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Immortalisation of normal human urothelial cells compromises differentiation capacity

Nikolaos T Georgopoulos^{1,3}, Lisa A Kirkwood¹, Claire L Varley¹, Nicola J MacLaine^{1,4}, Naveed Aziz² and Jennifer Southgate¹

¹Jack Birch Unit for Molecular Carcinogenesis and ²Technology Facility, Department of Biology, University of York, York, U.K. ³Department of Chemical and Biological Sciences, University of Huddersfield, Huddersfield, U.K; ⁴Edinburgh Cancer Research Centre, University of Edinburgh, Edinburgh, U.K.

Correspondence to:

Professor Jennifer Southgate, Jack Birch Unit for Molecular Carcinogenesis, Department of Biology, University of York, York YO10 5YW, U.K.

Tel: +441904328705, Fax: +441904328704, E-mail: js35@york.ac.uk

Short title: hTERT over-expression compromises urothelial differentiation

Keywords: Urothelium, bladder cancer, immortalisation, telomerase, differentiation

Abbreviations: CGH, comparative genomic hybridisation; EGF, Epidermal Growth Factor; EGFR, EGF Receptor; ERK, Extracellular signal-Related Kinase; FGFR3, Fibroblast Growth Factor Receptor 3; hTERT, human Telomerase Reverse Transcriptase; NHU, Normal Human Urothelial; PI3-K, Phosphatidylinositol 3-Kinase; STAT, Signal Transducer and Activator of Transcription; TER, Trans-epithelial resistance; UC, Urothelial carcinoma.

Abstract

Background: The development of urothelial malignancy is not solely a consequence of loss of proliferation constraints, but also involves loss of cellular differentiation, defined histopathologically as grade. Although tumour grade is an independent prognostic marker for urothelial carcinoma (UC), the molecular events underpinning the loss of urothelial differentiation are poorly understood.

Objective: To examine the effect of gene alterations implicated in UC development on the ability of human urothelial cells to undergo molecular differentiation and form a functional urothelial barrier.

Design, Setting, and Participants: Laboratory study.

Intervention(s): Normal human urothelial (NHU) cell cultures were transduced with recombinant retroviruses to produce stable sub-lines over-expressing wild-type or oncogenic mutated FGFR3, or human telomerase (hTERT). Previously-generated NHU sublines carrying dominant-negative CDK4 and p53 mutant genes or immortalised with the human papillomavirus 16 E6 oncoprotein were included.

Measurements: The activity of introduced transgenes was demonstrated by comparing phenotypes of transgene-expressing and isogenic control NHU cells. Modified and control sub-lines were compared for changes in generational potential (lifespan) and capacity to respond to differentiation-inducing signals by transcript expression of uroplakins 2 and 3. The ability to form a barrier epithelium was assessed by measuring the transepithelial electrical resistance.

Results & Limitations: By contrast to tumour suppressor loss-of-function or oncogene over-activation, hTERT over-expression alone led to life-span extension and immortalisation. hTERT-immortalised cells carried no gross genomic alterations, but became progressively insensitive to differentiation signals and lost the ability to form an

epithelial barrier. Further characterisation of hTERT cells revealed a down-regulation of p16 cyclin-dependent kinase inhibitor expression and loss of responsiveness to peroxisome proliferator-activated receptor gamma, providing mechanistic explanations for the subjugation of senescence constraints and abrogation of differentiation capability, respectively. Although immortalised urothelial cell lines without karyotypic aberrations may be generated, such cell lines are compromised in terms of differentiation and functional capacity.

Conclusions: Over-expression of hTERT promotes development of an immortalised, differentiation-insensitive urothelial cell phenotype. Whereas such cells offer a useful insight into the grade/stage paradigm of UC, they have limited value for investigating normal urothelial cell/tissue biology and physiology.

