLA-G expression in the Swan 71 cells

Based on the observation that the Swan 71 cells express vimentin, which suggests that these cells are of extravillous trophoblast origin, our next aim was to further investigate this possibility. Therefore, the expression of HLA-G, a non-classical MHC class I molecule that is only expressed by extravillous trophoblasts (McMaster, Librach et al. 1995), was evaluated in the Swan 71 cells. The primary transcript of the HLA-G gene is known to generate at least seven alternative splice variants with the potential to encode four membrane-bound isoforms (HLA-G1, HLAG2, HLA-G3 and HLA-G4) and three soluble isoforms (HLA-G5, HLA-G6 and HLA-G7) (Carosella, Moreau et al. 2003). Using an antibody, which recognizes HLA-G5 (or soluble HLA-G1) and HLA-G6 (or soluble HLA-G2), we evaluated the expression of sHLA-G in the Swan 71 cells by Western Blot analysis. As Figure 4A demonstrates, the Swan 71 cells as well as a 6-week placenta were positive for sHLA-G expression (36 kDa), whereas the trophoblast-derived choriocarcinoma cell line, JAR, did not express sHLA-G as previously described (Kovats, Main et al. 1990; Yang, Geraghty et al. 1995). Moreover, the expression of sHLA-G did not appear to change with the number of passages since similar expression levels were observed in the Swan 71 cells over time.

Low levels of hCG is secreted by the Swan 71 cells

During pregnancy, the primary source of human Chorionic Gonadotrophin (hCG) detected in maternal serum and urine is the trophoblast (Hussa 1987). To determine whether the Swan 71 clone secreted hCG, the culture supernatants from the Swan 71 cells and the HTR-8 and 3A trophoblast cell lines were assayed for hCG secretion using the ISDT decidual stromal cell line as a negative control. As shown in Figure 4B, low levels of hCG were detected in the conditioned media of the Swan 71 cells (3.7 ± 0.14 mIU/ml) as well as the HTR-8 (5.3 ± 0.27 mIU/ml) and 3A (1.6 ± 0.08 mIU/ml) trophoblast cell lines. In contrast, the amount of hCG detected in the culture supernatant of the ISDT decidual cell line was similar to the background.

Fetal fibronectin (FFN) is secreted by the Swan 71 cells

Fetal fibronectin (FFN) is a uniquely glycosylated form of fibronectin that is thought to be released by trophoblast cells (Feinberg, Kliman et al. 1991; Guller, Wozniak et al. 1995) and glucocorticoids such as dexamethasone have been shown to differentially regulate the expression of FFN in placental tissues (Rosen, Krikun et al. 1998; Yuehong, D'Antona et al. 2001). To determine whether the Swan 71 cells secrete fibronectin and respond to dexamethasone treatment, fibronectin levels in the culture supernatants of the Swan 71 cells and the HTR-8 trophoblast cell line was evaluated by ELISA. In the absence of serum, approximately 8.4 ng/ μ g total protein of FFN was detected in the conditioned media of the Swan 71 cells and this amount nearly doubled with the addition of charcoal-stripped FBS to the culture media (Figure 5). Moreover, a significant increase ($p < 0.05$) in FFN secretion was also observed in the Swan 71 cells following treatment with 100 nM of dexamethasone for 48 hours, regardless of whether the cells were cultured in serum free media (22.4 ng/ μ g total protein) or with FBS (46.2 ng/ μ g total protein). Similar results were obtained with the HTR-8 trophoblast cell line in the absence (Figure 5) and presence of serum (data not shown).

Characterization of the cytokine/growth factor profile of the Swan 71 cells

Trophoblast cells have been reported to express a variety of cytokines and growth factors both *in vitro* (Kameda, Matsuzaki et al. 1990; Librach, Feigenbaum et al. 1994; Shore, Wang et al.

