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Genes involved in differentiation, stem cell renewal and tumorigenesis are modulated in telomerase-immortalized human urothelial cells

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Abstract

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 analysed the effects of telomerase on gene expression in isogenic mortal and hTERT-transduced NHUC. hTERT expression led to consistent alterations in 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, HPGD) include 8 previously identified as polycomb group targets. TERT-NHUC showed overexpression of the polycomb-repressor complex 1 (PRC1) and PRC4 components BMI1 and SIRT1 and downregulation 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 downregulated in UC cell lines and tumours. 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.

Keywords

hTERT; telomerase; bladder; microarray

Introduction

The primary and well-documented role of telomerase is as a reverse transcriptase that acts in the maintenance of telomere length and structure. Upregulation of telomerase expression occurs in the majority of urothelial carcinoma (UC) irrespective of stage or grade (1)

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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 immortalisation 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}) is 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 upon the assumption that lack of telomerase activity will result in continued cell division and telomere attrition, which will eventually lead to a 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 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 expression of telomerase with 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 classical actions in telomere maintenance.

hTERT-immortalised NHUC (TERT-NHUC) are generally diploid and have no chromosomal alterations (detectable by array CGH or karyotyping) (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-immortalised counterparts was performed 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 cell-type specific pathways involved in immortalization. This is mirrored by the different combinations of alterations seen in tumours 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 3 biological replicates (derived from 3 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 uncharacterised 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 ability to elongate telomeres does not immortalize NHUC

It has been unclear how expression of hTERT immortalises 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 immortalisation (14). To determine whether immortalisation 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 additional proteins required (15). Despite induction of telomerase activity (7.2 fold compared to empty vector transduced cells), expression of hTERT-HA did not lead to significant extension in replicative lifespan of NHUC, confirming that telomere-dependent effects are required for immortalization of NHUC (Figure 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, B and A respectively (Figure 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 comparison of hTERT-transduced and isogenic mortal strains of NHUC from 3 donors. This analysis was performed soon after transduction with hTERT when cells were still within their normal mortal lifespan (<18 population doublings) and also when cells were deemed immortal and had undergone approximately 100 and 250 population doublings (PD). Expression of hTERT led to statistically significant downregulation of 104 probe sets, early after transduction, in at least 2 of the 3 donors (Table 1). These comprised 87 genes and 17 unknown transcripts or open reading frames (orfs) or hypothetical genes. In cases where a change in expression in cells from one donor of the three was not statistically significant, this was often due to higher 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) are those previously identified as possible polycomb gene (PCG) targets by Bracken et al.(16). Bracken et al., performed genome-wide identification of human promoters bound by PCG and identified a list of >1000 potential PCG targets. PCG proteins form multiprotein complexes called polycomb repressive complexes (PRC) that play a key role in regulation of transcription during development and differentiation. SIRT1, a PRC4 component was upregulated in cells from donors A and B and upregulation of PRC1 component BMI1 was detected in cells from donor N. This was confirmed by quantitative real time PCR (QRT-PCR) (Figure 2B).

Some genes modulated soon after expression of telomerase may be transient changes or those involved in a stress response following retroviral transduction. Therefore, TERT-NHUC that had undergone approximately 100PD (the point at which they were deemed immortal) were also examined for changes in gene expression. This second analysis (Table 2) demonstrated that 11 of the genes that were identified as acute alterations still showed consistent alteration. These genes (NME5, PSCA, TSPYL5, LY75, IGF2, IGF2, CEACAM6, XG, NOX5, KAL1 and HPGD) are therefore considered a "telomerase signature" of expression in NHUC. Of these 11 genes, 8 are those 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 to 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 timepoint when cells had undergone approximately 250-300PD (Supplementary Table 1).

