Immortalization of human urothelial cells by human papillomavirus type 16 E6 and E7 genes in a defined serum-free system

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Abstract. Normal human epithelial cell cultures exhibit a limited (although different between tissues) lifespan *in vitro*. In previous studies, urothelial cell cultures were immortalized using retroviral transformation with human papillomavirus type 16 E6 and E7 genes, in undefined culture systems containing serum or bovine pituitary extract. **Objective:** Due to the variability of results in such systems, we instead developed a procedure for the immortalization of urothelial cells using a defined, serum-free culture system. **Method and results:** Immortalization through retroviral transformation with human papillomavirus type 16 E6 and E7 was successful, and transformation of urothelial cells conferred an extended over normal lifespan and restored telomerase activity. Transformed cells retained typical morphology and exhibited a similar growth rate, cytokeratin immunoreactivity pattern, and response to growth factors as observed in untransformed cells. Karyotype analysis revealed a gradual accumulation of genetic mutations that are consistent with previously reported mutations in epithelial cells transformed with human papillomavirus type 16 E6 and E7. **Conclusion:** The ability to extend the *in vitro* lifespan of cells holds the potential to reduce the continuous need for tissue samples and to enable complete investigations with one cell line.

INTRODUCTION

In vitro cultures of normal human cells proliferate for a limited number of population doublings before entering replicative senescence (Hayflick & Moorhead 1961). While the exact mechanism triggering this senescence is yet to be elucidated, a current hypothesis is that cells enter senescence when telomeres, which shorten with successive cell divisions, reach a critically short threshold length (Harley *et al.* 1990) due to loss of telomerase function (Vaziri & Benchimol 1998). This hypothesis is supported by studies that have shown that exogenous expression of human telomerase reverse transcriptase (hTERT) restores telomerase activity and confers an

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extended lifespan in several types of human cells (Bodnar *et al.* 1998; Yang *et al.* 1999; Forsythe *et al.* 2002). Restoration of telomerase is not sufficient to immortalize some types of cells, however, suggesting that telomere shortening alone cannot completely explain the replicative lifespan limit (Kiyono *et al.* 1998; Dickson *et al.* 2000; O'Hare *et al.* 2001; Di Donna *et al.* 2003). This disparity suggests there are fundamental differences in the control of senescence in various cell types.

In some epithelial cell types, including keratinocytes (Kiyono *et al.* 1998; Dickson *et al.* 2000), mammary epithelial cells (Brenner *et al.* 1998; Kiyono *et al.* 1998; Romanov *et al.* 2001), prostate epithelial cells (Jarrard *et al.* 1999) and urothelial cells (Puthenveetil *et al.* 1999), there is evidence of an association between senescence and the increased expression and accumulation of p16^{INK4A}. Recent studies have shown that loss of p16 function does not extend urothelial cell lifespan in cultures derived from adult tissue specimens (Shaw *et al.* 2005; Chapman *et al.* 2006), but does extend lifespan in cultures derived from child tissue specimens (Chapman *et al.* 2006). Thus, the exact relationship between p16 accumulation and senescence in urothelial cell cultures remains unknown. It has been reported that the immortalization of some keratinocytes and epithelial cells requires a combination of telomerase activity restoration and a disruption in the retinoblastoma tumour suppressor protein (RB) and p16 pathway (Reznikoff *et al.* 1996a; Kiyono *et al.* 1998; Jarrard *et al.* 1999; Dickson *et al.* 2000). Some groups have reported that when cultured on fibroblast feeder layers, mammary epithelial cells (Herbert *et al.* 2002) and keratinocytes (Herbert *et al.* 2002; Fu *et al.* 2003) do not exhibit elevated p16 associated senescence. Others have reported that p16 is elevated and is associated with senescence in keratinocytes in both a serum-free and the feeder layer system (Rheinwald *et al.* 2002). These differences highlight the importance of recognizing the influence of the culture system in which an investigation is performed. Different culture conditions are known to result in differential expression of p16 and hTERT, as well as different levels of telomerase activity in keratinocytes that were immortalized with human papillomavirus type 16 (HPV-16) oncogenes E6 and/or E7 (Fu *et al.* 2003). E7 binds to the pRB (Dyson *et al.* 1989), which is known to play an important role in cell cycle control (Dyson 1998). pRB binds to and represses certain E2F transcription factors (Helin *et al.* 1993), which are involved in the transcriptional activation of many genes, including cell cycle control genes that enable G₁-S cell-cycle progression (Dyson 1998). Thus, through interfering with pRB/E2F complex formation, E7 could override pRB-mediated G₁ cell cycle arrest. E6 binds to and induces the degradation of p53 (Scheffner *et al.* 1990), as well as inducing telomerase activity by activating transcription of the hTERT gene (Veldman *et al.* 2001). The exact mechanism of transcriptional activation is not fully known, and there is discrepancy in the literature regarding what is known about the mechanism. Some report an association between E6 and c-Myc in activating hTERT transcription (McMurray & McCance 2003; Veldman *et al.* 2003), and others report activation of hTERT expression is not c-Myc dependent (Gewin & Galloway 2001). Another hypothesis proposed is that a novel telomerase repressor, NFX-91, is targeted for ubiquitination and degradation by E6-E6AP complexes, resulting in the de-repression of the hTERT promoter (Gewin *et al.* 2004).