Introduction

Bladder cancer is the fourth commonest cancer in men and arises predominantly as urothelial carcinoma (UC). Clinical evidence has revealed two major pathways (reviewed in [1]). Most UC are low-grade and recurrent, with low rates of malignant progression, whereas high-grade UC are associated with a high risk of progression to muscle invasive disease, with attendant poor prognosis. Carcinoma in-situ, a high-grade urothelial lesion representing ~10% of newly-diagnosed UC, is considered the principal precursor of muscle invasive disease. Despite the clear association between grade and prognosis [2], current models of bladder carcinogenesis concentrate on the impact of molecular changes for tumour growth, rather than differentiation.

The majority (>70%) of low-grade tumours exhibit activating mutations of Fibroblast Growth Factor Receptor 3 (FGFR3), compared to <20% of invasive tumours [3]. The most common mutation is a S249C substitution, which allows ligand-independent dimerisation and constitutive auto-phosphorylation. As a result, FGFR3 enhances proliferation by activating Ras-Raf-Extracellular signal-Related Kinase (ERK), Phosphatidylinositol 3-Kinase (PI3-K) and Signal Transducer and Activator of Transcription (STAT) proteins [4]. The p53/p21 and p16/Retinoblastoma (Rb) tumour suppressor pathways are frequently altered in muscle-invasive disease, with p53 loss associated with muscle-invasive disease and p16/Rb loss linked to tumours of higher metastatic potential (reviewed in [5]).

A further failing of current models is the lack of inclusion of critical genes/proteins despite strong evidence for a role in cancer progression. Activation of telomerase reverse transcriptase (hTERT) occurs in >90% of cancers [6], including UC [7]. hTERT is expressed by stem cells, but repressed in most normal somatic cells where over-

expression leads to immortalisation by overcoming senescence pathways. This has contributed towards both supporting the concept of the cancer stem cell and recognising telomerase activation as a key-stage in cancer progression [8].

We have developed methodologies to propagate normal human urothelial (NHU) cells as finite cell lines *in vitro* [9]. In low-calcium, serum-free medium, NHU cells adopt a rapidly-proliferating, non-differentiated regenerative phenotype [10] driven by autocrine EGFR signalling [11]. *In vitro*-propagated NHU cell lines retain differentiation capacity and can be induced to stratify [10], express genes associated with late/terminal urothelial cytodifferentiation [12] and form a functional, barrier-epithelium [13]. The genetic manipulation of NHU cells with retroviruses has enabled the generation of “para-malignant” [14] human urothelial sub-lines carrying defined genetic alterations, such as inactivated p53 or p16 functions [15-17]. We have shown that tumour suppressor inactivation has differential effects on NHU cell responses to genotoxic damage or non-genotoxic pro-apoptotic signals [16] and in modulating sensitivity to inhibitors of growth-promoting pathways [17].

In this study, we have used para-malignant human urothelial cells to investigate how defined genetic alterations associated with carcinogenesis affect not only proliferation and life-span, but also capacity for cytodifferentiation and barrier formation. Alongside previously-generated p53 and p16 functional knock-out cells, we have engineered urothelial cells with constitutively-activated mutant FGFR3 or over-expression of hTERT. Differentiated urothelial barrier function is contributed to by both paracellular barrier function (attributed to tight junctions) and trans-cellular barrier function (contributed to by the uroplakins, UPKs). Of the uroplakins, UPK2 and UPK3a represent the two most highly-restricted late/terminal differentiation stage and urothelium-specific markers available

[18]. Here, we have assessed both functional and molecular aspects of differentiation by investigating the ability of transduced cells to generate a functional tight barrier and to express uroplakin transcripts.

Materials and Methods

Cell culture

NHU cell cultures established from urological specimens obtained with informed consent from patients with no UC history were maintained as finite cell lines as detailed elsewhere [9,10].

