Genes Involved in Differentiation, Stem Cell Renewal, and Tumorigenesis Are Modulated in Telomerase-Immortalized Human Urothelial Cells

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Abstract

The expression of hTERT, the catalytic subunit of telomerase, immortalizes normal human urothelial cells (NHUC). Expression of a modified hTERT, without the ability to act in telomere maintenance, did not immortalize NHUC, confirming that effects at telomeres are required for urothelial immortalization. Previous studies indicate that inhibition of telomerase has an immediate effect on urothelial carcinoma (UC) cell line viability, before sufficient divisions to account for telomere attrition, implicating non–telomere effects of telomerase in UC. We analyzed the effects of telomerase on gene expression in isogenic mortal and hTERT-transduced NHUC. hTERT expression led to consistent alterations in the expression of genes predicted to be of phenotypic significance in tumorigenesis. A subset of expression changes were detected soon after transduction with hTERT and persisted with continued culture. These genes (*NME5, PSCA, TSPYL5, LY75, IGFBP2, IGF2, CEACAM6, XG, NOX5, KAL1*, and *HPGD*) include eight previously identified as polycomb group targets. TERT-NHUC showed overexpression of the polycomb repressor complex (PRC1 and PRC4) components, BMI1 and SIRT1, and down-regulation of multiple PRC targets and genes associated with differentiation. TERT-NHUC at 100 population doublings, but not soon after transduction, showed increased saturation density and an attenuated differentiation response, indicating that these are not acute effects of telomerase expression. Some of the changes in gene expression identified may contribute to tumorigenesis. Expression of NME5 and NDN was down-regulated in UC cell lines and tumors. Our data supports the concept of both telomere-based and non–telomere effects of telomerase and provides further rationale for the use of telomerase inhibitors in UC. (Mol Cancer Res 2008;6(7):1154–68)

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Introduction

The primary and well-documented role of telomerase is as a reverse transcriptase that acts in the maintenance of telomere length and structure. Up-regulation of telomerase expression occurs in the majority of urothelial carcinoma (UC) irrespective or stage or grade (1), suggesting that this may be an early event in tumorigenesis. Normal human urothelial cells (NHUC) are immortalized by expression of hTERT, the catalytic subunit of telomerase. In contrast to requirements for immortalization in other epithelial cell types and despite the common loss of expression of p16 in UC, inactivation of the *CDKN2A* locus (encoding p16 and $p14^{ARF}$) was not observed (2).

Non–telomere effects of hTERT expression have been described in other cell types, some of which may be relevant to tumorigenesis *in vivo* (3, 4). Inhibition of telomerase as a therapeutic strategy is generally based on the assumption that lack of telomerase activity will result in continued cell division and telomere attrition, which will eventually lead to replicative senescence or apoptosis (5). However, inhibition of telomerase has an immediate effect on UC cell line viability, before sufficient divisions to account for telomere attrition (6). This strongly implicates non–telomere effects of hTERT in bladder tumorigenesis and suggests that telomerase inhibition may be of rapid therapeutic benefit. Thus, identification of the genes and pathways involved in the non–telomere effects of telomerase in bladder and other cancers may highlight novel therapeutic or diagnostic targets. There is also data that links the expression of telomerase with the inhibition of cellular differentiation (7, 8). This may be a non–telomere event and is an example of how telomerase expression could contribute to tumorigenesis by mechanisms discrete from its classic actions in telomere maintenance.

hTERT-immortalized NHUC (TERT-NHUC) are generally diploid and have no chromosomal alterations (detectable by array CGH or karyotyping; ref. 2). However, changes in gene expression after telomerase expression have not been investigated. Microarray analysis of gene expression in isogenic mortal NHUC and their hTERT-immortalized counterparts was done to examine the hypothesis that expression of telomerase contributes to tumorigenesis in ways that are additional to its effect on telomere length and structure. As

TERT-NHUC had no detectable genetic alterations, we aimed to identify changes in gene expression that may have occurred via transcriptional mechanisms. Additionally, as TERT-NHUC provide the basis for an *in vitro* model of urothelial transformation, it is important to determine whether alterations in gene expression are present before other genes are experimentally manipulated.

Several previous experiments have examined the effect of hTERT on gene expression (9-12). There is little concordance between genes identified in these studies, which perhaps reflects the cell type–specific pathways involved in immortalization. This is mirrored by the different combinations of alterations seen in tumors arising within a particular tissue. No previous study has examined changes in gene expression after telomerase expression in matched pairs of mortal and immortal epithelial cells from multiple donors. By repeating the experiment in three biological replicates (derived from three cell donors) and looking for changes in expression consistent to multiple donors relative to their isogenic controls, inter-cell line differences should be minimal. This experimental design should increase the power to detect genes whose expression is consistently altered after expression of telomerase. We propose that the effects of telomerase *in vivo* are likely to be a combination and potentially synergistic effect of the classic actions in maintenance of telomere length and structure coupled to its currently uncharacterized non–telomere effects. For this reason, we chose to investigate the putative non–telomere effects of telomerase in the biologically relevant context of fully functional telomerase.

Results

Expression of a Modified hTERT without the Ability to Elongate Telomeres Does Not Immortalize NHUC

It has been unclear how expression of hTERT immortalizes NHUC, as profound shortening of telomere length is not observed in NHUC at replicative senescence (2, 13). However, it is possible that more subtle effects on telomere structure such as at the 3′ overhang are required for immortalization (14). To determine whether immortalization of NHUC was due to effects on telomere maintenance, cells were transduced to express hTERT-HA. hTERT-HA has a carboxyl-terminal hemagglutinin (HA) tag and induces soluble telomerase activity but cannot act in telomere maintenance, probably due to an inability to interact with the additional proteins required (15). Despite the induction of telomerase activity (7.2-fold compared with empty vector–transduced cells), the expression of hTERT-HA did not lead to a significant extension in the replicative life span of NHUC, confirming that telomere-dependent effects are required for the immortalization of NHUC (Fig. 1).

Telomerase Activity

A low level of telomerase activity was detected in mortal NHUC strains. Telomerase activity in each TERT-NHUC line was quantified relative to that in the isogenic NHUC cell strain. The ratios were 17, 9, and 6 for TERT-NHUC N, TERT-NHUC B, and TERT-NHUC A, respectively (Fig. 2A).

Expression of hTERT Leads to Consistent and Stable Changes in Gene Expression

Changes in gene expression of 2.0-fold or greater were identified following the comparison of hTERT-transduced and isogenic mortal strains of NHUC from three donors. This analysis was done soon after transduction with hTERT when cells were still within their normal mortal life span [<18 population doublings (PD)] and also when cells were deemed immortal and had undergone ~100 and 250 PDs. The expression of hTERT led to a statistically significant down-regulation of 104 probe sets, early after transduction, in at least two of the three donors (Table 1). These comprised 87 genes and 17 unknown transcripts or open reading frames (orf) or hypothetical genes. In cases in which a change in expression in cells from one of the three donors was not statistically significant, this was often due to a higher

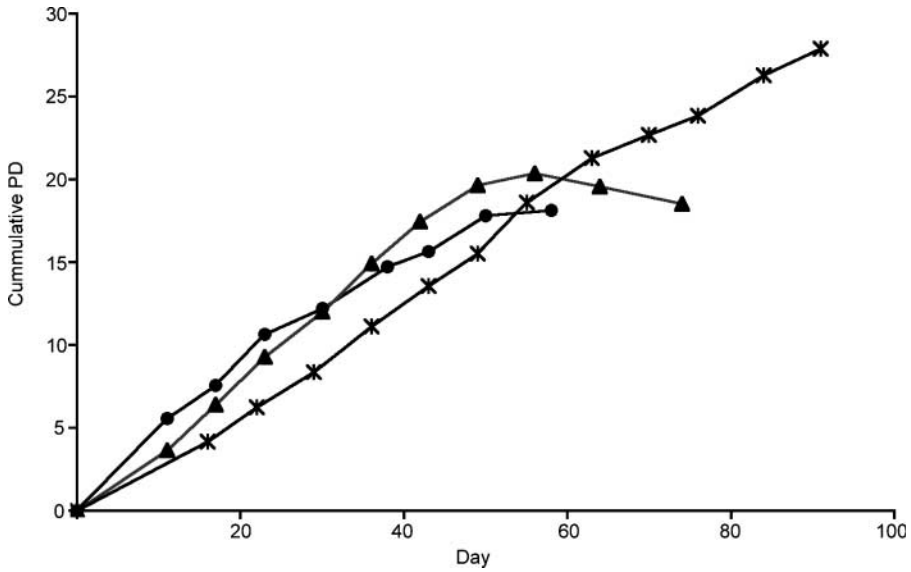

FIGURE 1. Transduction with TERT-HA does not immortalize NHUC, indicating that telomere-dependent effects are required for immortalization. *, wild-type hTERT; ▲, hTERT-HA; ●, empty vector-transduced cells. Data is derived from cells seeded in triplicate wells and is representative of that obtained using cells from two independent donors.

