7-2011

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Abstract

Background

Dyskerin, which is an important component of the telomerase complex and is needed for normal telomerase activity, is frequently overexpressed in neoplasia. Dyskerin also plays an essential role in ribosome biogenesis. Because protein synthesis increases during tumorigenesis, this led us to hypothesize that dyskerin expression would be upregulated independently of the cell immortalization mechanism.

Methods

Dyskerin and telomerase reverse transcriptase (TERT) expression were examined in oral squamous cell carcinomas (OSCC) and patient-matched controls, as well as in a panel of telomerase-positive and telomerase-negative cells. Antisense inhibition of TERT was used to test the effects of downregulation of telomerase on dyskerin expression.

Results

Dyskerin was frequently overexpressed in OSCC and in immortalized and transformed keratinocytes relative to primary cells, independently of TERT and telomerase activity. Instead, dyskerin expression strongly correlated with cell proliferation rates.

Conclusions

The role of dyskerin in tumorigenesis does not correlate with its function within the telomerase complex.

Keywords

TERT, ALT, telomere, ribosome biogenesis, oralcarcinogenesis

CORRELATION OF DYSKERIN EXPRESSION WITH ACTIVE PROLIFERATION INDEPENDENT OF TELOMERASE

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Accepted 9 July 2010
Published online 8 December 2010 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/hed.21579

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Methods. Dyskerin and telomerase reverse transcriptase (TERT) expression were examined in oral squamous cell carcinomas (OSCC) and patient-matched controls, as well as in a panel of telomerase-positive and telomerase-negative cells. Antisense inhibition of TERT was used to test the effects of downregulation of telomerase on dyskerin expression.

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Conclusions. The role of dyskerin in tumorigenesis does not correlate with its function within the telomerase complex.
 Head Neck **33:** 1041–1051, 2011

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Telomeres are specialized nucleoprotein structures that cap and protect the ends of eukaryotic chromosomes, thereby preserving genomic integrity.[1] To extend cellular replicative lifespan, human tumors use either 1 of 2 mechanisms for telomere maintenance. The most common is mediated by the telomerase ribonucleoprotein (RNP) complex, which replicates telomeres through the addition of a variable number of hexanucleotide TTAGGG repeats to the chromosome ends.[2–4] The other mechanism is the telomerase-independent, alternative lengthening of telomeres (ALT) pathway, which mediates telomere replication by homologous DNA recombination; the exact mechanisms remain poorly understood.[5]

Additional Supporting Information may be found in the online version of this article.

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Telomerase activity is undetectable in most somatic cells except for those that are highly regenerative and undergo constant self-renewal, including oral keratinocytes. However, upregulation of telomerase activity is common to most forms of neoplasia, including 80% to 90% of oral squamous cell carcinomas (OSCC); telomerase levels increase early in oral carcinogenesis.[3] In contrast, only a small subset of tumors, and only rarely OSCC and other head and neck squamous cell carcinomas, use the ALT mechanism for telomere homeostasis.[6]

The telomerase RNP has 2 core components, including telomerase reverse transcriptase (TERT), which is the catalytic subunit, and telomerase RNA (TERC), which is the template used to prime telomere replication.[4] But several additional factors are also required for full activity of the complex, including dyskerin (encoded by the *DKC1* gene), which directly binds to and stabilizes TERC within the complex.[2,7,8]

Dyskerin is a highly conserved and vital, 58-kDa nucleolar RNA binding protein that is required for the biogenesis of a subtype of RNP.[7,9] Together with 3 other conserved factors, dyskerin forms a core complex that binds noncoding H/ACA RNA, including TERC and subsets of small nucleolar RNA (snoRNA) and Cajal body RNA.[9] There are more than 100 known human H/ACA RNA, and the specific function of each H/ACA RNP is dependent on the RNA that it incorporates. Loss of dyskerin function reduces steady-state levels of TERC, decreases telomerase activity, and leads to premature telomere shortening.[7,8] However, dyskerin has also been shown to play important roles in ribosome biogenesis and function. Through binding to H/ACA snoRNA, dyskerin is required for posttranscriptional processing of precursor rRNA.[10,11] A recent report suggests that dyskerin also regulates translation of a subset of mRNAs via an internal ribosome entry site.[12] Thus dyskerin is a key component of 2 molecular pathways that are fundamentally important to the tumor cell phenotype; neoplasms require not only a mechanism for telomere homeostasis, but also have a high

demand for protein synthesis.[4,13] To that end, *DKC1* mRNA is frequently and significantly overexpressed in a variety of human cancers, including lymphoma, melanoma, neuroblastoma, and adenocarcinomas of the breast, colon, and ovary.[14–21]

We and others previously demonstrated that human dyskerin expression is increased in experimental conditions that promote cell growth and proliferation.[22–24] Gu et al[25] recently reported that a mutant form of murine dyskerin significantly impaired normal cell proliferation by a mechanism that was dependent on the presence of a functional telomerase complex. They observed that in the absence of either Tert or Terc, the dyskerin-mutant and wild-type cells grew similarly. However, it is unknown whether dyskerin also requires an active telomerase complex to exert its influence in human cells.