Retrovirus preparation and transduction

Wild-type FGFR3 (FGFR3WT) and FGFR3^{S249C} mutant (FGFR3mut) sequences (Dr Francois Radvanyi, Institut Curie, France) were PCR-amplified to incorporate *EcoRI*/*Bam*HI for sub-cloning into pLXSP, thus generating pLXSP-FGFR3WT and pLXSP-FGFR3mut, respectively. The pLXSP vector was derived from pLXSN (Clontech) by replacing the neomycin resistance gene with a puromycin expression sequence, as detailed elsewhere [16]. hTERT cDNA (Dr Jerry Shay, Southwestern Medical Center, USA) was sub-cloned into the *EcoRI* site of pLXSN, thus generating pLXSN-hTERT.

Retrovirus transductions were performed as described [16]. PT67 fibroblasts (Clontech) were transfected with pLXSN-hTERT, pLXSN-p53DD, pLXSP-CDK4^{R24C} [16], pLXSP-FGFR3WT or pLXSP-FGFR3mut, as well as pLXSN and pLXSP vectors. Three independent NHU lines were transduced with control or recombinant retroviruses resulting in control (HU-neo/HU-puro) and para-malignant (HU-hTERT, HU-p53DD, HU-CDK4mut, HU-FGFR3WT and HU-FGFR3mut) sub-lines. Immortalised, post-crisis HU-E6P cells were derived from NHU cells transduced with human papillomavirus 16 E6 oncoprotein as previously described [15].

Comparative genomic hybridisation (CGH) arrays

CGH was carried out using *Homo sapiens* AROS v4.0 OpArrays (Operon Biotechnologies) and analysed using ArrayAssist Expression software v5.5 (Stratagene). A list of 3439 genes was generated and imported into CGH-Explorer (v2.5) software [19] for copy number analysis.

Immunoblotting

Immunoblotting was performed as described [16] with the following primary antibodies: rabbit anti-phospho-ERK (#9101, Autogen Bioclear), mouse anti-panERK (clone#16, BD Biosciences), rabbit anti-FGFR3 (B-9; Autogen Bioclear), rabbit anti-FOXA1 (D-20; Autogen Bioclear), mouse anti-PPAR γ (E8, Autogen Bioclear) and mouse anti- β -actin (KJ43A, Sigma-Aldrich). Secondary antibodies were goat anti-rabbit immunoglobulin conjugated with IRDye[®] 800 (Tebu-Bio) or anti-mouse immunoglobulin conjugated with Alexa Fluor[®] 680 (Invitrogen). Immunoblots were analysed on a Li-Cor Odyssey (Cambridge).

Telomerase

Telomerase activity was measured using a TRAPEze XL telomerase detection kit (Millipore). For immunocytochemistry, anti-hTERT antibody (rabbit polyclonal, #600-401-252, Rockland Immunochemicals) was followed by goat anti-rabbit immunoglobulin conjugated to Alexa Fluor[®] 488 (Invitrogen).

Urothelial cytodifferentiation

Urothelial cells were induced to differentiate by co-activation of PPAR γ and inhibition of EGFR signalling as described [12,20,21]. RNA was isolated, cDNA synthesised and real-time PCR performed using TaqMan[™] PCR primers with UPK2, UPK3a and GAPDH probes

[22]. Data were analysed using ABI Prism 7700 SDS software (Applied Biosystems) and normalised against GAPDH (internal control).

Development and assessment of barrier function

Transepithelial electrical resistance (TER) was measured by electrophysiology. Urothelial cells were grown in KSFMc containing 5% (v/v) adult bovine serum (ABS) before sub-culture onto Snapwell™ membranes. TER measurements were made as described [13].

Results

Verification of para-malignant cell lines

Immunoblotting corroborated transgene expression in HU-FGFR3WT and HU-FGFR3mut cells, with control HU-puro cells showing little FGFR3 expression (Figure 1A).

HU-FGFR3WT and HU-FGFR3mut expressed higher basal p-ERK levels than controls (Figure 1B); addition of FGF1 did not further increase phosphorylation. Treatment with EGFR inhibitor (PD153035) blocked ERK-phosphorylation in HU-puro and HU-FGFR3 cells, yet only weakly down-modulated p-ERK in HU-FGFR3mut (Figure 1B). Exposure to FGF1 in PD153035-treated HU-FGFR3WT, but not PD153035-treated HU-puro cells, rescued p-ERK levels, whilst FGF1 treatment did not further increase p-ERK expression in HU-FGFR3mut cells (Figure 1 and supplementary Figure 1). This confirmed the ligand-independent, constitutively-activated status of the FGFR3mut construct and the ligand-dependent functionality of FGFR3WT.