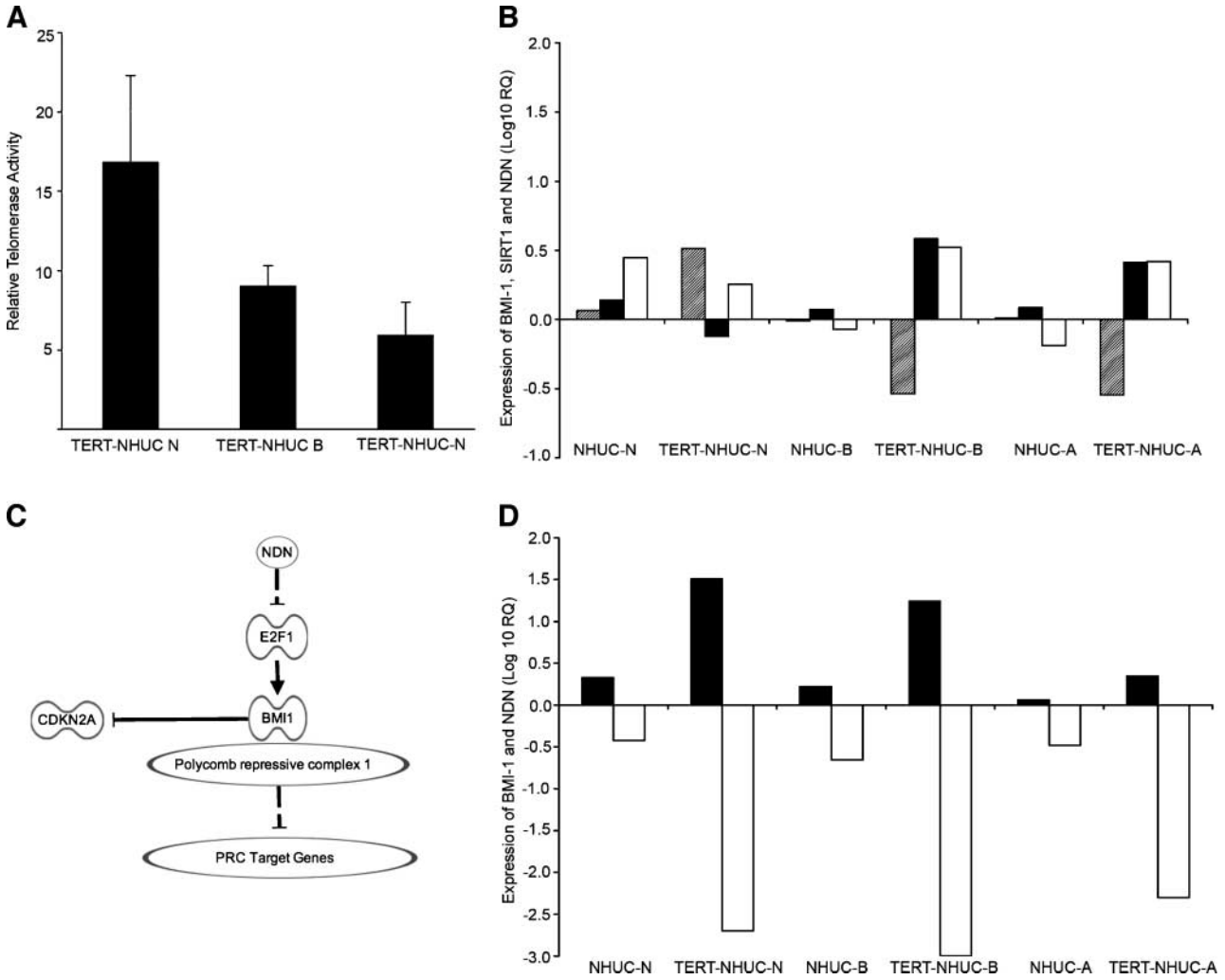

FIGURE 2. Characterization of TERT-NHUC. **A.** Quantification of telomerase activity in TERT-NHUC. Columns, mean ratio of telomerase activity relative to the empty vector–transduced isogenic NHUC. Data is derived from duplicate analyses. **B.** Relative quantification (*RQ*) of expression of BMI1 (*gray columns*), SIRT1 (*black columns*), and NDN (*unshaded columns*) in TERT-NHUC soon after transduction with hTERT. Expression is calculated relative to SDHA and normalized to a reference sample of pooled NHUC. **C.** The proposed role of NDN in controlling the expression of a subset of genes altered in TERT-NHUC. All interactions are known effects described in the literature. TERT-NHUC attenuated NDN expression. Down-regulation of NDN-dependent inhibition of E2F-1 could lead to an increase in the E2F-1 target gene BMI1. Up-regulation of BMI1 was detected in TERT-NHUC with down-regulated NDN. BMI1 forms part of the PRC1 which modulates the expression of a number of PCG targets. Multiple PCG targets were down-regulated in TERT-NHUC. **D.** Confirmation at 100 PD of down-regulated expression of NDN (*unshaded columns*) and up-regulated expression of BMI1 (*black columns*) in all three TERT-NHUC lines compared with the isogenic NHUC. Expression of NDN was undetectable after 40 cycles in TERT-NHUC B; this sample is assigned an arbitrary value of $\log_{10}$ (RQ) = −3.

variation in signal for that probe between the triplicate arrays for that donor or a fold change less than the stringent cutoff of 2.0-fold. Examination of raw data often showed that the trend in gene expression was followed.

Twenty-two of these 104 genes (indicated by asterisks in Table 1) have previously been identified as possible polycomb gene (PCG) targets by Bracken et al. (16). Bracken et al. did a genome-wide identification of human promoters bound by PCG and identified a list of >1,000 potential PCG targets. PCG proteins form multiprotein complexes called polycomb-repressive complexes (PRC) that play a key role in the regulation of transcription during development and differentiation. SIRT1, a PRC4 component, was up-regulated in cells from donors A and B and up-regulation of PRC1 component BMI1 was detected in cells from donor N. This was confirmed by quantitative real-time reverse transcriptase PCR (QRT-PCR; Fig. 2B).

Some genes modulated soon after the expression of telomerase may be transient changes or those involved in a stress response following retroviral transduction. Therefore, TERT-NHUC that had undergone ~100 PD (the point at which they were deemed immortal) were also examined for changes in gene expression. This second analysis (Table 2) showed that 11 of the genes that were identified as acute alterations still showed consistent alteration. These genes (*NME5, PSCA, TSPYL5, LY75, IGFBP2, IGF2, CEACAM6, XG, NOX5, KAL1*, and *HPGD*) are therefore considered a "telomerase signature" of expression in NHUC. Of these 11 genes, 8 have been

described as PRC targets. This is a statistically significant ($P = 0.0007$, Fischer's exact test) overrepresentation of PCG targets in the telomerase signature genes compared with the other genes altered soon after telomerase expression. Modulated expression of these genes (with the exception of *CEACAM6*) is stable because these alterations persisted to the final time point when cells had undergone ~250 to 300 PD (Supplementary Table S1).

At 100 PD, there was more consistency in the genes modulated in TERT-NHUC. Twenty-two genes or transcripts, including hTERT, were modulated in all three TERT-NHUC lines, and apart from hTERT, all were down-regulated (Table 2). This suggests that a general mechanism of gene repression had been activated. The gene that showed most consistent (all three lines) and profound down-regulation in TERT-NHUC at 100 PD was necdin (NDN; ref. 17). A significant down-regulation of NDN was not detected soon after the transduction with hTERT by array analysis. However, QRT-PCR showed some down-regulation of NDN in early passage TERT-NHUC N (but not in TERT-NHUC B or TERT-NHUC A; Fig. 2B). We hypothesize that down-regulation of NDN, in turn, leads to the down-regulation of a significant subset of genes in NHUC with long-term expression of hTERT (Fig. 2C). As it is known to bind to E2Fs and repress E2F-dependent transcription (18), down-regulation of NDN is predicted to lead to the increased expression of BMI1, a known E2F target gene (19). Up-regulation of BMI1 was identified on array analyses of TERT-NHUC at 100 PD and was confirmed by QRT-PCR in cells from all three donors (Fig. 2D). Of note, TERT-NHUC N, the only cell line to show early NDN down-regulation soon after transduction with hTERT, also showed BMI1 up-regulation. BMI1 forms part of the PRC1 and many of the genes (highlighted by asterisks in Tables 1 and 2) are PCG targets as identified by Bracken et al. (16).

As the expression of telomerase is an early event in tumorigenesis and its expression persists from premalignancy to tumor development, we were interested in the changes in gene expression that persisted in hTERT-immortalized cells as these may confer a selective advantage and might be important in tumorigenesis. Examination of the gene expression signature of TERT-NHUC at both <18 PD and 100 PD time points using Ingenuity Pathway Analysis software showed that cancer was the disease or disorder most closely associated with many of the alterations in gene expression (Fig. 3). This confirmed our observation in which altered expression of several of these genes has previously been associated with bladder or other cancers, e.g., CXADR and tumor suppressor candidates such as NDN and NME5. Analysis of gene ontology groups identified the overrepresentation of 15 genes involved in 31 often overlapping gene ontology categories in these cells (Supplementary Table S2).

Expression array analysis was done on TERT-NHUC that had undergone an additional period of proliferation of at least 150 PD (Supplementary Table S1). This third analysis confirmed that all but nine of the genes altered at the 100 PD time point still showed altered expression (>2.0-fold in cells from at least two donors) after this prolonged culture period. The genes for which expression was no longer significantly altered were *CEACAM6, DNAJC15, GALNTL4, HOXC4, C1orf115, EXOSC6, TPST1, COL12A1*, and *NOS1*. Of the 11 "telomerase signature genes" all, with the exception of CEACAM6, were still significantly altered at this final analysis time point.