At 100PD there was more consistency in the genes modulated in TERT-NHUC. Twenty-two genes or transcripts including hTERT were modulated in all 3 TERT-NHUC lines and apart from hTERT, all were downregulated (Table 2). This suggests that a general mechanism of gene repression had been activated. The gene that showed most consistent (all 3 lines) and profound downregulation in TERT-NHUC at 100PD was *neclin* (*NDN*) (17). A significant downregulation of *NDN* was not detected soon after transduction with hTERT by array analysis. However, QRT-PCR demonstrated some downregulation of *NDN* in early passage TERT-NHUC N (but not B and A) (Figure 2B). We hypothesise that downregulation of *NDN*, in turn leads to downregulation of a significant subset of genes in NHUC with long-term expression of hTERT (Figure 2C). As it is known to bind to E2Fs and repress E2F-dependent transcription (18), downregulation of *NDN* is predicted to lead to increased expression of *BMI1*, a known E2F target gene (19). Upregulation of *BMI1* was identified on array analyses of TERT-NHUC at 100PD and was confirmed by QRT-PCR in cells from all 3 donors (Figure 2D). Of note, TERT-NHUC N the only cell line to show early *NDN* downregulation soon after transduction with hTERT also demonstrated *BMI1* upregulation. *BMI1* forms part of 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 expression of telomerase is an early event in tumorigenesis and its expression persists from pre-malignancy 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 be important in tumorigenesis. Examination of the gene expression signature of TERT-NHUC at both < 18PD and 100PD timepoints, with Ingenuity Pathway Analysis software demonstrated that cancer was the disease or disorder most closely associated with many of the alterations in gene expression (Figure 3). This confirmed our observation that altered expression of several of these genes has previously been associated with bladder or other cancers e.g. *CXADR* and tumour suppressor candidates such as *NDN* and *NME5*. Analysis of gene ontology groups (GO) identified overrepresentation of 15 genes involved in 31 often overlapping GO categories in these cells. (Supplementary Table 2).

Expression array analysis was performed on TERT-NHUC that had undergone an additional period of proliferation of at least 150PD (Supplementary Table 1). This third analysis confirmed that all but 9 of the genes altered at the 100PD timepoint, still demonstrated altered expression (> 2.0 fold in cells from at least 2 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 timepoint.

Conditioned medium from TERT-NHUC does not affect proliferation of NHUC

Unlike the effect reported in human mammary epithelial cells (3), expression of telomerase in NHUC did not lead to alteration of expression of growth factors or receptors such as FGF and EGF receptor. In accordance with this, conditioned media 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 downregulated in TERT-NHUC at 100PD are 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 activator receptor γ (PPAR γ) signalling is involved in urothelial differentiation. Previously, treatment of NHUC with the PPAR γ agonist Troglitazone, together with inhibition of autocrine EGFR signalling by the small molecule inhibitor PD153035, has been shown to induce expression of the urothelial differentiation-associated markers, uroplakin II (UPK2) and cytokeratin 20 (CK20) (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 inter-donor variability, this assay was not sufficiently quantitative for assessment of differentiation in TERT-NHUC. Therefore, the ability to differentiate was assessed by QRTPCR for UPK2. Treatment of all three TERT-NHUC lines at the 100PD time point with Troglitazone and PD153035 resulted in an increase in UPK2 expression. In the case of TERT-NHUC N and 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 immortalisation by telomerase (Figure 4A). Induction of UPK2 was then measured in TERT-NHUC soon after expression of telomerase in comparison to those that had undergone approximately 100PD (Figure 4B). In these cells induction of UPK2 was greater than in those analysed at 100PD and 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. Morphological changes after treatment with Troglitazone and PD153035 have been described in NHUC (22). Neither early nor later passage TERT-NHUC demonstrated the characteristic “rosette” morphology observed in NHUC (Figure 4C), indicating that even soon after telomerase expression, differentiation may be attenuated to some extent.

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/cm² which is similar to that of the isogenic NHUC and the previously published value for NHUC of 1×10^5 /cm² (23) (Figure 5A). However, TERT-NHUC at 100PD had a higher saturation density compared to their paired isogenic NHUC and on average, reached contact inhibition at 1.7×10^5 cells/cm² compared to 0.91×10^5 cells/cm² for NHUC (Figure 5B). Genes involved in cell-cell signalling, such as ICAM2 were downregulated in these cells, which may have contributed to this phenotype.

Genes downregulated in TERT-NHUC are also downregulated in UC cell lines and tumours

NDN, NME5 and ADFP were selected for further analyses on the basis of profound and consistent downregulation in cells at 100PD. As discussed later, these genes have potential tumour suppressor functions and are implicated in mediating cellular differentiation. Expression was investigated in UC cell lines using Taqman QRTPCR. Expression of each gene was downregulated in the majority of UC cell lines compared to pooled NHUC. NDN was downregulated in 26/28 (92.9%) (Figure 6A), NME5 in 27/28 (96.4%) (Figure 6B) and ADFP in 16/28 (57.1%) (Figure 6C). Downregulation 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 downregulated in 35/58 (60%) (Figure 6D) and NME5 in 10/47 (21%) (Figure 6E), demonstrating that changes in 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 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, expression of a modified hTERT (hTERT-HA), that retains telomerase activity but is deficient in the ability to elongate telomeres, failed to confer any significant extension in replicative lifespan, confirming that the actions of telomerase in telomere maintenance are required for immortalization of NHUC. Recently, Choi et al., (2008) 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, which potentially could be directly modulated by expression of telomerase and our data for the first time identifies 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 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 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, though the mechanism by which telomerase expression may result in upregulation of SIRT1 is unknown.