Immunofluorescence microscopy confirmed increased expression of nuclear hTERT in HU-hTERT cells over HU-neo controls (Figure 1C). The demonstration of endogenous/basal hTERT by NHU cells was in agreement with previous reports [23]. hTERT activity was also confirmed by assaying telomerase activity (Table 1). HU-hTERT cells at different passages (see Table 1) consistently showed several-fold higher telomerase activity than HU-neo cells.

Effect of cancer gene transduction on proliferation and lifespan

We have previously reported that neither loss of p53 nor p16 alone caused an extension in NHU lifespan [16]. Similarly, over-expression of neither wild-type FGFR3 protein nor the constitutively-activated FGFR3 mutant had any effect on lifespan (not shown). Although isogenic HU-neo could only be cultured to p9-p10 before senescence, HU-hTERT cells

continued to proliferate until approximately p12 (Figure 2A). At this point, cells exhibited a lag-phase, characterised by reduced growth rates, but there was neither visible mass death nor signs of clonal recovery (Figure 2B). The lag-phase lasted from p11-p13, but by p15, HU-hTERT populations had resumed normal proliferation rates and were morphologically-similar to normal NHU cultures, showing regular epithelioid morphology. By p20, cells began to show subtle morphological changes and appeared heterogeneous, with a proportion of cells being of smaller size. Upon repeated sub-culture, and despite no changes in proliferation rates, HU-hTERT cells progressively adopted a smaller-size phenotype, which remains the same to date (>p45). These findings were reproducible with cells recovered from cryopreservation at different passages and indicate that over-expression of hTERT alone can efficiently immortalise NHU cells.

Coinciding with the lag in growth, expression of the cyclin-dependent kinase inhibitory p16 protein increased in hTERT-transduced cells up to passage 12, and reduced in later sub-cultures (Figure 3).

Genomic analysis of hTERT-immortalised cells

To exclude the possibility that the extension of NHU cell lifespan was due to accumulation of genetic changes and/or abnormalities, such as those reported for HPV16 E6-transduced cells [15], CGH arrays were used to assess the genomic integrity of HU-hTERT cells. When cells at p7 and p40 were compared, no significant alterations were seen across the genome (Figure 4 and supplementary data), including the frequently-modified regions of chromosomes 3p, 9q, 8q and 20 (reviewed in [14]). This absence of karyotypic alterations agrees with other reports [24].

Effect of para-malignant changes on differentiation capacity

Combined treatment of NHU cells with PD153035 (EGFR antagonist) and troglitazone (TZ, PPAR γ ligand) induces a programme of gene expression changes associated with terminal differentiation; this is mediated via the PPAR γ -dependent transcription of intermediary transcription factors, including FOX-A1 [21]. When tested within the lifespan of the isogenic lines, sub-lines transduced with hTERT, mutant or wild type FGFR3, p16-insensitive CDK4 mutant or dominant-negative p53, all responded to treatment with PD153035 and TZ by induction of UPK2 transcript expression (Figure 5); no attenuation of signal was seen compared to isogenic plasmid-only controls. By contrast, UPK2 expression was not induced in post-crisis HU-E6P cell cultures (Figure 5).

The response of HU-hTERT cells to TZ/PD153035 treatment was assessed over a range of passages. Although HU-hTERT cells were induced to express UPK2 and UPK3a transcripts at p10 (i.e. within the normal lifespan), by p17 the response was severely attenuated, as HU-hTERT cells showed only baseline UPK2 (Figure 7A) and UPK3a (Supplementary Figure 2) expression. Further examination revealed that although PPAR γ expression was retained, the responsiveness of cells to PPAR γ activation was curtailed, with FOX-A1 induction reduced by >50% in both HU-hTERT cells and post-crisis HU-E6P cells (Figure 7B).