Transformation with HPV-16 E6 and/or E7 has been used for the immortalization of many types of epithelial cells (Halbert *et al.* 1991; Shay *et al.* 1993; Reznikoff *et al.* 1994, 1996b; Foster & Galloway 1996; Kiyono *et al.* 1998; McMurray & McCance 2004), including urothelial cells (Reznikoff *et al.* 1994, 1996a), and it has proven to be a very effective system. However, the studies in which urothelial cells have been immortalized using either HPV-16 E6 or E6/E7 were performed in undefined cell culture systems that contained either bovine pituitary extract (Diggle *et al.* 2000, 2001) or foetal bovine serum (FBS) (Reznikoff *et al.* 1994, 1996a; Savelieva *et al.* 1997; Vieten *et al.* 1998), respectively. A recent study performed in a different culture system from ours and the HPV-16 E6/E7 studies, has reported the immortalization of urothelial cells through

retroviral expression of hTERT without the requirement for RB/p16 pathway alteration (Chapman *et al.* 2006). Due to previously reported differences in the HPV-16 E6/E7 immortalization of keratinocytes that were attributed to cell culture conditions (Fu *et al.* 2003), we have sought to immortalize urothelial cells in a defined, serum-free culture system via retroviral transformation with HPV-16 E6 and E7 genes. Here we describe our success and provide characterization and comparison between parental untransformed cultures and immortalized cultures.

MATERIALS AND METHODS

Retroviral vector

The pLXSN16E6E7 vector containing the HPV-16 E6 and E7 genes, under control of the Moloney murine leukaemia virus (MoMuLV) promoter-enhancer sequences, confers resistance to the antibiotic neomycin (G418) (Halbert *et al.* 1991). This vector was obtained from the American Type Culture Collection already packaged in the mouse amphotropic packaging cell line PA317 – this cell line is termed PA317 LXSN 16E6E7. Viral supernatants were collected, filtered through 0.45 µm pore cellulose acetate, and were stored at –70 °C until use.

Cell culture

Ureteric or bladder tissue was obtained with informed consent under a human subjects' protocol, approved by the Institutional Review Board of the Children's Hospital and Regional Medical Center. Tissue explants were processed in a protocol based on previously published methods (Bagai *et al.* 2002; Hudson *et al.* 2005; Delostrinos *et al.* 2006; Zhang *et al.* 2006). Surgical explant tissue was incubated in 0.1% ethylenediaminetetraacetic acid (EDTA) pH 8.0, 10 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulphonic acid (HEPES) (pH 7.2–7.5) in 1 mM phosphate-buffered saline (1 × PBS) at 4 °C for 16–24 h. After washing twice with 1 × Hank's balanced salt solution (HBSS), tissue was minced, and then was washed three times more with 1 × HBSS. Minced tissue pieces were then incubated in 1 × HBSS, 10 mM HEPES (pH 7.4), 0.0055 mM CaCl₂ and 200 U/ml type IV collagenase from *Clostridium* spp. (Sigma-Aldrich, St. Louis, MO, USA) for 10 min at 37 °C. Following incubation, minced tissue was washed twice with warmed Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Invitrogen), separating the minced tissue into equal aliquots upon the second washing. The tissue aliquots were then re-suspended into 3 ml of either non-infectious DMEM or infectious DMEM containing HPV-16 E6/E7 retrovirus. Cholera toxin and polybrene (Sigma) were added to final concentrations of 0.03 µg/ml and 4 µg/ml, respectively. The tissue re-suspensions were plated into three 6-well Primaria plates. After 24 h, all plating medium was replaced with a 50/50 (v/v) mixture of DMEM and Defined Keratinocyte Serum Free Medium supplemented with the manufacturer's growth supplement (DK-SFM + GS) (Invitrogen). After 36 h, this medium was removed and cells were fed with the selection medium DK-SFM + GS with 10 µg/ml G418 (Sigma). Selection was viewed as complete when the selection medium killed all cells in one of the non-infected control wells. All transformed cells continued to be cultured in DK-SFM + GS containing 10 µg/ml G418 after the selection period, and all untransformed cells were maintained in DK-SFM + GS, unless otherwise noted.

Telomerase activity assay

Telomerase activity of cell culture samples was determined using the TRAPeze® Gel-Based Telomerase Detection Kit (Chemicon, Temecula, CA, USA) (Feng *et al.* 1995). For the assay,

cell pellets of $\geq 1 \times 10^5$ cells were collected, and were lysed in the supplied $1 \times$ CHAPS lysis buffer with the addition of RNasin Plus (Promega, Madison, WI, USA) 200 units/ml final concentration for 30 min on ice. Lysates were then sedimented at $12\,000 \times g$ for 20 min at 4°C , and the cell extract supernatant was transferred to a clean tube. Protein concentrations were determined with the Pierce Bicinchoninic acid Protein Assay Kit and a Bio-Tek PowerWave XS plate reader. All extracts were aliquoted and stored at -80°C , and were thawed only for single use. Extracts were diluted to $0.5\text{ }\mu\text{g/ml}$ of total protein in $1 \times$ CHAPS buffer (with RNasin Plus) for the subsequent reactions.

Reaction mixtures contained $2\text{ }\mu\text{l}$ of test extract, AmpliTaq Gold DNA polymerase (Roche, Indianapolis, IN, USA), RNasin, and the deoxyribonucleotide triphosphates, primers, and TRAP reaction buffer provided in the TRAPeze kit. Reactions were incubated at 30°C for 30 min, then PCR amplified through 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min in a PerkinElmer GeneAmp 2400 thermocycler (Perkin Elmer, Waltham, MA, USA). PCR products were electrophoresed in a 12.5% non-denaturing polyacrylamide gel, stained with Sybr Green I (Molecular Probes, Eugene, OR, USA) and visualized with a Molecular Dynamics FluorImager SI (Sunnyvale, CA, USA).