We believe that examination of gene expression at 100PD (in an immortalized but non-transformed 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 timepoint there was again a high number of modulated genes described as PCG targets. The most downregulated gene in all three TERT-NHUC lines at 100PD 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 (Figure 2C). We found that expression of NDN was

downregulated 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 (PWS), where it is silenced by deletion, maternal uniparental disomy or translocation. Several observations suggest that NDN has a tumour suppressor role. Although not currently recognized as a cancer-prone syndrome, an increased risk of leukaemia has been reported in PWS (30). There are also reports linking PWS with solid tumours (31-33). NDN is a growth suppressor in post-mitotic neurons (34), is silenced in neuroblastoma (35) and has roles in differentiation (36, 37). NDN is involved in the interaction of NGF with its receptor p75NTR (18, 38). p75NTR signalling is implicated in control of epithelial cell growth and differentiation (39) and induction of apoptosis of bladder cells (40). NDN binds to and represses the activity of key cell-cycle-promoting proteins including SV40 large T (34, 38). It also interacts with p53 (41), antagonises 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 downregulation of NDN releases inhibition of E2F1 dependent transcription of BMI1. BMI1 forms part of PRC1, which is a chromatin modifying complex involved in control of gene expression and implicated in stem cell renewal (44) and in delaying senescence (45). Twenty-five of the genes altered in TERT-NHUC at 100PD 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. Upregulation of BMI1 and other polycomb genes occurs in a range of tumor types (46) and precancerous tissue (47), where 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 tumour 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. Upregulation of BMI1 was not associated with attenuation of p16 or p14^{ARF} transcription in TERT-NHUC. However, it is possible that BMI1 expression contributed to immortalization of NHUC by preventing significant upregulation of p16 expression, which plays a role in control of NHUC replicative lifespan.

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 100PD demonstrated reduced expression of many genes associated with differentiation. In many cases there is evidence that downregulation of these differentiation-associated genes also occurs in cancer. For example, expression of CXADR, which was consistently downregulated in TERT-NHUC, is known to be significantly reduced in invasive compared with superficial bladder cancers (53). A dramatic downregulation of UDP-glucuronosyltransferase family of detoxifying enzymes was observed in TERT-NHUC from donors N and B. In normal bladder, UGT staining appears to correlate with epithelial cell differentiation and is decreased in some UC (54). Also, PSCA is widely expressed in normal urothelium and non-invasive urothelial tumours, and is downregulated 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 is an endogenous ligand for PPAR γ , known to be pivotal to a key pathway 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 downregulated in various primary tumours and cell lines (56).

Several other genes that were downregulated after 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 differentiation of smooth muscle, adipocytes and neurons (36, 37, 57). NME5 is a homologue of nm23-H1, a tumour 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 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 differentiation of colorectal cell lines (60). ADFP is a transcriptional target of PPAR γ . Its expression is decreased in undifferentiated renal cell carcinoma, and ADFP positive tumours are associated with improved survival (61).

As TERT-NHUC at 100PD had a “de-differentiated” gene expression profile, it was of interest to determine whether TERT-NHUC like hTERT-immortalized bronchial epithelial cells (62) retain the ability to differentiate. TERT-NHUC (and NHUC) responded to a PPAR γ agonist with induction of UPK2. However, the fold-change of induction of UPK2 in TERT-NHUC at 100PD, was less than that seen in NHUC. Comparison of the differentiation response in TERT-NHUC soon after transduction to those that had undergone 100PD 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 100PD was not demonstrated 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 100PD 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, identification of genes that were consistently altered after expression of telomerase has led to robust filtering of gene-lists and identification of potential “telomerase signature” genes. We have confirmed that 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 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, as in addition to the known actions at telomeres, it would be predicted that multiple molecules would be targeted by a single intervention, leading to immediate therapeutic effect. Further investigations into the non-telomere effects of telomerase may provide valuable insights into processes relevant both during normal development and cancer pathogenesis.