1997) and *in vivo* (Kameda, Matsuzaki et al. 1990; Paulesu, King et al. 1991; Sharkey, Charnock-Jones et al. 1993), which may influence their overall survival and the growth and survival of surrounding cells or tissues (Hanna, Hanna et al. 2000). Therefore, our next objective was to determine the cytokine/growth factor profile of the Swan 71 cells and the HTR-8 trophoblast cell line by cytokine array. Using an array that contains a total of 42 proteins, we evaluated Swan 71 cell lysates and observed the expression of Interleukin-1 beta (IL-1 β), IL-6, IL-8, Macrophage Colony Stimulating Factor (MCSF), Macrophage Inflammatory Protein-1 delta (MIP-1 δ), Oncostatin M (OSM) and the growth factors, Thrombopoietin (Tpo) and Vascular Endothelial Growth Factor (VEGF). A similar expression pattern was observed in the HTR-8 trophoblast cell line, although the HTR-8 cells expressed IL-6 to a much lesser extent than the Swan 71 cells (Figure 6A).

Since trophoblast cells do not necessarily secrete the cytokines that they express (Abrahams, Visintin et al. 2005), the cytokine profile in the conditioned media from both the Swan 71 cells and the HTR-8 trophoblast cell line was also assayed using a cytokine array with a total of 120 proteins. As Figure 6B indicates, a variety of cytokines and growth factors were detected in the culture supernatant of the Swan 71 cells, with the secretion of IL-1 β , IL-6, IL-8, Monocyte Chemoattractant Protein-1 (MCP-1), GRO (CXCR2), GRO- α (CXCL1), Intercellular Adhesion Molecule-1 (ICAM-1), Osteoprotegerin (OPG), Tissue Inhibitor of Metalloproteinase-1 (TIMP-1), TIMP-2 and VEGF being the most notable. A similar cytokine secretion profile was observed in the conditioned media of the HTR-8 trophoblast cell line, except that little or no IL-1 β could be detected.

The Swan 71 cells are resistant to Fas-mediated apoptosis, but sensitive to TNF- α -induced apoptosis

Since primary first trimester trophoblast cells are normally resistant to Fas-mediated apoptosis, but sensitive to TNF- α -induced apoptosis (Yui, Garcia-Lloret et al. 1994; Payne, Smith et al. 1999; Knofler, Mosl et al. 2000; Aschkenazi, Straszewski et al. 2002), the effect of Fas stimulation and TNF- α on Swan 71 cell survival was evaluated. First, the Swan 71 cells were incubated with an agonistic anti-Fas monoclonal antibody (mAb) for 24 hours and cell viability was assessed using the Fas sensitive Jurkat T cell line as a positive control. As shown in Figure 7A, the Swan 71 cells actually proliferated upon Fas stimulation ($115.6 \pm 9.2\%$) in comparison to untreated control ($100 \pm 5.1\%$), whereas a significant decrease in cell viability ($62.4 \pm 1.6\%$) was observed in the anti-Fas mAb treated Jurkat cells ($p < 0.001$). In contrast, treatment with TNF- α for 48 hours induced a $39.6 \pm 7.9\%$ decrease in Swan 71 cell viability (Figure 7B; $p < 0.001$). Similar results were obtained with the HTR-8 ($65.3 \pm 11.6\%$) and 3A ($51.1 \pm 10.5\%$) trophoblast cell lines ($p < 0.001$). To determine whether this TNF- α -induced decrease in trophoblast cell viability was due to apoptosis, caspase-3 activity was evaluated in the Swan 71 cells following treatment with TNF- α for 24–72 hours. Indeed, TNF- α treatment induced a significant increase in caspase-3 activity ($p < 0.001$) over time (Figure 7C). This confirmed that the Swan 71 cell clone is Fas resistant, but sensitive to TNF- α -mediated apoptosis.