Analysis of each sample included two assays: one reaction with test extract and one reaction with heat-treated test extract. Heat-treatment at 85°C for 10 min inactivates telomerase because it is a heat-sensitive enzyme. Heat-treated samples served as negative controls. In addition to the positive control and primer-dimer/PCR contamination controls provided by the kit, cell extracts were generated from the UM-UC-3 transitional cell carcinoma cell line to use as an additional positive control (Grossman *et al.* 1986). These cells were cultured in Eagles' minimum essential medium supplemented with 2 mM L-glutamine, Earle's balanced salt solution adjusted to contain 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 10% FBS.

Growth curves

Cells were seeded at a density of $1.67\text{--}2.53 \times 10^5$ cells per 25 cm^2 flask in DK-SFM. When individual flasks approached 90% confluency, cells were harvested with trypsin and were counted using a haemocytometer. A fraction of counted cells was re-plated – this process was repeated for 30 days. All cells were accounted for in generating growth curves. Exponential trend lines, along with corresponding exponential equations and correlation coefficients, were generated with Microsoft Excel. Doubling times were calculated by solving for x at two y -values that differed by a factor of 2. Collection data for the growth curve was performed between passages 3 and 8, from day 19 to day 48 in culture for non-transformed cells, and between passages 53 and 58, from day 238 to day 263 in culture for transformed cells.

DNA synthesis assays

For the purposes of this assay, DK-SFM was used both with (DK-SFM + GS) or without (DK-SFM – GS) the manufacturer's growth factor supplement (GS). Untransformed and transformed urothelial cell cultures were grown in 96-well tissue culture plates (Costar, Corning, NY, USA) in DK-SFM + GS. At 80% confluency, cultures were washed twice with DK-SFM-GS and fed DK-SFM that contained $5\text{ }\mu\text{g/ml}$ heparin (Sigma), with or without GS, and with or without $1\text{ }\mu\text{M}$ PD-153035 (Roche), a potent epidermal growth factor receptor (EGFR) inhibitor. PD-153035 blocks autophosphorylation of EGFR, preventing any downstream signalling from this receptor (Fry *et al.* 1994). Cells were incubated under these conditions for 45 h, after which 5-bromo-2'-deoxyuridine (BrdU; Roche) was added to all wells to a $10\text{-}\mu\text{M}$ final concentration. After 2 h exposure to BrdU, cells were fixed for 0.5 h and were incubated for 1.5 h with a monoclonal mouse anti-BrdU antibody conjugated to horseradish peroxidase (Cell Proliferation ELISA,

Table 1. Cytokeratin expression in parental and immortalized cultures

Antibody	Source	Location	Species	Concentration used	Parental line	Immortal line
CK 7	Novocastra	Newcastle Upon Tyne, UK	mouse	1 µg/ml	+	+
CK 8	Zymed	San Francisco, CA, USA	mouse	2.1 µg/ml	+	+
CK 10	Chemicon	Temecula, CA, USA	mouse	1 : 200 dilution of ascites fluid	+	+
CK 13	Abcam	Cambridge, MA, USA	mouse	1 : 100 dilution of tissue culture supernatant	–	–
CK 14	Serotec	Oxford, UK	mouse	5 µg/ml	+	+
CK 17	Sigma	St. Louis, MO, USA	mouse	8.5 µg/ml	+	+
CK 18	Sigma	St. Louis, MO, USA	mouse	5.5 µg/ml	+	+
CK 19	Novocastra	Newcastle Upon Tyne, UK	mouse	1 : 100 dilution of tissue culture supernatant	+	+
CK 20	Fitzgerald	Concord, MA, USA	mouse	1 µg/ml	–	–

BrdU, Roche). Addition of tetramethyl-benzidine to each well produced a colorimetric reaction between the peroxidase and this substrate, and relative DNA synthesis was correlated with absorbance at 370 nm in each well.

Immunocytochemistry

Transformed and untransformed urothelial cells were screened for immunoreactivity with anti-cytokeratin antibodies (Table 1). Cells seeded onto coverslips were grown to 70% confluence, then were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in PBS (pH 7.4) (Sigma) for 15 min at room temperature. Following fixation, coverslips were washed three times, for 5 min each, with 0.01 M PBS (pH 7.4), 0.05% Tween 20 (Sigma) (PBS-T). Coverslipped cells were then blocked with 5% normal goat serum (Vector Laboratories, Burlingame, CA, USA) in PBS (pH 7.4) for 30 min at room temperature, and were rinsed briefly with PBS-T. Twelve to sixteen hours of incubation with a mouse monoclonal primary antibody was performed at 4 °C, followed by three 5-min washes with PBS-T. Cells were then incubated with 7.5 µg/ml goat anti-mouse immunoglobulin G conjugated with Cy2 fluor (Jackson Immuno Research, West Grove, PA, USA) for 1 h at room temperature, and were washed three times, for 5 min each, with PBS-T. Coverslips were then washed twice for 5 min each with reverse osmosis deionized water, then cells were stained with 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes) for 2 min at room temperature. Three 5-min washes with PBS-T were performed following DAPI staining, and coverslips were mounted with ProLong Antifade Mounting Media (Molecular Probes). Negative controls consisted of omitting the primary antibody step. Auto-fluorescence controls consisted of fixing and mounting cells with no further processing performed. Cells were viewed using a Leica DMI 6000B inverted fluorescence microscope with a Leica 40× correction collar objective. Images of 1300 × 1030 resolution were captured using a Leica DC 500 CCD digital camera.