Conditioned Medium from TERT-NHUC Does Not Affect the Proliferation of NHUC

Unlike the effect reported in human mammary epithelial cells (3), expression of telomerase in NHUC did not lead to the alteration of expression of growth factors or receptors such as fibroblast growth factor and epidermal growth factor receptors. In accordance with this, conditioned medium from TERT-NHUC had no effect on the proliferation of unmodified NHUC (data not shown).

Induction of Differentiation in TERT-NHUC

As several genes that were consistently down-regulated in TERT-NHUC at 100 PD were associated with differentiation (genes identified in italics in Tables 1 and 2), it was of interest to determine whether these cells retained a normal differentiation response. Peroxisome proliferator–activated receptor-γ (PPARγ) signaling is involved in urothelial differentiation. Previously, treatment of NHUC with the PPARγ agonist Troglitazone, together with the inhibition of autocrine epidermal growth factor receptor signaling by the small molecule inhibitor, PD153035, has been shown to induce the expression of urothelial differentiation–associated markers, uroplakin II (UPK2) and cytokeratin 20 (CK20; refs. 20-22). Treatment with PD153035 alone has no effect on UPK2 expression (20). We assessed the induction of CK20 expression by immunofluorescence microscopy in NHUC and found that as induction was restricted to a minority of cells in the culture (data not shown) and there was interdonor variability, this assay was not sufficiently quantitative for the assessment of differentiation in TERT-NHUC. Therefore, the ability to differentiate was assessed by QRT-PCR for UPK2. Treatment of all three TERT-NHUC lines at the 100 PD time point with Troglitazone and PD153035 resulted in an increase in UPK2 expression. In the case of TERT-NHUC N and TERT-NHUC B, isogenic NHUC were available and we observed that the magnitude of UPK2 induction was less than that seen in NHUC, indicating that the response is attenuated following immortalization by telomerase (Fig. 4A). Induction of UPK2 was then measured in TERT-NHUC soon after the expression of telomerase in comparison to those that had undergone ~100 PD (Fig. 4B). In these cells, the induction of UPK2 was greater than in those analyzed at 100 PD and was similar to that previously observed in isogenic NHUC. This suggests that attenuation in the ability to differentiate is not an acute effect of telomerase but rather is related to changes in gene expression that were detected after continued proliferation. Morphologic changes after treatment with Troglitazone and PD153035 have been described in NHUC (22). Neither early nor later passage TERT-NHUC showed the characteristic "grosetteh" morphology observed in NHUC (Fig. 4C), indicating that even soon after telomerase expression, differentiation may be attenuated to some extent.

Table 1. Genes Whose Expression Was Modulated in NHUC Soon (<18 PD) after Expression of hTERT

Probe set	TERT-NHUC			Symbol	Description
	A	B	N		
Down-regulated genes					
204351_at	−46.71		−43.58	S100P	S100 calcium-binding protein P
39248_at	−16.12	−9.64		AQP3	Aquaporin 3 (Gill blood group)
231259_s_at		−13.00	−5.69	CCND2*	Cyclin D2
205668_at	−4.27	−12.09		*LY75**	Lymphocyte antigen 75
202718_at	−5.19	−10.44		IGFBP2*	Insulin-like growth factor binding protein 2, 36 kDa
204818_at	−7.23	−8.17		HSD17B2	Hydroxysteroid (17-β) dehydrogenase 2
202409_at		−10.73	−9.22	IGF2*	Insulin-like growth factor 2 (somatomedin A)
211657_at	−8.10	−4.45	−6.86	CEACAM6*	Carcinoembryonic antigen-related cell adhesion molecule 6
231169_at	−4.50	−6.30	−7.73		
202295_s_at	−4.35	−5.58		CTSH	Cathepsin H
1554062_at		−11.71	−2.82	XG*	Xg blood group
229352_at		−10.11	−4.07	NOX5*	NADPH oxidase, EF-hand calcium binding domain 5
227554_at	−3.33	−5.96			MRNA; cDNA DKFZp686I18116
206197_at	−3.76	−5.57	−3.74	*NME5*	Nonmetastatic cells 5, protein expressed in prostate stem cell antigen
205319_at	−4.22	−4.29		PSCA	
242277_at		−7.32	4.44	PHACTR2	Phosphatase and actin regulator 2
230921_s_at	−3.19	−4.26	−3.60		
211548_s_at 203914_x_at					Hydroxyprostaglandin dehydrogenase 15-(NAD)
203913_s_at	−3.97	−5.29		HPGD*	
222240_s_at	−3.04	−3.17		ISYNA1	*myo*-inositol 1-phosphate synthase A1
213122_at	−3.27		−2.83	TSPYL5	TSPY-like 5
220494_s_at		−3.00	−3.04		
242844_at	−2.19	−3.12	−3.66	PGGT1B	Protein geranylgeranyltransferase type I, β subunit
240277_at		−3.21	−2.72	SLC30A7	Solute carrier family 30 (zinc transporter), member 7
225730_s_at		−3.25	−2.53	THUMPD3	THUMP domain containing 3
229128_s_at		−5.14	−3.39	ANP32E	Acidic (leucine-rich) nuclear phosphoprotein 32 family, member E
228443_s_at		−2.89	−2.57	SETD8	SET domain containing (lysine methyltransferase) 8
235863_at		−2.46	−2.94	JSRP1	Junctional sarcoplasmic reticulum protein 1
241669_x_at		−2.50	−2.80	PRKD2	Protein kinase D2
207306_at		−2.23	−3.04	TCF15*	Transcription factor 15 (basic helix-loop-helix)
213872_at		−4.12	−3.78	C6orf62	Chromosome 6 open reading frame 62
227338_at		−2.75	−2.52	LOC440983	Hypothetical gene supported by BC066916
238326_at		−3.66	−4.22	LOC440836	Similar to MGC52679 protein
240757_at		−3.79	−4.05	CLASP1	Cytoplasmic linker associated protein 1
214182_at		−2.58	−2.63	ARF6	ADP-ribosylation factor 6
213747_at		−4.58	−3.19	AZIN1	Antizyme inhibitor 1
235186_at		−2.68	−2.47	LOC644634	Hypothetical LOC644634
222614_at		−4.49	−3.22	RWDD2B	RWD domain containing 2B
238267_s_at		−2.45	−2.68		
213710_s_at		−4.59	−3.07		
211948_x_at 214055_x_at	−2.35	−2.51	−2.66	BAT2D1	BAT2 domain containing 1
208994_s_at	−2.51	−2.47		PPIG	Peptidylprolyl isomerase G (cyclophilin G)
227473_at		−3.78	−3.64		
231268_at		−2.27	−2.61	LOC645895	Hypothetical LOC645895
201120_s_at		−2.43	−2.41	PGRMC1	Progesterone receptor membrane component 1
241671_x_at		−2.43	−2.41	FLJ22536	Hypothetical locus LOC401237
240686_x_at		−2.33	−2.50	TFRC	Transferrin receptor (p90, CD71)
215454_x_at		−2.32	−2.49	SFTPC*	Surfactant, pulmonary-associated protein C
229676_at		−3.90	−3.29	PAPD1	PAP associated domain containing 1
229713_at		−3.79	−3.37		
217696_at		−2.15	−2.62	FUT7	Fucosyltransferase 7 (α (1,3) fucosyltransferase)
206042_x_at		−2.18	−2.55		SNRPN upstream reading frame
243297_at		−2.27	−2.39	VPS13D	Vacuolar protein sorting 13 homologue D (*S. cerevisiae*)
229747_x_at		−2.29	−2.38	MGC40489	Hypothetical protein MGC40489
241618_at		−2.32	−2.27	PACS1	Phosphofurin acidic cluster sorting protein 1
218094_s_at	−2.41		−2.17	DBNDD2	Dysbindin (dystrobrevin binding protein 1) domain containing 2
217040_x_at		−2.00	−2.57	SOX15	SRY (sex determining region Y)-box 15
210733_at		−3.05	−3.69	TRAM1	Translocation associated membrane protein 1
214630_at		−2.34	−2.07	CYP11B2*	Cytochrome P450, family 11, subfamily B, polypeptide 2
205212_s_at		−2.22	−2.15	CENTB1*	Centaurin, β1
1555226_s_at		−2.05	−2.23	C1orf43	Chromosome 1 open reading frame 43
215778_x_at		−2.15	−2.11	HAB1	B1 for mucin
227897_at		−3.87	−2.53	RAP2B	RAP2B, member of RAS oncogene family
238051_x_at		−2.01	−2.22	PWWP2	PWWP domain containing 2
217410_at		−2.05	−2.17	AGRN	Agrin
227781_x_at		−2.01	−2.19	FAM57B	Family with sequence similarity 57, member B
Up-regulated genes					
202237_at		37.34	11.82	NNMT*	Nicotinamide *N*-methyltransferase
1555271_a_at	12.59	8.68	12.91	TERT	Telomerase reverse transcriptase

Continued on the following page

Table 1. Genes Whose Expression Was Modulated in NHUC Soon (<18 PD) after Expression of hTERT (Cont'd)