Discussion

The growing number of studies on trophoblast biology in recent years has significantly increased the demand for suitable *in vitro* models. Although trophoblast cell lines have been established by SV40 or similar transformation, numerous are reported to exhibit karyotypic and/or phenotypic abnormalities (Aboagye-Mathiesen, Zdravkovic et al. 1996; Khoo, Bechberger et al. 1998; King, Thomas et al. 2000). Our group and others have successfully generated several cell lines by inducing telomerase activity without affecting the karyotype or phenotype of the cells (Jiang, Jimenez et al. 1999; Morales, Holt et al. 1999; Alvero, Fishman et al. 2004; Krikun, Mor et al. 2004; Krikun, Mor et al. 2005). Previously, Wang *et al.* reported

the successful immortalization of a telomerase-infected first trimester trophoblast cell line, B6Tert (Wang, Qiu et al. 2006). However, the primary parental cells used to generate the B6Tert cells were infected with hTERT at passage number 25 and almost 10 years after isolation, questioning whether these cells had acquired phenotypic and genotypic traits not characteristic of primary trophoblasts before immortalization (Rong-Hao, Luo et al. 1996; Wang, Qiu et al. 2006). In the present study, we describe a first trimester trophoblast cell line infected with telomerase at passage 3 and only a few days after isolation that retain similar characteristics as primary first trimester trophoblast cells.

Initially, we determined that the Swan 71 cells are positive for telomerase activity, confirming that the introduction of hTERT was successful. The induction of telomerase activity correlated with a prolonged lifespan, as evidenced by the number of passages that the Swan 71 cells have been maintained in culture. Interestingly, placentas and chorionic villi from normal pregnancies have been shown to exhibit telomerase activity (Kyo, Takakura et al. 1997; Izutsu, Kudo et al. 1998; Kudo, Izutsu et al. 2000). In addition, Kyo *et al.* also demonstrated that the trophoblast cell fraction of chorion was positive for telomerase activity, indicating that the trophoblast was the source of activity (Kyo, Takakura et al. 1997). This suggests that telomerase might be involved in maintaining trophoblast longevity *in vivo*.

After confirming that the Swan 71 cells had been successfully infected with hTERT, we determined that the Swan 71 cells are also positive for hCG and FFN secretion, cytokeratin 7, vimentin and HLA-G expression, but do not express CD45, CD68 or FSA. The relatively low level of hCG secretion suggests that the Swan 71 cells are either intravillous or extravillous cytotrophoblast in origin since early first trimester placentas contain a substantial amount of intravillous cytotrophoblasts (Suzuki, Nakayama et al. 1999) and neither undifferentiated intravillous or extravillous trophoblasts secrete hCG during pregnancy (Gosseye and Fox 1984). However, the expression of vimentin and HLA-G indicates that the Swan 71 cells are derived from extravillous cytotrophoblasts as extravillous trophoblasts are known to express vimentin and HLA-G, whereas villous trophoblast cells do not express vimentin or HLA-G. HLA-G is a non-classical MHC class I molecule that is expressed by the trophoblast to prevent an immune response from maternal Natural Killer cells (Chumbley, King et al. 1994). The expression of vimentin, on the other hand, is likely to be related to the invasive properties of extravillous trophoblasts (Aboagye-Mathiesen, Laugesen et al. 1996). Certain epithelial cancer cell lines also express vimentin and the expression of vimentin has been shown to be associated with the increased invasiveness of these cells both *in vitro* and *in vivo* (Birchmeier, Weidner et al. 1993). Therefore, the Swan 71 cells might express vimentin for invasion since they have been reported to exhibit invasive properties on matrigel (Aplin, Straszewski-Chavez et al. 2006; Fest, Aldo et al. 2007). Interestingly, the B6Tert extravillous first trimester trophoblast cell line described by Wang *et al.* (Wang, Qiu et al. 2006) was negative for vimentin expression, however, the expression of CD45, CD68 or FSA was not investigated in this study. Thus, this raises the possibility of immune cell, fibroblast or other potential cell contaminant in the B6Tert cell culture. This may also explain why the parental primary cells used to generate the B6Tert cells did not senesce until approximately 50 passages (Wang, Qiu et al. 2006), whereas in our experience, primary trophoblast cells undergo senescence after only a few passages (Straszewski-Chavez, Abrahams et al. 2004; Straszewski-Chavez, Visintin et al. 2007).