Effect of para-malignant changes on urothelial barrier function

Sub-culture of NHU cells in medium containing serum and 2mM calcium stimulates them to stratify and develop functional barrier properties equivalent to normal urothelia [13]. We examined the ability of para-malignant cell lines to form a tight barrier, as assessed by measurement of trans-epithelial resistance (TER).

Under normal growth conditions, all cultures showed limited TER ($<50 \Omega \cdot \text{cm}^2$). Within the normal lifespan, all para-malignant sub-lines responded to the serum/calcium protocol by formation of urothelial sheets with tight barrier properties; no significant differences were noted against the respective vector-only controls (Figure 6, panels A, B and C). By contrast, transformed HU-E6P cells failed to form a substantial barrier (Figure 6B).

When the ability of late-passage HU-hTERT to form a barrier was examined and, in accordance with UPK2 and UPK3a expression, we found that cells up to p10 were able to form a tight barrier with equivalent TER to non-transformed control cultures (Figure 7C). HU-hTERT cells produced a partial barrier at p17 and p28, and showed no barrier formation at p41, where TER values were similar to those of non-differentiated control cultures (Figure 7C).

Discussion

NHU cells with abrogated p53 (but not p16) function exhibited increased growth rates and reduced dependency on EGFR-driven proliferation [17], whereas p16 (but not p53) inactivation altered urothelial cell responses to pro-apoptotic signals [16]. We now show that neither loss of p53 nor p16 function had any direct effect on differentiation, in agreement with the normal differentiation potential of keratinocytes with inactivated p16 function [25]. Less expected was the lack of any association between loss of p53 and differentiation, as changes in p53 have been associated with carcinoma *in situ* [5], thus implying that other changes may contribute to the dysplastic phenotype.

FGFR3 mutation is closely-associated with benign urothelial hyperplasia [3]. Due to the low-grade nature of these tumours, it was not unexpected that HU-FGFR3mut cells showed no loss of differentiation capacity. Although mutant-FGFR3 had no overt effect on NHU proliferation despite an increase in ERK phosphorylation, it is likely that FGFR3mut-mediated proliferation signals would be masked, as NHU cultures are already in a high-proliferation state driven by autocrine/paracrine EGFR/ERK signalling [11,17]. Indeed, functional EGFR-blocking suggested cross-talk downstream of FGFR3 and EGFR signalling through ERK. Thus, FGFR3-driven ERK activation may contribute to tumour growth *in vivo*.

It is striking that, unlike inactivation of p53 or p16, hTERT over-expression alone could influence both the lifespan and differentiation potential of urothelial cells. Our findings are in agreement with a previous report demonstrating hTERT-mediated immortalisation of NHU cells [24]. In accordance with our study, Chapman et al. reported that immortalisation of NHU cells was not accompanied by any detectable genetic instability when the HU-hTERT derivatives were maintained under conditions optimised for the

culture of NHU cells, although they did observe a predisposition to genetic aberration when hTERT over-expressers were stressed under sub-optimal culture conditions [26].

Several epithelial types have been immortalised by hTERT, but this often requires concurrent inactivation of tumour suppressor function [27,28]. As shown in this study and reported elsewhere [24], urothelial cells, in common with prostate and small airway epithelial cells [29,30], were immortalised by hTERT over-expression alone. Prior to immortalisation, HU-hTERT populations displayed a lag-phase (p11-p13) characterised by reduced growth rates, without senescence or apoptosis. Importantly, there was no emergence of a rare, rapidly-growing cell population that would have indicated clonal recovery. Accumulation of p16 has been associated with urothelial senescence in culture and is recognised as an essential block to neoplastic transformation [25]. In agreement with this and observations by Chapman et al [24], we saw a dramatic increase in p16 protein associated with the growth lag around the normal senescence point at passage 12 and growth rate recovery was accompanied by a reduction in p16 expression. In the absence of genomic aberration, these observations fit a scenario in which p16 gene expression is silenced epigenetically, as shown for small airway epithelial cells [30]. However, p16 silencing does not provide a complete mechanism, as we have shown previously that direct abrogation of p16 function does not overcome senescence and is not immortalising in NHU cells [16].