Probe set	TERT-NHUC			Symbol	Description
	A	B	N		
235057_at	5.71	5.67	8.35	ITCH*	Itchy homologue E3 ubiquitin protein ligase
206230_at	4.37	7.46		LHX1*	LIM homeobox 1
244075_at	3.88	7.64		HSD17B7P2	Hydroxysteroid (17-β) dehydrogenase 7 pseudogene 2
218878_s_at	3.05	7.53		SIRT1	Sirtuin
232174_at		7.02	6.93	EXT1	Exostoses (multiple) 1
205932_s_at	3.14	5.41		MSX1*	msh homeobox 1
229574_at		6.59	4.90	TRA2A	Transformer-2α
215505_s_at	3.06	5.78	2.62	STRN3	Striatin, calmodulin binding protein 3
238735_at		3.80	3.54	TCF12	Transcription factor 12
204255_s_at		7.81	2.58	VDR*	Vitamin D (1,25-dihydroxyvitamin D_3) receptor
204837_at		3.39	3.25	MTMR9*	Myotubularin-related protein 9
209811_at 34449_at		3.50	3.11	CASP2	Caspase 2
1553111_a_at		2.49	3.44	KBTBD6	Kelch repeat and BTB (POZ) domain containing 6
219235_s_at		3.33	2.49	PHACTR4	Phosphatase and actin regulator 4
240145_at		2.87	2.92	DGKH*	Diacylglycerol kinase, ε
207078_at		2.98	2.73	MED6	Mediator complex subunit 6
204669_s_at		2.01	3.42	RNF24	Ring finger protein 24
203243_s_at		2.56	2.83	PDLIM5	PDZ and LIM domain 5
204730_at		3.39	4.66	RIMS3	Regulating synaptic membrane exocytosis 3
205206_at		1.93	3.15	KAL1*	Kallmann syndrome 1 sequence
225484_at		2.51	2.55	TSGA14	Testis-specific, 14
236816_at		2.41	2.60	C12orf30	Chromosome 12 open reading frame 30
223679_at	2.59		2.37	CTNNB1*	Catenin (cadherin-associated protein), β1, 88 kDa
239709_at	2.51	2.45		RP11-78J21.1	Heterogeneous nuclear ribonucleoprotein A1-like
235432_at		2.78	2.16	NPHP3	Nephronophthisis 3 (adolescent)
230779_at		4.38	3.03	TNRC6B	Trinucleotide repeat containing 6B
224595_at		4.29	3.08	SLC44A1	Solute carrier family 44, member 1
238738_at		4.33	2.80	PSMD7	Proteasome 26S subunit, non-ATPase, 7
204115_at	2.06	2.66		GNG11	Guanine nucleotide binding protein γ11
222557_at		3.17	3.77	STMN3	Stathmin-like 3
202723_s_at		3.19	3.66	FOXO1*	Forkhead box O1
203355_s_at		2.75	4.04	PSD3	Pleckstrin and Sec7 domain containing 3
208200_at	2.29	2.21	2.24	IL1A	Interleukin 1α
225662_at		4.09	2.61	ZAK	Sterile α motif and leucine zipper containing kinase AZK
214806_at		3.57	2.89	BICD1	Bicaudal D homologue 1 (Drosophila)
201040_at	2.07	2.04		GNAI2	Guanine nucleotide binding protein, α-inhibiting activity polypeptide 2
225961_at		2.84	3.32	KLHDC5	Kelch domain-containing 5

NOTE: Genes are ranked in order of average fold change. Genes previously identified as polycomb targets are highlighted with asterisks, and telomerase signature genes are in boldface. Genes with associations with differentiation are shown in italics.

TERT-NHUC Show Increased Culture Saturation Density

TERT-NHUC, soon after transduction, had an average saturation density (the confluent cell density at which cell proliferation is contact-inhibited) of 1.18×10^5 cells/cm^2, which is similar to that of the isogenic NHUC and the previously published value for NHUC of 1×10^5/cm^2 (ref. 23; Fig. 5A). However, TERT-NHUC at 100 PD had a higher saturation density compared with their paired isogenic NHUC and on average, reached contact inhibition at 1.7×10^5 cells/cm^2 compared with 0.91×10^5 cells/cm^2 for NHUC (Fig. 5B). Genes involved in cell-cell signaling, such as *ICAM2* were down-regulated in these cells, which may have contributed to this phenotype.

Genes Down-Regulated in TERT-NHUC Are Also Down-Regulated in UC Cell Lines and Tumors

NDN, NME5, and *ADFP* were selected for further analyses on the basis of profound and consistent down-regulation in cells at 100 PD. As discussed later, these genes have potential tumor suppressor functions and are implicated in mediating cellular differentiation. Expression was investigated in UC cell lines using TaqMan QRT-PCR. Expression of each gene was down-regulated in the majority of UC cell lines compared with pooled NHUC. NDN was down-regulated in 26 of 28 (92.9%; Fig. 6A), NME5 in 27 of 28 (96.4%; Fig. 6B), and ADFP in 16 of 28 (57.1%; Fig. 6C). Down-regulation of NDN protein expression in cell lines was confirmed by Western blotting (data not shown). Expression of NDN and NME5 was then investigated in a panel of primary UC. NDN was down-regulated in 35 of 58 (60%; Fig. 6D) and NME5 in 10 of 47 (21%; Fig. 6E), demonstrating that changes in the expression of genes that are modulated in TERT-NHUC also occur in bladder cancer *in vivo*.

Discussion

We have shown previously that expression of hTERT immortalizes NHUC *in vitro* with no detectable chromosomal alterations. It was not clear whether immortalization was due to the telomere-dependent effects of telomerase, as a low level of endogenous telomerase activity is detected in cultured NHUC, and profound telomere length-shortening is not seen at replicative senescence (13). However, the expression of a modified hTERT (hTERT-HA), that retains telomerase activity

Table 2. Genes Whose Expression Was Modulated in TERT-NHUC at 100 PD

Probe set	TERT-NHUC			Symbol	Description
	A	B	N		
Down-regulated genes					
209122_at	−64.52		−35.12	*ADFP*	Adipose differentiation-related protein
208596_s_at		−49.41	−14.48	*UGT1A3*	UDP glucuronosyltransferase 1 family, polypeptide A3
202409_at	−5.74	−45.69	−35.40	LOC492304	NA
209550_at	−26.41	−23.44	−28.06	*NDN*	Necdin homologue (mouse)
215125_s_at 206094_x_at		−39.47	−12.36	*UGT1A6**	UDP glucuronosyltransferase 1 family, polypeptide A6
215440_s_at	−26.23		−21.15	BEXL1	Brain expressed X-linked-like 1
214974_x_at 215101_s_at	−16.09	−21.40		CXCL5*	Chemokine (C-X-C motif) ligand 5
202157_s_at	−7.06	−27.65		CUGBP2	CUG triplet repeat, RNA binding protein 2
221024_s_at	−18.02		−16.26	SLC2A10	Solute carrier family 2 member 10
218694_at	−17.65		−15.59	ARMCX1	Armadillo repeat-containing, X-linked 1
203917_at 239155_at	−10.55	−32.8	−6.37	*CXADR**	Coxsackie virus and adenovirus receptor
205513_at	−10.50	−22.27		TCN1	Transcobalamin I
207126_x_at		−20.53	−11.30	*UGT1A1*	UDP glucuronosyltransferase 1 family, polypeptide A1
204532_x_at		−20.89	−8.99	*UGT1A9*	UDP glucuronosyltransferase 1 family, polypeptide A9
202075_s_at	−16.15		−11.20	PLTP	Phospholipid transfer protein
204083_s_at	−16.17	−10.34		TPM2	Tropomyosin 2 (β)
201348_at 214091_s_at	−10.33	−16.33		GPX3	Glutathione peroxidase 3 (plasma)
204417_at	−16.13		−9.38	GALC*	Galactosylceramidase (Krabbe disease)
235940_at	−14.27		−9.82	C9orf64	Chromosome 9 open reading frame 64
216623_x_at 215108_x_at	−16.36		−4.12	TNRC9	Trinucleotide repeat containing 9
212859_x_at	−6.81		−12.53	MT1E*	Metallothionein 1E (functional)
205856_at 229151_at	−15.67	−11.72		SLC14A1	Solute carrier family 14 (urea transporter), member 1
206714_at 1555416_a_at	−9.87	−8.94		*ALOX15B*	Arachidonate 15-lipoxygenase, second type
206197_at	−11.88	−5.67	−9.94	*NME5*	Nonmetastatic cells 5, protein expressed in
238021_s_at	−3.36		−14.61	LOC388279	NA
202888_s_at	−14.98		−2.43	ANPEP	Alanyl (membrane) aminopeptidase
205997_at	−11.33	−5.57		ADAM28*	ADAM metallopeptidase domain 28
202718_at	−5.27	−11.19		IGFBP2*	Insulin-like growth factor binding protein 2, 36kDa
211657_at	−10.37		−5.33	CEACAM6*	Carcinoembryonic antigen-related cell adhesion molecule 6
229352_at		−9.85	−5.64	NOX5*	NADPH oxidase, EF-hand calcium binding domain 5
205668_at	−5.20	−12.10	−5.33	*LY75**	Lymphocyte antigen 75
224435_at	−7.51	−6.12		C10orf58*	Chromosome 10 open reading frame 58
1554062_at	−2.86	−11.70	−4.61	XG*	Xg blood group
202410_x_at		−8.02	−4.62	IGF2*	Insulin-like growth factor 2 (somatomedin A)
1552566_at	−3.99	−8.62		C10orf87	Chromosome 10 open reading frame 87
203921_at	−5.75	−6.86		CHST2	Carbohydrate sulfotransferase 2
229095_s_at	−9.36	−6.71	−2.67	LIMS3	LIM and senescent cell antigen-like domains 3
221690_s_at		−6.27	−5.69	NALP2	NACHT, leucine rich repeat and PYD containing 2
1569110_x_at	−5.38		−6.56	PDCD6	Programmed cell death 6
221950_at	−5.58	−5.42	−6.52	EMX2*	Empty spiracles homologue 2 (Drosophila)
228843_at	−7.64	−3.29	−6.03		NA
213122_at	−7.53	−3.04	−5.68	TSPYL5	TSPY-like 5
228080_at	−8.18	−4.00	−3.29	LOC143903	NA
204984_at	−3.63	−6.61		GPC4	Glypican 4
205501_at	−7.38	−2.79			NA
1559827_at	−5.58	−4.50		LOC401074	NA
239082_at		−5.23	−4.73		NA
211732_x_at 204112_s_at	−4.04	−5.51		HNMT	Histamine *N*-methyltransferase
203274_at	−6.70	−2.40		F8A1	Coagulation factor VIII–associated (intronic transcript) 1
203423_at	−5.32		−3.67	RBP1	Retinol binding protein 1, cellular
223824_at	−5.48	−3.95	−3.94	C10orf59	Chromosome 10 open reading frame 59
210664_s_at	−4.04	−6.13	−3.03	TFPI	Tissue factor pathway inhibitor
223960_s_at	−3.40	−5.46		C16orf5	Chromosome 16 open reading frame 5
205493_s_at	−4.13	−4.65		DPYSL4	Dihydropyrimidinase-like 4
222592_s_at 218322_s_at	−5.91	−2.79		ACSL5	Acyl-CoA synthetase long-chain family member 5
1555564_a_at	−5.07		−2.85	IF*	I factor (complement)
205319_at	−5.20	−3.71	−2.49	*PSCA*	Prostate stem cell antigen
213620_s_at	−4.95	−3.31	−3.11	*ICAM2*	Intercellular adhesion molecule 2
1554079_at	−2.30	−5.04		GALNTL4	UDP-*N*-acetyl-α-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase-like 4
214077_x_at		−3.70	−3.20	MEIS4	Meis1, myeloid ecotropic viral integration site 1 homologue 4
231728_at 231729_s_at	−3.81		−2.99	CAPS	Calcyphosine
223832_s_at	−2.72	−2.78	−4.78	CAPNS2	Calpain, small subunit 2
203192_at	−3.79		−3.02	ABCB6	ATP-binding cassette, sub-family B (MDR/TAP), member 6
227554_at	−3.68		−3.10	LOC402560	NA
218435_at 227808_at	−3.64		−2.95	DNAJC15	DnaJ (Hsp40) homologue, subfamily C, member 15
205073_at		−3.41	−3.11	CYP2J2*	Cytochrome P450, family 2, subfamily J, polypeptide 2
203404_at	−3.79		−2.66	ARMCX2*	Armadillo repeat-containing, X-linked 2
211548_s_at 203913_s_at 203914_x_at	−3.84	−2.45		HPGD*	Hydroxyprostaglandin dehydrogenase 15-(NAD)