Materials and methods

Cell lines

TERT-NHUC A, B, and N and isogenic normal human urothelial cells (NHUC) were cultured as described (2). Analyses of TERT-NHUC were performed on cells soon after transduction with hTERT (recovered from frozen within 4 passages of selection and cultured for triplicate RNA extractions, PD level was < 18PD), on cells that had undergone approximately 100PD and at a final timepoint when cells had undergone at least an additional 150PD. Saturation density was assessed by plating 2×10^4 cells in duplicate 35mm diameter dishes and counting after 2, 5, 7, 9, 12 and 14 days. For conditioned medium experiments, medium was added to 100PD cells at 50% confluence. After 48h, medium was harvested, centrifuged at 100g for 10 min, filtered, and stored at -20°C. NHUC were seeded at 6×10^4 cells per 35mm 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, UK. Further information including RNA extraction methods is available at <http://bioinformatics.picr.man.ac.uk/mbcf/index.jsp>. 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 (< 18PD), after 100PD and after approximately 250PD. Microarray data were analysed using the Bioconductor (63) packages. cel files were RMA pre-processed (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 parameters 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 impact 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 2 of the 3 donors. Data were analyzed through the use of Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenuity.com). Functional analysis identified the biological functions and diseases that were most significant to the data set (list of genes altered in at least 2 of 3 donors). Genes from the dataset 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 an 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.

Quantitative-real-time-RT-PCR (QRTPCR)

To validate microarray data expression of selected genes was confirmed by QRTPCR. RNA from triplicate repeats for each condition on the array was pooled. One µg of total RNA was reverse transcribed using Advantage® RT-for-PCR kit (Clontech, Hampshire, UK). QRTPCR was carried out using an ABI 7500 Real Time PCR System and TaqMan® Gene Expression Assays (Applied Biosystems, Cheshire, UK); Hs00267349 (NDN), Hs00177499 (NME5), Hs00605340 (ADFP), Hs00180411 (BMI1) and Hs01009006 (SIRT1). Expression was quantified relative to Hs00417200 (SDHA) and normalised to pooled NHUC cDNA. TERT-NHUC RNA was that used for array analyses. Expression of NDN and NME5 was

also examined in UC cell lines and tumours. cDNA was prepared from these as described (67).

Induction of urothelial differentiation

Cells were plated at 2×10^5 cells/ml and when approximately 70% confluent, cells were treated for 24h with $1\mu\text{M}$ Troglitazone plus $1\mu\text{M}$ of the EGFR inhibitor, PD153035 (Merck, Nottingham, UK) (20) then maintained for 5 days in $1\mu\text{M}$ PD153035. RNA was extracted using GenElute mammalian total RNA mini-prep kit (Sigma, Dorset, UK) and cDNA was transcribed from $1\mu\text{g}$ RNA using SuperScript First-Strand Synthesis system (Invitrogen, Paisley, UK). UPK2 expression was quantified using Taqman QRT-PCR (assay Hs00171854).

Quantification of telomerase activity

Telomerase activity was measured using the TRAPeze-RT kit (Chemicon, Hampshire, UK) and Titanium Taq (Chemicon) according to the manufacturer's instructions. Triplicate reactions containing 1000 cells per reaction were carried out on an ABI 7500. The assay was repeated twice and an average value 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, wildtype hTERT or empty vector, (Addgene plasmids 1772, 1771 and 1764, www.addgene.org/pgvec1). Expression of hTERT-HA leads to telomerase activity but due to an HA tag on its C terminus lacks the ability to act in telomere elongation or maintenance (15).

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1. Transduction with TERT-HA did not immortalise NHUC indicating that telomere-dependent effects are required for immortalization. Crosses represent wildtype hTERT, triangles represent hTERT-HA and circles, 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.

Fig 2.

Characterisation of TERT-NHUC A). Quantification of telomerase activity in TERT-NHUC. Units shown are mean ratio of telomerase activity relative to the empty vector transduced isogenic NHUC. Data is derived from duplicate analyses. B) Relative quantification of expression of BMI1 (grey shaded columns), SIRT1 (black shaded columns) and NDN (unshaded columns) in TERT-NHUC soon after transduction with hTERT. Expression is calculated relative to SDHA and normalised to a reference sample of pooled NHUC. C) The proposed role of NDN in controlling expression of a subset of genes altered in TERT-NHUC. All interactions are known effects described in the literature. TERT-NHUC had attenuated NDN expression. Downregulation of NDN-dependent inhibition of E2F-1 could lead to an increase in the E2F-1 target gene BMI-1. Upregulation of BMI-1 was detected in TERT-NHUC with downregulated NDN. BMI-1 forms part of the polycomb repressor complex 1 (PRC1) which modulates expression of a number of polycomb gene (PCG) targets. Multiple PCG targets were downregulated in TERT-NHUC. D) Confirmation at 100PD of downregulated expression of NDN (unshaded columns) and upregulated expression of BMI1 (black shaded columns) in all three TERT-NHUC lines compared to 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

Fig 3. Ingenuity Pathway Functional Analysis identified the A) Diseases and Disorders and B) Cellular functions, that were most significant to the gene expression signature set of TERT-NHUC. The Vertical line on each chart represents the threshold level of $p=0.05$. Data from cells soon after transduction with hTERT (< 18PD) is shown in black and data from cells at 100PD in grey.