Proliferating NHU cells express (low level) endogenous hTERT expression, as shown in this study (Figure 1C) and elsewhere [23], as do some somatic cell types that have the capacity to switch from a quiescent to highly-proliferative phenotype. Urothelium is renowned as a low-turnover, mitotically-quiescent epithelium that has high regenerative capacity. With its ability to overcome proliferation constraints and block differentiation,

hTERT may facilitate tumorigenesis by promoting proliferation over differentiation, thus “priming” cells for a second-hit to a loss-of-function senescence block, although appropriate *in vivo* studies would be necessary to address this hypothesis. Our findings support the reported association of hTERT over-activation and bladder cancer [7] and provide a basis for explaining the grade/stage relationship.

Although the precise underlying mechanisms for this remain unclear, hTERT over-expression can progressively attenuate the molecular differentiation of NHU cells (shown in this study and reported elsewhere [31]) and diminish their capacity to form a functional urothelial barrier. A mechanism for hTERT-mediated suppression of differentiation would be in keeping with the stem cell maintenance function of hTERT, whose activity is known to extend beyond telomerase activation and includes chromatin reorganisation, indicating a potential role in epigenetic regulation of gene transcription [32]. The loss of differentiation capacity by hTERT-immortalised urothelial cells implies that immortalisation by hTERT and the loss of differentiation capacity are causally-linked. A likely scenario is that the hTERT-mediated chromatin reorganisation affects the efficacy of ligand-activated PPAR γ , as a nuclear receptor, to bind and initiate the cascade of transcriptional and chromatin reorganising events associated with urothelial differentiation. Further studies in this area of epigenetic regulation are likely to provide novel insight into the cancer evolution process.

Conclusions

Immortalised, cytogenetically-normal HU-hTERT cells should provide a valuable resource by overcoming some of the problems of working with NHU lines of finite lifespan. However, HU-hTERT cells can no longer be regarded as “normal” and cannot be

substituted in studies where normal regulatory controls are assumed. This may limit their application, although they nevertheless provide a valuable model for clarifying the role of telomerase activation in UC and its influence on proliferation and differentiation.

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Table 1 Measurement of telomerase activity in transduced NHU cells

Cell Line	*Telomerase activity (relative to HU-neo)
HU-neo	1.0 ± 0
HU-hTERT at p9	3.0 ± 0.2
HU-hTERT at p16	3.5 ± 0.1
HU-E6P	19.2 ± 8.2
HU-neo (heat inactivated)	-0.2 ± 0
HU-hTERT at p9 (heat inactivated)	-0.4 ± 0.2
HU-hTERT at p16 (heat inactivated)	0.0 ± 0.4
HU-E6P (heat inactivated)	-0.2 ± 0

Cell lysates were prepared and PCR performed according to the manufacturer's instructions, using the reaction profile of 30 min at 30°C, followed by 36 cycles of 30 s at 94°C, 30 s at 59°C and 1 min at 72°C. Following PCR, fluorimetric measurements were made at 495nm (telomerase activity) and 600nm (sulforhodamine internal control). Following background subtraction for each channel to determine net fluorescein (FL) and sulforhodamine (R), telomerase activity in HU-hTERT was expressed as the ratio of FL/R and normalised against the HU-neo signal (*). Immortalised HU-E6P cells were included as positive controls and heat-inactivated samples served as negative controls. Values are means of triplicate samples (\pm standard deviation, SD) and are representative of two independent experiments.