Cytogenetic analysis

Metaphase chromosome spreads from cultures of untransformed urothelial cells at passage 4 and from transformed cells at passages 3, 21 and 54 were harvested and G-banded (Wright's stain) using standard cytogenetic techniques (Priest 1997). Briefly, the culture medium was removed and cells were incubated with 0.06 µg/µl of colcemid (Invitrogen) at 37 °C for 2.5 h. They were then detached from the T-25 flasks with trypsin/EDTA (0.5%/0.1%) and were incubated in

10 ml of 1 : 1 (v/v) mixture of 0.8% sodium citrate and 0.075 M potassium chloride for 30 min at 37 °C. This was followed by the slow addition of 5 ml Carnoy's fixative (3 : 1 (v/v); methanol : acetic acid). Cells were centrifuged 10 min at 200 × *g*, then were re-suspended in 10 ml Carnoy's fixative, and were incubated at room temperature for 30 min. Following fixation, the cells were centrifuged and were re-suspended in 10 ml of Carnoy's fixative twice more. For analysis, suspensions of fixed nuclei were applied dropwise to a moistened microscope slide to yield metaphase spreads, which were treated with trypsin, followed by Wright's stain to give G-banded chromosomes. Cell parameters were analysed using a digital imaging system (Applied Imaging, Santa Clara, CA, USA), and karyotypes were described according to the recommendations of the International System for Human Cytogenetic Nomenclature (Shaffer & Tommerup 2005).

Single-cell sorting of transformed cells

Transformed cells at passage 49 were incubated in Versene (Invitrogen) at 37 °C for 5 min until the cells rounded up. These were then incubated in 0.25% (v/v) trypsin (Invitrogen) in Versene until they detached. Cells were pelleted by sedimentation at 180 × *g* for 5 min, then were washed once with DK-SFM. The final cell pellet was re-suspended in 1 ml of DK-SFM, and 1 ml of Accumax (Innovative Cell Technologies Inc., San Diego, CA, USA) was added to the cell suspension. The suspension was incubated for 10 min at 37 °C to disperse cell clumps. Cells were then single sorted into 96-well Primaria™ plates using an Influx flow cytometer cell sorter located in the Department of Pathology at the University of Washington, Seattle, WA, USA.

RESULTS

Results described in this section were obtained from work performed on two ureter cell lines and one bladder cell line. Unless otherwise mentioned, urothelial cells described in this section refer to the ureter cell lines.

Telomerase activity in parental, untransformed cells

Untransformed human urothelial cell lines were tested for telomerase activity by the TRAP assay. Cultures with telomerase activity add telomeric repeats on to the 3' end of an oligonucleotide substrate. Subsequent PCR amplification of these extension products generated a ladder that increased by 6 bp starting at 50 bp. A 36-bp internal standard product was also generated in each reaction. This internal control band indicated some PCR inhibition present in telomerase positive samples; however, PCR inhibition was minimal in telomerase negative samples. All telomerase activity levels observed in untransformed urothelial cell cultures were lower than activity levels in the UM-UC-3 transitional cell carcinoma (TCC) line tested (Fig. 1, lanes 2 and 3). This TCC line was used as a positive control as it has been documented that TCC, like many tumour cells, exhibit high levels of telomerase activity (Belair *et al.* 1997; Kinoshita *et al.* 1997). Both ureter and bladder cell cultures exhibited low levels of telomerase activity (Fig. 1, lanes 4 and 10, respectively). This finding supports previous reports of telomerase activity in cultured human urothelial cells (Belair *et al.* 1997; Puthenveetil *et al.* 1999). In our assays, telomerase activity level diminished rapidly with time in culture. A steady reduction of extension products is observed in a cell culture with continued passage (Fig. 1, lanes 4, 6 and 8). For comparison, a bladder cell culture is shown at passage 4, 33 days total, which exhibits extremely low activity (Fig. 1, lane 10). In a ureter cell culture, at passage 6, 60 days total, telomerase activity was almost undetectable (Fig. 2, lane 4).

Figure 1. Telomerase activity levels in untransformed cells as detected by TRAP assay. Shown is a 12.5% polyacrylamide gel stained with Sybr Green I. 1: molecular weight marker bp. 3, 5, 7, 9 and 11: heat inactivated samples. 2 and 3: UM-UC-3 transitional bladder carcinoma. 4 and 5: ureter cells, P4, 19 days in culture. 6 and 7: ureter cells, P5, 23 days in culture. 8 and 9: ureter cells P6, 28 days in culture. 10 and 11: bladder cells, P4, 33 days in culture.

Transformation of normal human urothelial cultures

Transformation with HPV-16 E6/E7 at initial plating produced foci of rapidly multiplying cells. During the selection period, 60–70% of cells succumbed to antibiotic treatment, while 30–40% remained healthy and continued to proliferate under selection conditions, yielding a culture of transformed cells with extended lifespan.