Fig 4.

A) Expression of differentiation-associated uroplakin II was induced in TERT-NHUC (at 100PD) after treatment with the PPAR γ agonist Troglitazone and the EGFR inhibitor PD153035. The magnitude of the response was less than that seen in isogenic NHUC. Data shows Log10 RQ (relative quantification) relative to SDHA control gene and normalised 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 (arrowed). Size bars indicate 200 μ m.

Fig 5.

A) Maximum saturation density obtained in TERT-NHUC soon after transduction with hTERT (< 18PD) was less than that seen in TERT-NHUC that had undergone approximately 100PD. TERT-NHUC soon after transduction with hTERT had a saturation density similar to that of NHUC. Error bars indicate range of values within triplicate donors. B) TERT-NHUC (at 100PD) reached a higher density before contact inhibition compared to their matched NHUC. Data shown is mean of cells from the three donors. Filled symbols represent TERT-NHUC (at approximately 100PD) and open circles NHUC. Error bars indicated standard deviations of values from triplicate donors.

Fig 6.

Expression of candidate telomerase signature genes was altered in UC cell lines and tumors. Data shows Log10 RQ relative to pooled NHUC cDNA and normalised to SDHA. The reference sample (pooled NHUC) therefore has a RQ value =1 and Log10 RQ value of 0. Samples with a Log 10 RQ value < 0 have downregulation of expression and those with Log10 RQ > 0, overexpression relative to pooled NHUC. Where transcript was undetectable after 40 cycles of PCR, Log10 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 non-malignant 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, E) NME5. Tumors are ranked in order of expression of each gene.

Table 1. Genes whose expression was modulated in NHUC soon (<18 PD) after expression of hTERT. Genes are ranked in order of average fold change. Genes previously identified as polycomb targets are highlighted with asterisks and "Telomerase signature" genes are shaded. Genes with associations with differentiation are shown in italics.

TERT-NHUC					
Probe set	A	B	N	Symbol	Description
DOWNREGULATED 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, 36kDa
204818_at	-7.23	-8.17		HSD17B2	hydroxysteroid (17-beta) 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	non-metastatic cells 5, protein expressed in
205319_at	-4.22	-4.29		PSCA	prostate stem cell antigen
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					
203913_s_at	-3.97	-5.29		HPGD*	hydroxyprostaglandin dehydrogenase 15-(NAD)
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, beta 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 (alpha (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 homolog 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, beta 1
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
UPREGULATED GENES					
202237_at		37.34	11.82	NNMT*	nicotinamide N-methyltransferase

1555271_a_at	12.59	8.68	12.91	TERT	telomerase reverse transcriptase
235057_at	5.71	5.67	8.35	ITCH*	itchy homolog E3 ubiquitin protein ligase
206230_at	4.37	7.46		LHX1*	LIM homeobox 1
244075_at	3.88	7.64		HSD17B7P2	hydroxysteroid (17-beta) 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-2 alpha
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 D3) 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, eta
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), beta 1, 88kDa
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 gamma 11
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 1, alpha
225662_at		4.09	2.61	ZAK	sterile alpha motif and leucine zipper containing kinase AZK
214806_at		3.57	2.89	BICD1	bicaudal D homolog 1 (Drosophila)
201040_at	2.07	2.04		GNAI2	guanine nucleotide binding protein, alpha inhibiting activity polypeptide 2
225961_at		2.84	3.32	KLHDC5	kelch domain containing 5

Table 1.

Table 2. Genes whose expression was modulated in TERT-NHUC at 100PD. Genes are ranked in order of average fold change. Genes previously identified as polycomb targets are highlighted with asterisks and "Telomerase signature" genes are shaded. Genes with associations with differentiation are shown in italics.

TERT-NHUC					
Probe set	A	B	N	Symbol	Description
DOWNREGULATED 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 homolog (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 (beta)
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	non-metastatic 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 metalloproteinase 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 homolog 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-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 4
214077_x_at		-3.70	-3.20	MEIS4	Meis1, myeloid ecotropic viral integration site 1 homolog 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) homolog, 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)

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
UPREGULATED 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, beta 4
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, alpha 1
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

Table 2.