Continued on the following page

Table 2. Genes Whose Expression Was Modulated in TERT-NHUC at 100 PD (Cont'd)

Probe set	TERT-NHUC			Symbol	Description
	A	B	N		
227006_at	−2.48	−2.73	−4.22	PPP1R14A	Protein phosphatase 1, regulatory (inhibitor) subunit 14A
220911_s_at	−3.25	−3.06	−2.75	KIAA1305	KIAA1305
227803_at	−2.32		−3.60	ENPP5*	Ectonucleotide pyrophosphatase/phosphodiesterase 5
242856_at	4.52	−7.70	−5.62		NA
203799_at	−3.32		−2.44	CD302	CD302 antigen
218094_s_at	−3.33		−2.29	C20orf35*	chromosome 20 open reading frame 35
227892_at	−2.45		−3.16		NA
228721_at	−3.20	−2.65	−2.27	LOC339903	NA
206194_at		−2.62	−2.69	HOXC4	Homeo box C4
220027_s_at	−2.12	−2.76	−2.61	RASIP1*	Ras interacting protein 1
225841_at	−2.15		−2.17	C1orf59	Chromosome 1 open reading frame 59
205827_at	−2.10		−1.93	CCK	Cholecystokinin
Up-regulated genes					
205952_at		40.00	26.67	KCNK3*	Potassium channel, subfamily K, member 3
1555271_a_at	8.14	24.16	9.30	TERT	Telomerase reverse transcriptase
238846_at		15.67	10.07	TNFRSF11A*	Tumor necrosis factor receptor superfamily, member 11a
227307_at		5.18	13.49	TSPAN18	Tetraspanin 18
202265_at		8.02	9.50	BMI1	Polycomb group ring finger 4 (BMI1)
203895_at		12.93	2.41	PLCB4	Phospholipase C, β4
227641_at	5.31		8.84	FBXL16*	F-box and leucine-rich repeat protein 16
202555_s_at 224823_at		5.47	7.49	MYLK	Myosin, light polypeptide kinase
239132_at	5.45		4.43	NOS1	Nitric oxide synthase 1 (neuronal)
209493_at		4.02	5.42	PDZK3	PDZ domain containing 3
227123_at		4.40	3.93	RAB3B	RAB3B, member RAS oncogene family
231766_s_at	3.20		4.43	COL12A1	Collagen, type XII, α1
205206_at		2.81	4.60	KAL1*	Kallmann syndrome 1 sequence
202957_at	4.21	3.19		HCLS1	Hematopoietic cell-specific Lyn substrate 1
204140_at		3.65	3.75	TPST1	Tyrosylprotein sulfotransferase 1
231916_at	3.62		3.73	EXOSC6	Exosome component 6
218546_at	2.23	2.00		C1orf115	Chromosome 1 open reading frame 115

NOTE: Genes are ranked in order of average fold change. Genes previously identified as polycomb targets are highlighted with asterisks, and telomerase signature genes are in boldface. Genes with associations with differentiation are shown in italics.

but is deficient in the ability to elongate telomeres, failed to confer any significant extension in replicative life span, confirming that the actions of telomerase in telomere maintenance are required for the immortalization of NHUC. Recently, Choi et al. have described the effects of a reverse transcriptase–defective hTERT in transcriptional regulation of multiple genes converging on developmental pathways in skin progenitor cells (24). This and data presented here supports the concept of non–telomere effects of hTERT on gene expression.

Identification of changes in gene expression that occur soon after transduction with hTERT and persist with continued culture of TERT-NHUC identifies genes which can be considered a telomerase signature of gene expression. These are genes that could potentially be directly modulated by the expression of telomerase. Our data, for the first time, identifies the involvement of polycomb gene pathways. As TERT-NHUC had no detectable chromosomal alterations and these changes in expression occur in a timescale which makes spontaneous mutation unlikely, we suggest that this telomerase signature is of epigenetic origin. The fact that not all those alterations identified soon after transduction with hTERT persist with continued culture supports the concept that these changes are due to transcriptional rather than permanent genetic changes. However, the possibility that a proportion of the changes in gene expression are due to currently unidentified mutations cannot be discounted.

Twenty-two of the 104 (21%) transcripts whose expression was modulated soon after the expression of telomerase are reportedly PCG targets. As it is estimated that between 1% and 5% of all genes are PCG targets (25), there is a significant bias towards PCG target genes in this expression profile. TERT-NHUC, soon after transduction with hTERT, had overexpression of SIRT1, a NAD+-dependent deacetylase. SIRT1 with EED2 forms part of PRC4 (26) and is involved in epigenetic silencing by PCG proteins (27) and aberrant methylation of tumor suppressor proteins (28). SIRT1 promotes transcriptional repression by deacetylating specific histone proteins, recruiting histone H1b and modulating the activity of SUV39H1 (29), the enzyme responsible for the accumulation of trimethylated histone H3 (H3K9me) in a region of chromatin. Thus, SIRT1 is a good candidate for causing acute telomerase-associated modulation of gene expression, although the mechanism by which telomerase expression may result in up-regulation of SIRT1 is unknown.