Immortalization results in restoration of telomerase activity

Urothelial cell cultures transformed with HPV-16 E6/E7 exhibited strong telomerase activity (Fig. 2). Untransformed cells after as little as 28 days in culture showed very little telomerase activity (Fig. 1, lanes 8 and 10), while transformed cells at 27 and 47 days in culture showed much higher telomerase activity levels in comparison (Fig. 2, lane 6). TRAP assays performed on transformed cells generated DNA ladders with as many as nine clearly visible extension products (Fig. 2, lane 6), which was an activity level similar to that of the transitional cell carcinoma positive control (Fig. 2, lane 2).

Retention of cellular phenotype in immortalized urothelial cultures

Cultures of transformed and non-transformed cells passaged at equivalent densities, exhibited highly similar attachment and spreading kinetics (not shown) and displayed cobblestone morphology typified by 'giant' cells surrounded by smaller basal cells (Fig. 3a,b,e–h). After 40–60 doublings, untransformed cells between passages 4–10 exhibited the following characteristics of senescence: increased blebbing, larger cytoplasmic areas, decreased cobblestone appearance, and had a larger, more flattened appearance (Fig. 3c,d). In contrast, HPV-16 E6/E7-transformed urothelial cells had not exhibited senescent morphological characteristics at 641 days in culture. Transformed cells at passages 10, 29 and 59 (73, 139 and 266 days in culture, respectively) (Fig. 3f–h), exhibited morphology almost identical to healthy, early passage, non-transformed

Figure 2. Restoration of telomerase activity by transformation with HVP-16 E6/E7 detected by TRAP assay. Shown is a composite 12.5% polyacrylamide gel stained with Sybr Green I. 1: molecular weight marker bp. 3, 5 and 7: heat inactivated samples. 2 and 3: UM-UC-3 transitional bladder carcinoma cell line. 4 and 5: normal, untransformed cells at passage 6, 60 days in culture. 6 and 7: cells transformed with E6/E7, passage 4, 27 days in culture.

Figure 3. Urothelial cells transformed with HPV-16 E6/E7 exhibit a normal morphology by phase contrast microscopy. (a–d) Untransformed cells. (e–f) Transformed cells. (a) Ureter cells, P2, 17 days in culture. (b) Ureter cells, P4, 31 days in culture. (c) Ureter cells, P10, 73 days in culture. (d) Bladder cells, P4, 46 days in culture. (e) Ureter cells, P4, 31 days in culture. (f) Ureter cells, P10, 73 days in culture. (g) Ureter cells, P29, 139 days in culture. (h) Ureter cells, P59, 266 days in culture. 10× magnification; size bar is 40 µm.

Figure 4. Growth curve of untransformed and transformed urothelial cells. (a) Log scale. (b) Non-log scale. Cells were seeded into a T75 cm² flask in DK-SFM, harvested at 90% confluency and counted. All cells were accounted for in calculations. Time 0 represents passage 3, day 19 in culture for untransformed cells, and passage 53, day 238 in culture for transformed cells.

cells (Fig. 3a,b). At the time of submission of this manuscript, the transformed cell mass culture had reached passage 127, which is 641 days in culture.

Growth rates of transformed and untransformed cultures

A growth curve was constructed of the untransformed and transformed cultures over a 30-day period (Fig. 4). For the first 15–17 days, both types of cell culture exhibited similar growth, with doubling times roughly constant over the first 20 days. Beginning at day 20, untransformed cell culture growth slowed with increased passage number and time in culture (Fig. 4). Doubling times were calculated as 57 h for the untransformed cells, and 37 h for the transformed cells, over the same time period. Our data and some comparative examples are provided in Table 2.

Table 2. Comparison of doubling time amongst various epithelial cell lines

Cell line	Time of 1 cell cycle	Medium used ¹	Reference
Parental flask 1 – normal urothelial	52 h	DK-SFM, defined, serum-free, with epidermal growth factor (EGF) and proprietary growth supplement	Data from Fig. 3
Parental flask 2 – normal urothelial	52 h	DK-SFM, defined, serum-free, with EGF and proprietary growth supplement	Data from Fig. 3
Transformed flask 1 – normal urothelial	37 h	DK-SFM, defined, serum-free, with EGF and proprietary growth supplement	Data from Fig. 3
Transformed flask 2 – normal urothelial	37 h	DK-SFM, defined, serum-free, with EGF and proprietary growth supplement	Data from Fig. 3
Normal human urothelial primary cultures	14.7 h	K-SFM, undefined, serum-free, with EGF and bovine pituitary extract	Southgate <i>et al.</i> 1994; Delostrinos <i>et al.</i> 2006
Normal human urothelial primary cultures	51 h	HMRI-1, undefined, serum-free, with EGF and bovine pituitary extract	Kirk <i>et al.</i> 1985
Foetal oral epithelial line	90, 55 and 26.5 h for different lines	DK-SFM, defined, serum-free, with EGF and proprietary growth supplement	Gilchrist <i>et al.</i> 2000
Conjunctiva epithelial line	16.5 h	DMEM, with 10% serum	Girjes <i>et al.</i> 2003
Conjunctiva epithelial line	26.5 h	DMEM, with 2% serum	Girjes <i>et al.</i> 2003

¹DK-SFM, defined keratinocyte-serum free medium; K-SFM, keratinocyte-serum free medium; HMRI-1, modified MCDB 153 medium; DMEM, Dulbecco's modified Eagle medium.

Figure 5. Removal of growth supplements (GS) and addition of EGFR inhibitor PD-153035 renders transformed and untransformed cultures quiescent. Cultures were fed DK-SFM with or without growth supplements and with or without EGFR inhibitor as previously described in the Materials and methods. The absorbance at 370 nm represents BrdU incorporation into cellular DNA. For each condition, $n = 8$, and error bars represent SEM. (a) Parental, untransformed cells. (b) Immortalized cells.