Figure Legends

Figure 1 Verification of functional FGFR3 and hTERT over-expression

(A) Human urothelial cells over-expressing wild-type (HU-FGFR3WT), constitutively-activated mutant FGFR3 (HU-FGFR3mut) and empty virus (control) cells (HU-puro) were established by retroviral transduction. To confirm transgene expression, cell lysates were prepared and FGFR3 expression assessed by SDS-PAGE and immunoblotting. Alongside untreated controls, replicate cultures were maintained in medium containing 1 μ M EGFR inhibitor PD153035 for 24 hours, followed by treatment with or without 1nM FGF1 ligand (FGF1) in 2 μ g/mL heparin for a further 24 hours.

(B) Urothelial cells were treated as above and expression of phosphorylated ERK (p-ERK1/2) protein was assessed by SDS-PAGE and immunoblotting using a rabbit polyclonal antibody. To ensure equal loading, membranes were simultaneously probed with a mouse monoclonal antibody for detection of total ERK. This was followed by treatment with fluoro-chrome-conjugated goat anti-rabbit antibody (green) and anti-mouse (red) antibodies, respectively, and immunolabelling was visualised by infrared imaging as described in the Methods section. The relative expression of pERK2 was calculated from the densitometry readings and is stated below each track (representative of two experiments).

(C) Expression of the catalytic unit of telomerase (hTERT) was assessed in isogenic control (HU-neo) and hTERT over-expressers (HU-hTERT) cells by indirect immunofluorescence microscopy. Urothelial cells were fixed, permeabilised and labelled with primary anti-hTERT antibody followed by Alexa Fluor 488-conjugated secondary antibody; nuclei were visualised using Hoechst 33258. Scale bar: 50 μ M.

Figure 2 Effects of long-term hTERT over-expression on urothelial cell morphology and growth

(A) Retrovirus-transduced control (HU-neo) and over-expressing hTERT (HU-hTERT) cells were cultured in standard culture medium (KSFMc) and representative phase contrast micrographs taken at various passage numbers. For HU-neo: a) p5, b) p10. For HU-hTERT: i) p5, ii) p10, iii) p11, iv) p12, v) p13, vi) p15, vii) p20, viii) p30. Scale bar: 50µM.

(B) HU-hTERT cells were maintained as above and consistently sub-cultured upon reaching confluency at a 1:3 ratio. The passage number of HU-hTERT cultures was recorded over a total sequential culture period of 299 days (40 passages) and thus the graph is representative of the time-length between each passage. The labels (i-viii) correspond to the passages where the representative images in (A) were taken.

Figure 3 The effect of long term culture on p16 protein expression in HU-hTERT cells

Protein lysates were prepared from HU- hTERT cells at passage 6, 12, 15 and 27.

A) p16 protein expression was assessed by immunoblotting with a p16 mouse monoclonal antibody and compared to isogenic control (HU-neo) at passage 6. To ensure equal loading, membranes were also probed with an antibody to β -actin.

B) Densitometry readings for p16 were normalised to β -actin and displayed as a bar graph.

Figure 4 Effects of hTERT expression on urothelial cell karyotype

Genomic DNA was isolated from HU-hTERT cultures at p7 and p40 and comparative genomic hybridisation (CGH) was carried out as detailed in the Methods. The generated data was filtered for background correction and subsequently normalised by mean scale normalisation using the ArrayAssist Expression software. The list of genes from the above

analysis was generated with expression ratios of sample1/sample2. The generated list, including gene identities, chromosome number and location was imported into the CGH-Explorer software for copy number analysis (see supplementary data).

Figure 5 Assessment of differentiation potential of transduced NHU sub-lines

NHU-derived sub-lines HU-FGFR3WT, HU-FGFR3mut, HU-p53DD, HU-CDK4mut and HU-hTERT, alongside control cell lines HU-neo and HU-puro (all at <p7) and post-crisis, immortalised HU-E6P cells were induced to differentiate by treatment with 1 μ M troglitazone (TZ) and 1 μ M PD153035 (PD) in combination (TZ+PD). DMSO controls (Con) were also included. RNA was extracted, cDNA generated and quantitative PCR was performed. UPK2 expression values were initially normalized relatively to those of the internal control (GAPDH) to calculate relative UPK2 expression. Results were then expressed as fold UPK2 expression with respect to isogenic untransduced NHU controls. Each bar thus represents mean fold UPK2 expression (\pm SD) of three replicates for one representative cell line for each transduction.