Once we had determined which phenotypic markers the Swan 71 cells expressed, the cytokine and growth factor profile of the Swan 71 cells was evaluated. The Swan 71 cells exhibit a profile that is consistent with the type of cytokines and growth factors that primary trophoblast cells have been shown to express and/or secrete. In particular, analogous to the Swan 71 cells, the expression and secretion of IL-1 β , IL-6 and VEGF, as well as the lack of TNF- α or IFN- γ secretion from primary human trophoblasts has been previously demonstrated (Kameda, Matsuzaki et al. 1990; Librach, Feigenbaum et al. 1994; Jokhi, King et al. 1997; Shore, Wang

et al. 1997). The high level of TIMP secretion by the Swan 71 cells may explain the occasional multi-nucleated cell clusters observed in culture given that first trimester extravillous trophoblasts have been reported to undergo fusion *in vitro* via the TGF- β /TIMP pathway (Graham and Lala 1991; Graham, Lysiak et al. 1992). Moreover, the expression and/or secretion of several chemokines such as IL-8, MCP-1 and GRO- α was also detected, which may be due to the ability of trophoblasts to recruit immune cells as previously described (Abrahams, Visintin et al. 2005). This cytokine and growth factor expression and secretion profile does not appear to change with the number of passages or time since previous studies from our laboratory have demonstrated that the Swan 71 cells express and/or secrete similar levels of cytokines and growth factors by several different methods (Costello, Joyce et al. 2007; Fest, Aldo et al. 2007; de la Torre, Mulla et al. 2009; Mulla, Yu et al. 2009).

Finally, the sensitivity of the Swan 71 cells to anti-Fas and TNF- α was assessed. Although they are resistant to Fas-mediated apoptosis, the Swan 71 cells are sensitive to TNF- α -induced apoptosis, attributes that are characteristic of primary first trimester trophoblast cells (Yui, Garcia-Lloret et al. 1994; Payne, Smith et al. 1999; Knofler, Mosl et al. 2000; Aschkenazi, Straszewski et al. 2002; Straszewski-Chavez, Abrahams et al. 2004). Therefore, the Swan 71 cells might be useful for studying the intracellular pathways regulating trophoblast apoptosis and survival.

Previous attempts to establish trophoblast cell lines that are representative of primary trophoblasts have been hindered by the lack of an appropriate immortalization approach. This study describes a novel telomerase-infected first trimester trophoblast cell line, which retained the characteristics of primary trophoblast cells. Since the Swan 71 cells have been maintained in culture for over 100 passages without exhibiting any signs of senescence, these cells can be considered immortal. The Swan 71 cells have already proven to be valuable in several *in vitro* studies (Costello, Joyce et al. 2007; Fest, Aldo et al. 2007; Fest, Brachwitz et al. 2008; Fraccaroli, Alfieri et al. 2009; Fraccaroli, Alfieri et al. 2009; Mulla, Yu et al. 2009) and should continue to represent a useful model for future trophoblast studies.

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(A) **(B)**

Figure 1. The Swan 71 cells are positive for telomerase activity

(A) Light microscopy (20X) of the 7-week parental trophoblast cells, PL129, which senesced at passage 4, and the telomerase-transfected trophoblast cells, Swan 71. (B) Telomerase activity in passage 15 Swan 71 cells was determined by a PCR-ELISA method. The PL129 parental trophoblast cells and heat-inactivated cell lysates (+Heat) served as negative controls.