Control of EGF-dependent cell-cycle progression in parental and immortalized cultures

Based on the reduced doubling times of immortalized cell cultures, we decided to investigate the extent to which untransformed and transformed cell cultures could be rendered quiescent. BrdU assays were performed to quantify DNA synthesis after subjecting both the transformed and untransformed cells to varying growth conditions. Cells were grown in DK-SFM either with or without GSs, and either with or without PD-153035. DNA synthesis in untransformed and transformed cultures grown in DK-SFM with GSs and without PD-153035 is shown in Fig. 5 (a and b, respectively, bars with horizontal lines). These bars represent normal growth. Removal of GSs caused a 61% reduction of DNA synthesis in the untransformed cells (Fig. 5a, grid bar) and a 45% reduction in the transformed cells (Fig. 5b, grid bar). Addition of PD-153035 to a

Figure 6. Retention of cytokeratins 7, 14 and 17 expression by immortalized cells shown by immunocytochemistry. (a–c) Normal untransformed ureter cells, passage 4, 31 days in culture. (d–f) Ureter cells transformed with E6E7, passage 4, 31 days in culture. (a, d) Cytokeratin 7. (b, e) Cytokeratin 14. (c, f) Cytokeratin 17. 40 $\times$ magnification; size bar is 20 μ m.

final concentration of 1 μ M resulted in a 10% reduction in DNA synthesis in untransformed cells (Fig. 5a, grey bar) and 45% reduction of DNA synthesis in the transformed cells (Fig. 5b, grey bar). When cells were subjected to removal of GSs in addition to EGFR pathway inhibition with PD-153035, the level of DNA synthesis in untransformed cells was reduced by 99% (Fig. 5a, black bar) and in transformed cells by 93% (Fig. 5b, black bar).

Cytokeratin expression

By immunocytochemistry, untransformed and transformed cells were screened for differences in cytokeratin expression. Cytokeratin expression is a marker of differentiation in urothelial cells (Moll *et al.* 1988). A complete list of the cytokeratins tested, along with the results, can be found in Table 1. Both untransformed and transformed urothelial cells were immunoreactive with antibodies raised against cytokeratins 7, 8, 10, 14, 17, 18 and 19 and nonimmunoreactive for cytokeratins 13 and 20. There were no differences in the staining patterns between the transformed and untransformed cells, as exemplified by staining for cytokeratins 7, 14 and 17 shown in Fig. 6.

Karyotypes of parental and immortalized cells

To assess the extent that immortalization induced chromosomal rearrangement, cytogenetic analysis of metaphase spreads by G-banding was performed (Table 3). Figure 7 describes data collection for parallel untransformed and transformed cell cultures. The number of metaphase cells available was limited, and only five metaphase cells were scored for each culture analysed. Untransformed parental cells at passage 4 (23 days in culture) yielded a normal 46, XY karyotype. Transformed cells at passage 3 (27 days in culture) also yielded a normal 46, XY karyotype. Upon analysis of passage 21 (101 days in culture) transformed cells, four, related, abnormal karyotypes were detected. All five of these metaphases had at least one extra copy of

Table 3. Karyotypes of transformed and untransformed urothelial cells

Cells	Karyotype
Parental, passage 4, 23 days in culture	46, XY [5] ^a
Transformed, passage 3, 27 days in culture	46, XY [5]
Transformed, passage 21, 101 days in culture	47, XY, +20 [2] 48, XY, +6, +20 [1] 49, XY, +6, +20, +20 [1] 50, X, -Y, +5, +9, +9, +20, +20 [1]
Transformed, passage 54, 241 days in culture	52, XY, +5, +6, +7, +9, t(9; 15)(q34; q22), +20, +20 [3] 51, XY, +5, +6, +7, +9, -13, +der(15)t(9; 15)(q34; q22), +20 [1] 51, XY, +5, +6, +7, +9, +20 [1]

^anumber of cells with this karyotype are indicated in brackets.

Figure 7. Time line of cell culture growth. 'Parental' refers to the untransformed culture. 'Immortalized' refers to the parallel culture transformed with HPV-16 E6E7. Both cultures originated from the same surgical explant.

chromosome 20. In two cells (by definition a clonal abnormality) this was the sole abnormality and in the other three cells aneuploidy was present. More complex karyotypes, indicating evolution of the previously abnormal cells, were detected in passage 54 (241 days in culture). Again, all five of these metaphases had at least one extra copy of chromosome 20, and in addition to aneuploidy for several chromosomes, a structural rearrangement, an apparently balanced translocation between the long arm of a chromosome 9 and the long arm of a chromosome 15, was present in three cells (again, by definition a clonal abnormality).

Generation of single cell clones from immortalized cell cultures

Passage 49 cells were trypsinized and were single cell sorted into each of 96-well plates. Only 18/96 wells seeded survived and grew into colonies that could be further passaged from the 96-well plate into a 48-well plate. With each passage, the size of the tissue culture wells was steadily increased until the single cell clones were in T25 flasks at passage 55. Of the initial 18 colonies

Figure 8. Restoration of telomerase activity to single cell clones detected by TRAP assay. Shown are two 12.5% polyacrylamide gels stained with Sybr Green I. 1: molecular weight marker bp. 3, 5, 7, 9 and 11: heat inactivated samples. 2, 4 and 6: telomerase activity level in 3 different single cell clones at P57, 266 days in culture. 8: normal untransformed culture, passage 6, 60 days in culture. 10: transformed mass culture, passage 59, 266 days in culture. All single cell clones were sorted from the mass culture at passage 50.

that survived, only 14 survived to passage 55, and the length of time it took for the colonies to arrive at that passage number varied from 36 days post-sorting to 62 days post-sorting. All single cell-derived clones exhibited cell morphology similar to both mass transformed cell cultures and parental cultures. The clones remain cryopreserved for characterization at a later date.