In this study, we investigated the dependency of the interaction between dyskerin and telomerase in human cells and during tumorigenesis. TERT expression is commonly used as a surrogate marker for telomerase.[26,27] However, we show for the first time that dyskerin expression is frequently overexpressed in sporadic OSCC and that upregulation of dyskerin does not require the presence of TERT or an active telomerase complex. Instead, dyskerin expression correlates with active cell proliferation.

MATERIALS AND METHODS

Cell Culture. Primary oral keratinocytes OKF4, OKF6, and their respective TERT-immortalized derivatives, OKF4-TERT1F and OKF6-TERT2, were cultured in Keratinocyte-Serum Free Media (K-SFM; Invitrogen, Carlsbad, CA) supplemented with bovine pituitary extract 25 μg/mL, epidermal growth factor 0.2 ng/mL, and $CaCl_2$ 0.4 m*M*. OKF4 and OKF6 cells and their
TERT-derivatives have been well characterized.[28] These cells originate from the normal oral epithelium of 2 distinct individuals. K-SFM is not designed to grow cells to high density. Thus, to enable us to expand the cultures to densities high enough for our assays, cells were plated in rapid growth media composed of a 1:0.5:0.5 mix of K-SFM: calcium-free, glutamine-free Dulbecco's minimal essential mediun (Invitrogen): Ham's F-12 (Invitrogen), supplemented with 0.2 ng/ml epidermal growth factor, 25 μg/ml bovine pituitary extract, 0.75 m*M* L-glutamine, and 0.2 m*M* $CaCl_2$. OKF6-Δp53D1 cells were grown using Defined K-SFM (Invitrogen) with a pre-formulated growth supplement supplied by the manufacturer. OKF6-Δp53D1 is a telomerase-negative, ALT-immortalized derivative of OKF6 and has been previously described.[29] The OSCC cell line, UM-SCC-1, and U2OS human osteosarcoma cells (purchased from American Type Culture Collection, Manassas, VA) were grown in D-MEM containing high glucose (4,500 μg/mL), 1 mmol/L of glutamine, and 10% fetal bovine serum. All media formulations were supplemented with 100 IU/mL penicillin and 100 IU/mL streptomycin.

MRNA Analysis and Quantitation. Thirteen distinct, fresh frozen OSCC and patient-matched normal oral mucosal tissue samples were obtained from the University of Pennsylvania Oral, Head and Neck Tumor, Tissue and Saliva Bank with appropriate informed consent and institutional regulatory approval. Total RNA was extracted from 20 to 110 mg of tissue using TRIzol Reagent (Invitrogen), as per the manufacturer's recommendations. For RNA extraction from cell lines, we used the Rneasy technique (Qiagen, Valencia, CA). Quantitative RT-PCR was performed as previously outlined.[22] Quantitect Primer Assays (Qiagen) for *DKC1*, *TERT*, β-actin and Tata binding protein (*TBP*) were used, respectively. Relative *DKC1* and *TERT* expression levels, respectively, were normalized to *TBP* or actin using the $2^{-\Delta\Delta CT}$ method. For *DKC1* and *TERT* quantitation from the tissue samples, the reactions were performed three times. For analysis of cellular mRNA expression, 3 independent samples were used.

Western Blot Analysis. Whole cell lysates were extracted as described previously.[22] Polyclonal antibodies against dyskerin, β-actin, GAPDH and secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All Western blot analyses were performed at least three times. NIH Image J was used to quantitate relative dyskerin expression.

Cell Proliferation and Cell Cycle Distribution Assays. Equivalent numbers of cells were seeded and cell proliferation was assessed using the Cell Proliferation Reagent WST-1 (Roche Applied Science, Indianapolis, IN). Absorbance was measured using a Multiskan Ascent microplate photometer (ThermoScientific Labsystems, Hudson, NH). The relative absorbances were measured for up to 4 days after plating of the cells. For cell cycle analysis, cells were washed and fixed for at least 60 minutes with cold 80% ethanol. The cells were then resuspended in 1 mL cold phosphate-buffered saline solution and stained with 10 μg/mL propidium iodide containing 1 mg/ml RNase A (Sigma-Aldrich) for 30 minutes. Samples were analyzed on a Becton-Dickinson Facstar^{PLUS} flow cytometer (BD Biosciences). A minimum of 5000 events were collected on each sample; cell cycle analysis was performed with Modfit (Verity Software House; Topsham, ME).