*=statistical significance between the non-heat inactivated PL129 and Swan 71 cells;

**=statistical significance between the non-heat inactivated and heat inactivated Swan 71 cells.

Figure 2. Cytokeratin 7 expression in the Swan 71 cells

The expression of cytokeratin 7 was evaluated in the Swan 71 cells at passage 10 by immunocytochemistry (A2) and at passage 27 by Western Blot analysis (B) using the HTR-8 (H8) and 3A trophoblast cell lines (B) as positive controls. Swan 71 cells incubated without primary antibodies served as a negative control (A1). The blot was re-probed for β -actin as a loading control.

Figure 3. The Swan 71 cells express vimentin, but not CD45, CD68 or FSA

Swan 71 cells were assessed for the expression of CD45 (A2) and CD68 (B2) at passage 10 by immunocytochemistry using phorbol 12-myristate 13-acetate (PMA)-differentiated monocytic THP-1 cells as a positive control (A3 and B3). THP-1 cells (A1 and B1) incubated without primary antibodies served as negative controls. The expression of vimentin (C) and the Fibroblast Specific Antigen (FSA/CD90/Thy-1) (D) was evaluated at passage 14 by Western Blot analysis. As positive controls, the HTR-8 trophoblast cell line and HeLa cells (+C) were used for vimentin expression, while third trimester placental fibroblasts (+C) served as a positive control for the expression of FSA. β -actin was used as a loading control.

Figure 4. The Swan 71 cells express HLA-G and secrete low levels of human Chorionic Gonadotrophin (hCG)

(A) HLA-G expression was assessed in different passages (numbers refer to month and year of pass) of the Swan 71 cells between passage 14 and 52 by Western Blot analysis. The trophoblast-derived choriocarcinoma cell line, JAR, was used as a negative control, while a 6-week placenta served as a positive control (+C). The blot was re-probed for β -actin as a loading control. (B) Conditioned media from the Swan 71 cells was analyzed for hCG secretion at passage 11 by an automated immunometric assay using the ISDT decidual stromal cell line and the 3A and HTR-8 trophoblast cell lines as negative and positive controls, respectively. Bar graph shows the concentration of hCG expressed as mIU/ml.

Figure 5. Fetal fibronectin (FFN) secretion by the Swan 71 cells

The level of FFN in the culture media of the Swan 71 cells was measured by ELISA at passage 31 using the HTR-8 trophoblast cell line as a positive control. Note that the amount of FFN secretion from the Swan 71 cells increased in the presence of both FBS and dexamethasone containing medium. Bar graph shows the concentration of FFN expressed as ng/μg of total protein. NT=No Treatment

Figure 6. The cytokine/growth factor profile of the Swan 71 cells

Cytokine and growth factor expression (A) and secretion (B) was evaluated in Swan 71 cell lysates and culture supernatants at passage 22 and 25, respectively, by cytokine array. The HTR-8 first trimester trophoblast cell line was used as a comparison. Signal intensities were normalized against the positive controls on each array membrane and a value greater than 0.2 was considered significant.

Figure 7. The Swan 71 cells are resistant to Fas-mediated apoptosis, but sensitive to TNF- α -induced apoptosis

(A) Swan 71 cells were incubated with 500 ng/ml of an agonistic anti-Fas (α -Fas) monoclonal antibody or (B) 25 ng/ml of TNF- α for 24 hours at passage 44 using the Fas-sensitive Jurkat T cell line and the HTR-8 and 3A trophoblast cell lines as positive controls, respectively. Cell viability was evaluated using a colorimetric assay. Bar graphs show percent cell viability relative to the untreated control (NT). (C) The cell lysates of passage 39 Swan 71 cells were analyzed for caspase-3 activity using a luminescent assay following treatment with 25 ng/ml of TNF- α for 24, 48 and 72 hours (H). Bar graph shows caspase activity expressed as Relative Light Units (RLU). NT=No Treatment