Telomerase activity in single cell-sorted clones

After single cell-derived clones were established, three different clones were assayed for telomerase activity at passage 57 (266 days in culture). While all three clones exhibited telomerase activity, the level of activity varied between them (Fig. 8, lanes 2, 4 and 6). The clone with the highest telomerase activity level generated a DNA ladder with six easily detectable extension products (Fig. 8, lane 2) similar to the level exhibited by the mass transformed culture assayed at passage 59 (266 days in culture) (Fig. 8, lane 10). The other two clones exhibited lower levels of activity: one clone with a DNA ladder of only three distinct bands and three faint bands (Fig. 8, lane 6), and one clone that generated a ladder with only two clearly visible bands and two very faint bands (Fig. 8, lane 4). The weakest telomerase activity level present in the clones was still greater than the activity level detected in untransformed parental cells assayed at passage 6 (60 days in culture) (Fig. 8, lane 8).

DISCUSSION

Telomerase activity has been detected in a high percentage of malignant tumours, including in 98% of bladder cancers, and it has been shown that both TCC biopsies and TCC *in vitro* cell cultures exhibit telomerase activity (Belair *et al.* 1997). Interestingly, it has been reported that *in vitro* cultures of urothelial cells exhibited telomerase activity that diminished with continued passage, while telomerase activity was not detected in uncultured specimens of normal urothelial

cells (Belair *et al.* 1997). However, there are reports of low level telomerase activity in tissues that are self-renewing (Forsythe *et al.* 2002), and reports of the detection of telomerase expression in some epithelial cells and lymphocytes while proliferating *in vivo* (Holt *et al.* 1997). In addition, it has been reported that some types of telomerase-positive cells become telomerase-negative when arrested in G₀, and that this down-regulation of telomerase activity is reversible when cells re-enter the cell cycle and begin proliferation (Holt *et al.* 1996). Taken together, these reports have led to the hypothesis that telomerase activity is more closely associated with cell proliferation, rather than with malignant transformation (Belair *et al.* 1997; Holt *et al.* 1997). As urothelial cells *in vitro* adopt a highly proliferative phenotype (Southgate *et al.* 1994), it is not unexpected that telomerase activity might be detected in earlier passage cultures. In agreement with previous findings (Belair *et al.* 1997), our early passage untransformed urothelial cells exhibited telomerase activity that was markedly reduced to almost undetectable levels with continued passage of the cultures. In contrast, urothelial cells transformed with HPV-16 E6/E7, did not exhibit a reduction in telomerase activity with continued passage. Transformation with HPV-16 E6/E7 yielded not only the restoration of telomerase activity, but also an increase in telomerase activity to nearly the levels exhibited by the UM-UC-3 TCC cell line.

It has been shown that culture conditions can alter the doubling times for urothelial cells grown *in vitro*. Factors that have been shown to influence this are calcium concentration, growth factor supplementation and serum supplementation, although there is some discrepancy within the literature regarding which conditions are more favourable for cell proliferation. This is most likely due to the studies investigating the effects of these factors being carried out in different culture media systems. The literature lists doubling times ranging from 24 h to 77 h (Kirk *et al.* 1985; Reznikoff *et al.* 1986; Dubeau & Jones 1987; Petzoldt *et al.* 1994). In this study, doubling time for untransformed cells fell in the middle of this range, at 52 h, while the transformed cells exhibited a doubling time of 37 h. The overall slower doubling time for untransformed cells could be attributed to the inclusion of days 20–27 in the growth curve. Beginning at day 20, untransformed cells had started to exhibit morphology consistent with senescence and did not maintain the growth rate of transformed cells.

While difference in doubling times could be attributed to the beginning of senescence in untransformed cells, we decided to also confirm that transformed cells had not acquired a phenotype characterized by uncontrollable growth. The removal of GSs from the culture medium reduced DNA synthesis in untransformed and transformed cultures alike, although untransformed cultures showed 16% greater reduction than the transformed cells. The combination of GS removal and EGF pathway inhibition rendered both the untransformed and transformed cultures nearly quiescent. This suggests the mechanisms by which the HPV-16 E6 and E7 genes immortalize urothelial cells do not confer uncontrollable cell-cycle progression. The signals required for continued cell cycling *in vitro* have not been 'overridden' or negated. The minor DNA synthesis that remained may be due to residual growth factors still bound to cells, incomplete inhibition of the EGF pathway by PD-153035 or transforming growth factor- α (TGF- α).

Both transformed and untransformed cell cultures exhibited the same cytokeratin expression profiles, indicating that the immortalized cells were not exhibiting uncharacteristic differentiation. In agreement with previous findings for normal human urothelial cells (Southgate *et al.* 1994), both the transformed and non-transformed cells were immunoreactive for CKs 7, 8, 14, 17, 18 and 19, while testing negative for CKs 13 and 20. A CK 16 immunoglobulin G was not used. Immortalized cells exhibited previously reported markers of transitional (Moll *et al.* 1988; Southgate *et al.* 1994) and of squamous (Southgate *et al.* 1994; Harnden & Southgate 1997) epithelia, suggesting that an immature phenotype resulted as a consequence of reversal of differentiation. While complete loss of CK 13 expression has not been previously reported, it has been shown

that in serum-free growth conditions urothelial cultures switch to a more squamous phenotype evidenced by the reduced CK 13 expression and increased CK 14 expression (Cross *et al.* 2005).