We believe that examination of gene expression at 100 PD (in an immortalized but nontransformed cell population) may identify those changes in gene expression that confer a phenotypic advantage and that may be relevant to tumorigenesis *in vivo*. Changes in gene expression at this time point are stable as further microarray expression analysis after a prolonged culture period found that nearly all changes in expression were still present. TERT-NHUC have no identifiable chromosomal alterations, and therefore, it is likely that these genes have been silenced by transcriptional or epigenetic mechanisms. At this second time point, there was again a high number of modulated genes described as PCG targets. The

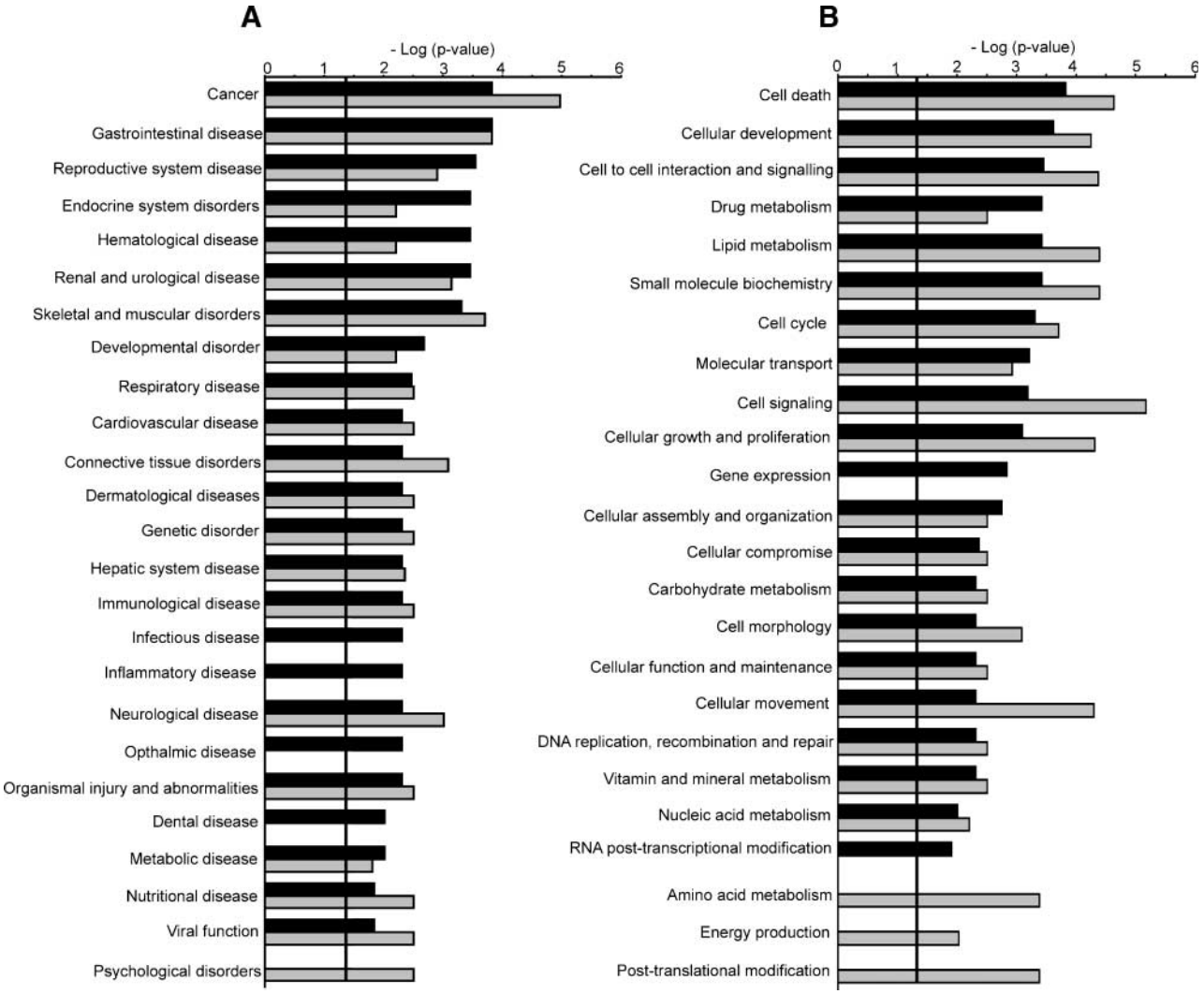

FIGURE 3. Ingenuity Pathway Functional Analysis identified the diseases and disorders (**A**) and cellular functions (**B**) that were most significant to the gene expression signature set of TERT-NHUC. Vertical line on each chart, threshold level of $P = 0.05$. Data from cells soon after transduction with hTERT (<18 PD; *black columns*) and data from cells at 100 PD (*gray columns*).

most down-regulated gene in all three TERT-NHUC lines at 100 PD was NDN. NDN is of interest as it is both a novel candidate tumor suppressor gene and a potential modulator of a subset of other changes in gene expression via its interaction with E2F1, and consequently, BMI1 and PRC1 target genes (Fig. 2C). We found that the expression of NDN was down-regulated in a high proportion of UC examined suggesting that this may indeed be relevant to tumor development *in vivo*.

NDN maps within an imprinted region on 15q11 implicated in the pathogenesis of the neurodevelopmental disorder Prader-Willi syndrome, where it is silenced by deletion, maternal uniparental disomy or translocation. Several observations suggest that NDN has a tumor suppressor role. Although not currently recognized as a cancer-prone syndrome, an increased risk of leukemia has been reported in Prader-Willi syndrome (30). There are also reports linking Prader-Willi syndrome with solid tumors (31-33). NDN is a growth suppressor in postmitotic neurons (34), is silenced in neuroblastoma (35), and has roles in differentiation (36, 37). NDN is involved in the interaction of nerve growth factor with its receptor p75NTR (18, 38). p75NTR signaling is implicated in the control of epithelial cell growth and differentiation (39), and induction of apoptosis in bladder cells (40). NDN binds to and represses the activity of SV40 large T (34, 38). It also interacts with p53 (41), antagonizes E2F1-mediated transcription, inhibits apoptosis, and suppresses colony formation of osteosarcoma cells (18, 34, 41, 42). NDN also directly binds to specific DNA sequences and acts as a transcriptional repressor (43).

We hypothesize that down-regulation of NDN releases the inhibition of E2F1-dependent transcription of BMI1. BMI1 forms part of PRC1, which is a chromatin-modifying complex involved in the control of gene expression and is implicated in stem cell renewal (44) and in delaying senescence (45). Twenty-five of the genes altered in TERT-NHUC at 100 PD

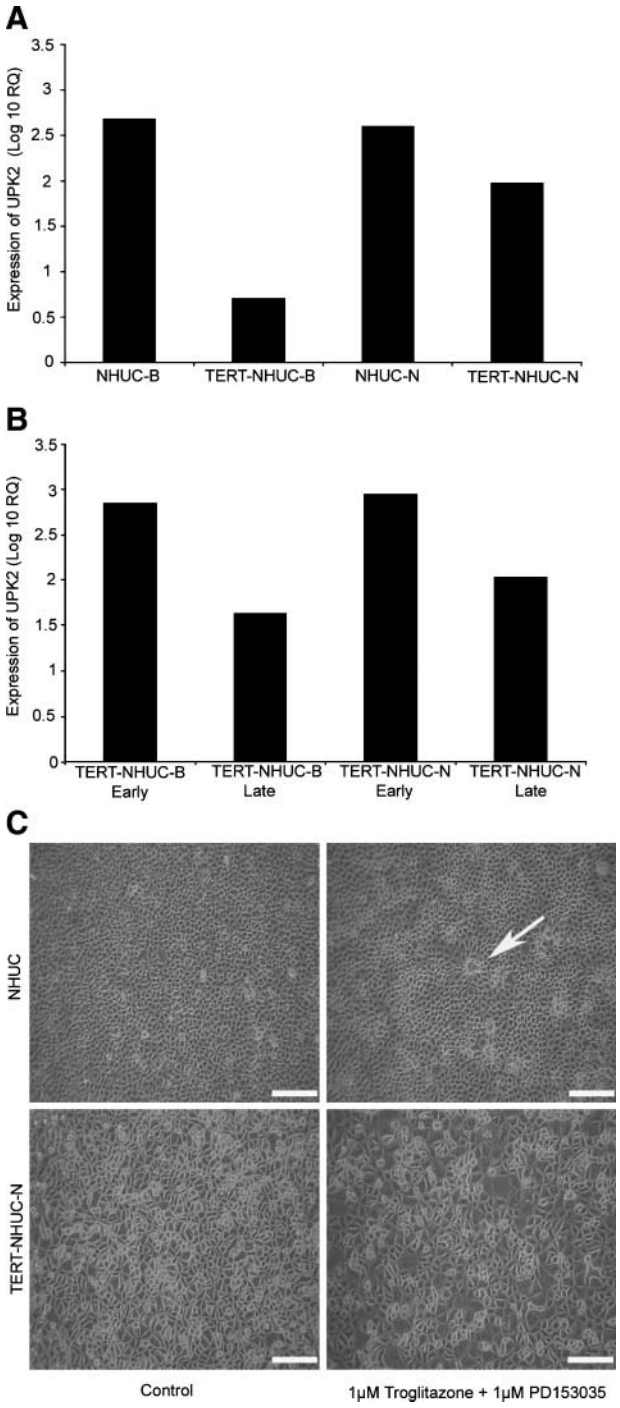

FIGURE 4. **A.** Expression of differentiation-associated uroplakin II was induced in TERT-NHUC (at 100 PD) after treatment with the PPARγ agonist Troglitazone and the epidermal growth factor receptor inhibitor PD153035. The magnitude of the response was less than that seen in isogenic NHUC. Data shows $\log_{10}$ relative quantification (RQ) relative to SDHA control gene and normalized to pooled NHUC cDNA. Expression data was derived from the average of duplicate experiments. **B.** Comparison of induction of UPK2 in TERT-NHUC at early and late passage shows that attenuation of differentiation is not an acute effect of telomerase expression. **C.** Troglitazone and PD153035 treatment of TERT-NHUC did not result in the characteristic "rosette" morphology seen in NHUC (*arrow*). Bars, 200 μm.

are putative polycomb gene targets (16), as are 8 of the 11 genes defined as the telomerase signature that was induced and persisted throughout the period of study. Up-regulation of BMI1 and other polycomb genes occurs in a range of tumor types (46) and precancerous tissue (47), in which they are thought to promote tumorigenesis by transcriptional repression of tumor suppressor genes and possibly via effects on stem cell maintenance (48). BMI1 is also associated with a stem-like expression profile that predicts poor outcome and treatment failure in multiple tumor types including bladder cancer (49). BMI1 expression is required for immortalization of some cell types by telomerase, possibly due to its effects in silencing p16/p14^{ARF} transcription. Up-regulation of BMI1 was not associated with the attenuation of p16 or p14^{ARF} transcription in TERT-NHUC. However, it is possible that BMI1 expression contributed to the immortalization of NHUC by preventing significant up-regulation of p16 expression, which plays a role in the control of the NHUC replicative life span.