Figure 6 Assessment of barrier function capacity of transduced NHU sublines

Transduced control cell lines HU-neo and HU-puro, and para-malignant sub-lines HU-FGFR3WT, HU-FGFR3mut (A), HU-hTERT and post-crisis, immortalised HU-E6P (B), as well as HU-p53DD and HU-CDK4mut cells (C) (all at <p7) were grown on Snapwell™ chambers in the presence of 5% ABS and 2mM Ca²⁺, and trans-epithelial electrical resistance (TER) was measured (as detailed in the Methods). Bar graphs represent mean TER value (\pm SD) of three replicate samples following background subtraction.

Figure 7 Effects of long-term hTERT over-expression on molecular differentiation and barrier function of urothelial cell cultures

(A) Control virus-transduced (HU-neo) and HU-hTERT cells at the indicated passage number were induced to undergo differentiation following treatment with combination of 1 μ M PPAR γ agonist troglitazone and 1 μ M EGFR inhibitor PD153035 (TZ+PD). Total RNA was extracted, cDNA generated and quantitative real-time RT-PCR was performed to assess UPK2 mRNA expression. For each datum point, UPK2 mRNA level was normalised relative to that for the internal control (GAPDH) and each bar represents mean UPK2/GAPDH ratio (\pm SD) for three replicates.

(B) HU-neo (p7), late-passage HU-hTERT (p23) and HU-E6P cells were exposed to DMSO vehicle control (-) or treated with TZ+PD (+) as described above and expression of PPAR γ and FOXA1 were assessed by immunoblotting. Densitometry values for the relevant protein bands are indicated, following normalisation against β -actin.

(C) Control (HU-neo) cells, HPV16 E6-expressing (HU-E6P) derivatives and hTERT over-expressers (HU-hTERT) at the indicated passage numbers were treated with medium containing 5% serum in the presence of 2mM Ca²⁺ (ABS+Ca) for induction of differentiation and trans-epithelial resistance assays were performed. Untreated, non-differentiated, isogenic HU-neo cells (ND Con) were included as negative controls. Bars represent mean TER value (\pm SD) of three replicate samples for each cell line following background subtraction.

Supplementary Figure 1**Effect of FGF1 and EGFR inhibitor PD153035 on ERK1/2 phosphorylation in human urothelial cells over-expressing wild-type (HU-FGFR3WT), constitutively-activated mutant FGFR3 (HU-FGFR3mut) and empty virus (control) cells (HU-puro).**

Cell lysates were prepared after 24h treatment with or without PD153035 (1 μ M) or FGF1 (1 nM) and phosphorylated ERK (p-ERK1/2) protein was assessed by SDS-PAGE and immunoblotting using a rabbit polyclonal antibody. To ensure equal loading, membranes were simultaneously probed with a mouse monoclonal antibody for detection of total ERK. This was followed by treatment with fluorochrome-conjugated goat anti-rabbit antibody (green) and anti-mouse (red) antibodies, respectively, and immunolabelling was visualised by infrared imaging as described in the Methods section.

Supplementary Figure 2**Effects of long-term hTERT over-expression on UPK3a expression.**

Control virus-transduced (HU-neo) and HU-hTERT cells at the indicated passage number were induced to undergo differentiation following treatment with combination of 1 μ M PPAR γ agonist troglitazone and 1 μ M EGFR inhibitor PD153035 (TZ+PD). Total RNA was extracted, cDNA generated and quantitative real-time RT-PCR was performed to assess UPK3a mRNA expression. For each datum point, UPK3a mRNA level was normalized relative to that for the internal control (GAPDH) and each bar represents mean UPK3a/GAPDH ratio (\pm SD) for three replicates.