Genetic alterations seen in our immortalized cells were not unexpected. A variety of genetic alterations has been reported in human urothelial cells immortalized with either HPV-16 E6 and/or E7 genes (Reznikoff *et al.* 1994, 1996b; Savelieva *et al.* 1997; Vieten *et al.* 1998; Cuthill *et al.* 1999). It has been shown that urothelial cells immortalized with HPV-16 E6 or E7, have exhibited combinations of clonal losses of chromosome regions 3p, 6q, 9p, 11p, 18q and Y, and gains of chromosome regions 3q, 7, 9q and 20q (Reznikoff *et al.* 1996a,b). Some studies have suggested a 20q gain as an event associated with immortalization of urothelial cells after transformation with HPV-16 E7 (Savelieva *et al.* 1997; Cuthill *et al.* 1999). It is noteworthy that gain of at least one copy of chromosome 20 was seen in every abnormal cell. An association between chromosome 3 deletion and the immortalization of urothelial cells by transformation with HPV-16 E6 has been described, but chromosome 3 deletion in HPV-16 E7 transformed urothelial cells has also been observed (Vieten *et al.* 1998). The significance of other genetic alterations in relationship to the immortalization of urothelial cells is not fully known, although some of the alterations have been studied within the context of malignant transformation. It is thought that loss of a chromosome indicates the possibility of that chromosome containing tumour suppressor gene(s), and gain of a chromosome indicates the possibility that the chromosome may contain oncogene(s) (Reznikoff *et al.* 1996b). We recognize that the karyotypic alterations seen in immortalized cell lines are likely to alter or compromise some functions within the cell. While this is indeed a potential shortcoming for investigations aimed at elucidating specific mechanisms or pathways in normal cells, immortalized cell lines can still provide a useful tool for the investigation of diseases in which altered karyotypes are observed, such as cancer. For our purposes, our data indicate that the genetic variance in our transformed cells does not alter their phenotype. In addition, the genetic variance neither inhibited culture propagation, nor conferred uncontrollable cell-cycle progression.

After establishing transformed cultures, we performed multiple cell-sorting experiments in an attempt to obtain homogeneous cultures, each derived from a single cell. Although we finally did obtain such cultures, it was not without great difficulty. The low success rate experienced during the single cell-sorting process was not surprising. It has been reported that establishment of primary cultures requires a relatively high initial plating density (Southgate *et al.* 1994), and we routinely seed primary cultures at a plating density of 1×10^4 cells/cm². While plating density requirements can be as low as 2.5×10^2 cells/cm² when sub-culturing established cell lines (Southgate *et al.* 1994), this density is equivalent to 80 cells in one well of a 96-well plate. Being alone in a well is extremely stressful for a cell, in part due to minimal opportunity for growth signals from the autocrine loop involving epidermal growth factor receptor (EGFR) and heparin-binding EGF-like growth factor (HB-EGF) (Varley *et al.* 2005). In addition, it has been reported that cell density has an effect on the expression of cell adhesion molecules that are involved in cell-cell and cell-matrix interactions (Stanley *et al.* 1995). If this is the case for urothelial cells, lower cell density could result in reduced expression of integrins that are important for cell adhesion and mobility, affecting cell proliferation.

While not presented in this report, we have been successful in extending the lifespan of several bladder urothelial cell cultures up to 30 passages before cryogenically storing them. It is therefore apparent that urothelial cells derived from embryonic endoderm (ureter) and embryonic mesoderm (bladder) are not refractory to immortalization by our methods.

While the HPV-16 E6 and E7 immortalization of urothelial cells has previously been reported, the culture system used in those studies was an undefined system containing FBS (Reznikoff *et al.* 1994, 1996a). In the present study, we have demonstrated that transformation of urothelial cells with

HPV-16 E6 and E7 genes in a defined, serum-free culture system results in the restoration of telomerase activity and the immortalization of the transformed cell cultures. In our experiments, retroviral expression of hTERT alone did not confer an extended lifespan on urothelial cells. This is consistent with work published reporting the necessity for RB/p16 pathway alteration or inactivation for the immortalization of some epithelial cells (Reznikoff *et al.* 1996a; Kiyono *et al.* 1998; Jarrard *et al.* 1999; Dickson *et al.* 2000). However, a recent study reported that retroviral expression of hTERT resulted in the immortalization of urothelial cells without the inactivation of the RB/p16 pathway (Chapman *et al.* 2006). While this may seem contradictory, a direct comparison cannot be made due to key differences – different retroviral systems, different retroviral expression vectors and different culture systems were used. Of these three variables, the most interesting difference is cell culture system. We have employed a defined serum-free culture system, while the contrasting study used an undefined serum-free system containing bovine pituitary extract. As cell culture systems have been shown to influence the results of *in vitro* cell senescence studies, it must be considered that cultures systems could influence the immortalization of cells as well. The discrepancies within the current literature regarding urothelial cell senescence and immortalization bolster the need for continued efforts in developing a defined and reproducible system for urothelial cell immortalization.

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