Although not a widely acknowledged function of telomerase, other studies have shown a reciprocal relationship between telomerase activity and differentiation (7, 8, 50-52). Bracken et al. (16) reported that many putative polycomb gene targets such as those identified here, have roles in differentiation and development. TERT-NHUC at 100 PD showed reduced expression of many genes associated with differentiation. In many cases, there is evidence that down-regulation of these differentiation-associated genes also occurs in cancer. For example, the expression of CXADR, which was consistently down-regulated in TERT-NHUC, is known to be significantly reduced in invasive compared with superficial bladder cancers (53). A dramatic down-regulation of UDP-glucuronosyltransferase family of detoxifying enzymes was observed in TERT-NHUC from donors N and B. In normal bladder, UGT staining seems to correlate with epithelial cell differentiation and is decreased in some UCs (54). Also, prostate stem cell antigen is widely expressed in normal urothelium and noninvasive urothelial tumors, and is down-regulated in undifferentiated bladder carcinomas, leading to its description as a potential molecular marker of dedifferentiation in urothelial cells (55). ALOX15B is also of interest as one of its products, 15-*S*-hydroxyecosatetraenoic acid, an endogenous ligand for PPARγ, is known to be pivotal in urothelial cell differentiation (20). Its expression in mature squamous but not basal keratinocytes also hints at a role in cellular differentiation and it is down-regulated in various primary tumors and cell lines (56).

Several other genes that were down-regulated after the expression of telomerase have not yet been shown to have roles in urothelial cell differentiation, but there is evidence that they may play this role in other cell types. NDN has roles in the differentiation of smooth muscle cells, adipocytes, and neurons (36, 37, 57). NME5 is a homologue of nm23-H1, a tumor suppressor previously linked to bladder cancer (58). The function of NME5, also known as nm23 H5, is not well-described, although it is implicated in the differentiation of spermatozoa (59). We detected altered NME5 expression in UC. To our knowledge, this is the first investigation of NME5 expression in any tumor type. Expression of LY75 (gp200-MR6) has been linked to the differentiation of colorectal cell

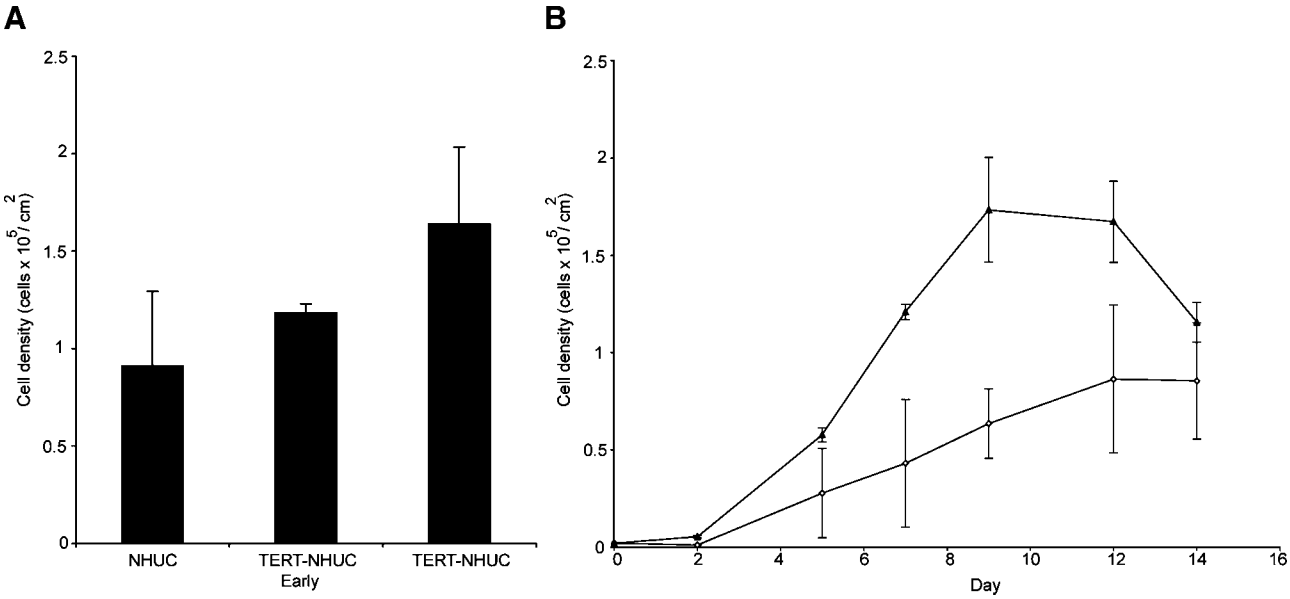

FIGURE 5. **A.** Maximum saturation density obtained in TERT-NHUC soon after transduction with hTERT (<18 PD) was less than that seen in TERT-NHUC that had undergone ~100 PD. TERT-NHUC soon after transduction with hTERT had a saturation density similar to that of NHUC. Columns, range of values within triplicate donors. **B.** TERT-NHUC (at 100 PD) reached a higher density before contact inhibition compared with their matched NHUC. Points, mean of cells from three donors, values from triplicate donors: filled symbols, TERT-NHUC (at ~100 PD); open circles, NHUC; bars, SD.

lines (60). *ADFP* is a transcriptional target of PPARγ. Its expression is decreased in undifferentiated renal cell carcinoma, and ADFP-positive tumors are associated with improved survival (61).

As TERT-NHUC at 100 PD had a "de-differentiated" gene expression profile, it was of interest to determine whether TERT-NHUC such as hTERT-immortalized bronchial epithelial cells (62) retain the ability to differentiate. TERT-NHUC (and NHUC) responded to a PPARγ agonist with the induction of UPK2. However, the fold-change of induction of UPK2 in TERT-NHUC at 100 PD was less than that seen in NHUC. Comparison of the differentiation response in TERT-NHUC soon after transduction to those that had undergone 100 PD found that low passage TERT-NHUC had a response similar to that of mortal NHUC. Similarly, the increased saturation density observed in TERT-NHUC at 100 PD was not shown soon after transduction with hTERT. Thus, attenuation of differentiation and cell-cell contact inhibition are not acute effects of telomerase expression but occur after telomerase-mediated immortalization. The attenuation of these responses in TERT-NHUC at 100 PD suggests that caution is required if using these cells as a platform to study gene function in normal urothelial cells. However, these cells may be a good model in which to study gene function in the context of premalignancy.

In summary, the identification of genes that were consistently altered after the expression of telomerase has led to robust filtering of gene lists and the identification of potential telomerase signature genes. We have confirmed that the telomere maintenance effects of telomerase are required for immortalization of NHUC. However, additional non–telomere effects include profound and consistent alterations in gene expression which might be predicted to contribute to tumorigenesis. Thus, further analysis of telomerase signature genes in bladder and other cancers is merited. Genes altered after acute and prolonged expression of telomerase include both PRC components and PCG target genes. These non–telomere actions of hTERT may explain the predominance of activation of telomerase in bladder and other epithelial cancers rather than the alternative lengthening of telomeres' pathway.

These considerable alterations in gene expression should not preclude the use of TERT-NHUC as experimental tools *in vitro* but do argue for the consideration of these changes when using these in place of normal unmodified cells. We suggest that TERT-NHUC are not suitable for long-term tissue replacement strategies in patients. Our data provides support for the use of telomerase inhibitors in UC, because in addition to its known actions at telomeres, it could be predicted that multiple molecules would be targeted by a single intervention, leading to an immediate therapeutic effect. Further investigations into the non–telomere effects of telomerase may provide valuable insights into processes relevant both during normal development and in cancer pathogenesis.

Materials and Methods

Cell Lines

TERT-NHUC A, TERT-NHUC B, and TERT-NHUC N and isogenic NHUC were cultured as described (2). Analyses of TERT-NHUC were done on cells soon after transduction with hTERT (recovered from frozen samples within four passages of selection and cultured for triplicate RNA extractions; PD level was <18 PD) on cells that had undergone ~100 PD and at a final time point when cells had undergone at least an additional 150 PD. Saturation density was assessed by plating 2×10^4 cells in duplicate 35-mm diameter dishes and counting after 2, 5, 7, 9, 12, and 14 days. For conditioned medium experiments, medium was added to 100 PD cells at 50% confluence. After

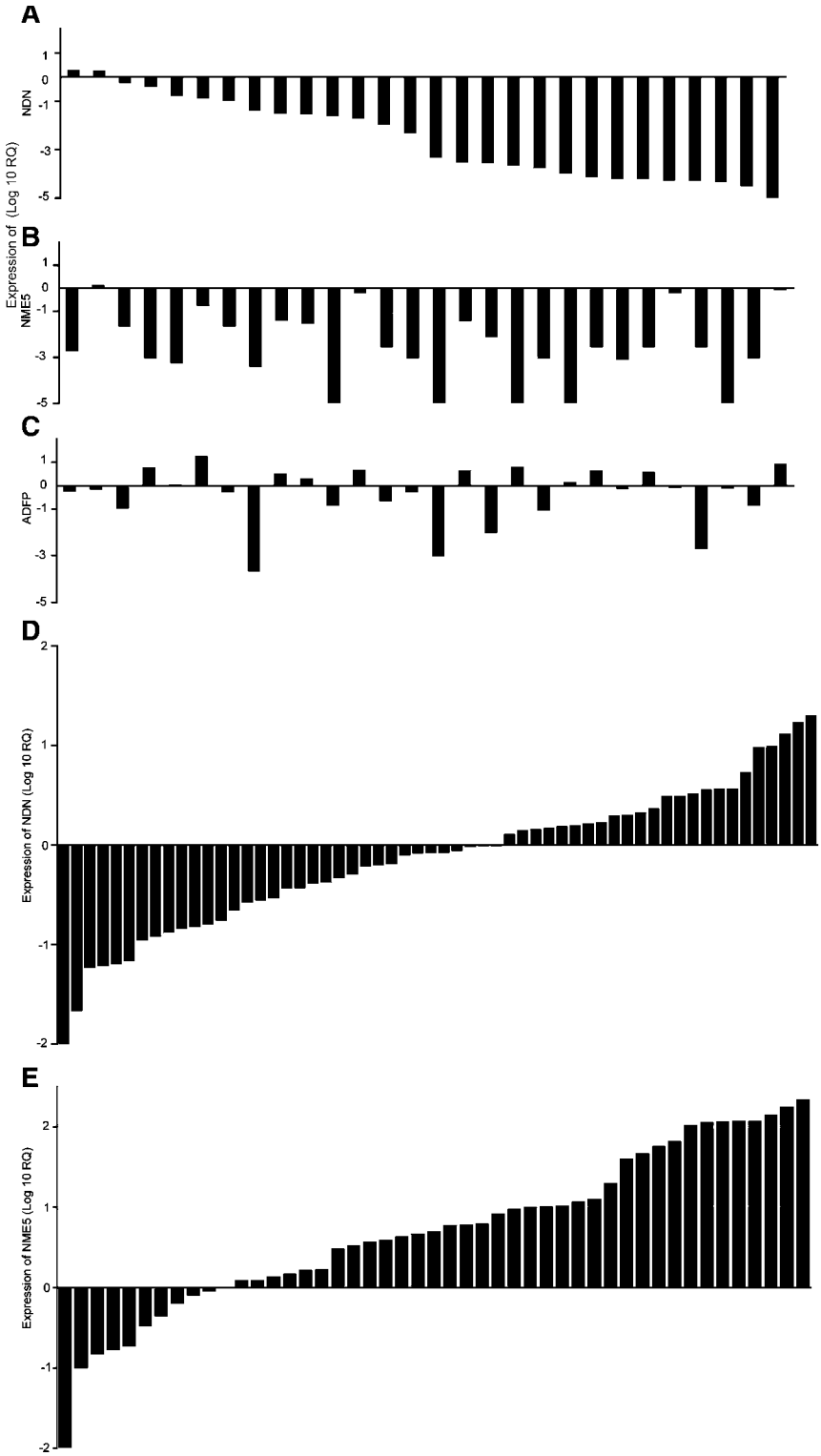

FIGURE 6. Expression of candidate telomerase signature genes was altered in UC cell lines and tumors. Log_{10} RQ relative to pooled NHUC cDNA and normalized to SDHA. The reference sample (pooled NHUC) therefore has a RQ value of = 1 and log_{10} RQ value of 0. Samples with a log_{10} RQ value < 0 have down-regulation of expression and those with log_{10} RQ > 0, overexpression relative to pooled NHUC. Where transcript was undetectable after 40 cycles of PCR, log_{10} RQ is shown as −5. Bladder cell lines from left to right are; CAL29, 253J, DSH1, HT1376, TCCSUP, J82, KU1919, RT112, JO'N, LUCC1, 97-7, 5637, 96-1, 97-6, SCaBER, SVHUC, 94-10, RT4, T24, UMUC3, JMSU, 97-1, SD, SW1170, 92-1, BFTC905, VMCUB2, and HCV29. SVHUC was derived from urothelium transformed *in vitro*, HCV29 was established from nonmalignant ureteric urothelium of a patient with bladder cancer and the remainder are UC cell lines. **A.** NDN, **B,** NME5, and **C,** ADFP. Expression of candidate telomerase -regulated genes was altered in primary UC. **D,** NDN; and **E,** NME5. Tumors are ranked in order of expression of each gene.

48 h, medium was harvested, centrifuged at 100 × *g* for 10 min, filtered, and stored at −20°C. NHUC were seeded at 6×10^4 cells per 35 mm well in triplicate, fed every 3 days with the conditioned medium and counted weekly.

Microarray Processing and Data Analysis

Affymetrix HG_U133 Plus 2.0 oligonucleotide arrays were hybridized at the Patterson Institute for Cancer Research, Manchester, United Kingdom. Further information including RNA extraction methods is available at their web site.[3] Two micrograms of total RNA was used to prepare biotinylated target RNA, according to the Affymetrix One Cycle Target Preparation Protocol, driven by T7-linked oligo(dT) primers. RNA was extracted from TERT-NHUC, soon after transduction with hTERT (<18 PD), after 100 PD, and after ~250 PD. Microarray data were analyzed using the Bioconductor (63) packages. cel files were robust means analysis preprocessed (64) using the Limma package (65). A linear model was constructed to fit, within each donor, a baseline NHUC level and offsets for telomerase expression. The Limma package allows these variables to then be tested, using an empirical Bayes method that adjusts the per-gene replicate variance towards the global average variance across genes, to lessen the effect of variance underestimation. Differentially expressed genes were selected on the basis of the false discovery rate being controlled to 0.0001; this test was applied to discover separate gene lists for each comparison of TERT-NHUC to the baseline NHUC, within cells from each donor. Gene lists were compared to identify genes consistently altered in cells from at least two of the three donors. Data were analyzed through the use of Ingenuity Pathway Analysis (Ingenuity Systems).[4] Functional analysis identified the biological functions and diseases that were most significant to the data set (list of genes altered in at least two of three donors). Genes from the data set that met the criteria of >2-fold change and were associated with biological functions and/or diseases in the Ingenuity Pathways Knowledge Base were considered for the analysis. Fischer's exact test was used to calculate a *P* value determining the probability that each biological function or disease assigned to the data set is due to chance alone. For gene ontology analysis, Affymetrix probes were mapped to a unique set of Entrez IDs. GOstats (66) was then used to test for overrepresentation at all the "biological process" gene ontology nodes, using a hypergeometric test.

QRT-PCR

To validate microarray data, the expression of selected genes was confirmed by QRT-PCR. RNA from triplicate repeats for each condition on the array was pooled. One microgram of total RNA was reverse-transcribed using Advantage RT-for-PCR kit (Clontech). QRT-PCR was carried out using an ABI 7500 Real-time PCR System and TaqMan Gene Expression Assays (Applied Biosystems); Hs00267349 (NDN), Hs00177499 (NME5), Hs00605340 (ADFP), Hs00180411 (BMI1), and Hs01009006 (SIRT1). Expression was quantified relative to Hs00417200 (SDHA) and normalized to pooled NHUC cDNA. TERT-NHUC RNA was similar to that used for array analyses. Expression of NDN and NME5 was also examined in UC cell lines and tumors. cDNA was prepared from these cell lines as previously described (67).

Induction of Urothelial Differentiation

Cells were plated at 2×10^5 cells/mL and when ~70% confluent, cells were treated for 24 h with 1 μmol/L of Troglitazone plus 1 μmol/L of the epidermal growth factor receptor inhibitor, PD153035 (Merck; ref. 20), then maintained for 5 days in 1 μmol/L of PD153035. RNA was extracted using GenElute mammalian total RNA mini-prep kit (Sigma) and cDNA was transcribed from 1 μg of RNA using SuperScript First-Strand Synthesis system (Invitrogen). UPK2 expression was quantified using TaqMan QRT-PCR (assay Hs00171854).

Quantification of Telomerase Activity

Telomerase activity was measured using the TRAPeze-RT kit (Chemicon) and Titanium Taq (Chemicon) according to the manufacturer's instructions. Triplicate reactions containing 1,000 cells per reaction were carried out on an ABI 7500. The assay was repeated twice and an average value was calculated.

TERT-HA NHUC

Retroviruses were produced using pBabe-puro vectors and ecotrophic packaging cells. NHUC expressing the ecotrophic retroviral receptor (2) were transduced to express hTERT-HA, wild-type hTERT, or empty vector (Addgene plasmids 1772, 1771, and 1764).[5] The expression of hTERT-HA leads to telomerase activity but, due to an HA tag on its COOH terminus, lacks the ability to act in telomere elongation or maintenance (15).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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