TERT Inhibition and Telomerase Repeat Amplification Protocol Assay. A *TERT* antisense DNA oligonucleotide, 5′-TTGAAGGCCTTGCGGACGTG-3′ (TERT-ANTI) and a control, nonspecific oligonucleotide, 5′-TAAGCTGTTCTATGTGTT-3′ (TERT-CTRL), were synthesized by Invitrogen and prepared as 1 m*M* stocks in sterile water. Both molecules were

protected by phosphorothioate linkages at their terminal ends. The TERT-ANTI and TERT-CTRL sequences were previously described in studies by Kraemer et al.[30]

OKF6-TERT2 cells were seeded in 24-well plates and grown for 2 days. On the third day, cells were transfected in quadruplicate with either TERT-ANTI or TERT-CTRL 10 μ*M* with Lipofectamine 2000 (Invitrogen) as the carrier. Twenty-four hours after transfection, the cells were pelleted, washed with phosphate-buffered saline solution, and resuspended in ice-cold CHAPS lysis buffer (0.5% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate [CHAPS], benzamidine 0.1 m*M*, 10% glycerol, Tris-HCl 10 m*M*, pH 7.5, $MgCl_2$ 1 m*M*, 1 EGTA m*M*, and 2-mercaptoethanol 5 m*M*). Total protein concentrations were measured with the BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL). Each of the respective protein extracts 5 μg were then used to assess telomerase activity levels with the TRAPeze RT Telomerase Detection Kit (Millipore, Billerica, MA) as per the manufacturer's recommendations. This highly sensitive assay makes use of fluorimetric probes, which allow for real-time quantification of telomerase activity. In a parallel set of experiments, the cells were similarly transfected with the TERT-ANTI and TERT-CTRL oligonucleotides, except the cells were harvested for total RNA. All transfection experiments were performed twice. Where indicated, OKF6-TERT2 and OKF6-Δp53D1 cells were harvested in exponential growth phase and assayed for telomerase activity. However, for illustration purposes, the TRAP PCR products were resolved on a precast 10% Novex TBE polyacrylamide gel (Invitrogen).

Mutation Screening. Genomic DNA was extracted from each of the 13 OSCC samples, as previously described.[31] With previously published primer sets[32] with some modifications (Supporting Information Table 1), the complete translated and untranslated regions of the *DKC1* gene were amplified. This included amplification of all 15 exons and associated intron splice sites. The reaction conditions were genomic DNA 40 to 100 ng, each primer 10 pmol; $MgCl_2$ 4–8 m*M*, each dNTP 2 m*M*, 0.5 unit of AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA), and the manufacturer's buffer in a 50-μL final volume. Amplifications were carried out as follows: an initial denaturation for 10 minutes at 95°C followed by 35 cycles of 94°C for 30 seconds, 55°–63°C for 30 seconds, 72°C for 1 minute, and a final extension for 10 minutes at 72°C. The annealing temperatures and $MgCl_2$ concentrations were optimized for each specific primer set. All PCR products were analyzed by agarose gel electrophoresis, purified with either the QIAQuick PCR Purification Kit or QIAQuick Gel Extraction Kit (Qiagen), and then sequenced on the ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Sequencing was done in the sense and anti-sense directions. All sequence analyses were performed with Sequence Analysis 3.7 and Sequencher 4.0.5 software (Gene Codes Corporation, Ann Arbor, MI).

Correlation of DKC1 and TERT Expression in Human Cell Lines. Relative *DKC1* and *TERT* mRNA expression levels were correlated in 93 unique, transformed human cell lines using the NCI60 on U133A dataset available from the open access Gene Expression Atlas (http://symatlas.gnf.org/SymAtlas).[33] As we previously described,[22] we first log-transformed the average relative mRNA expression levels for both genes. Then we performed a linear regression analysis of the log transformed values, characterizing *DKC1* as the dependent variable and *TERT* expression as the independent variable. As controls, we also examined the relationship between *DKC1* and *MYC*, *NHP2*, *MKI67* and *CASP3*. All statistical analyses were performed with Sigma Plot 11.0 (SYSTAT Software, San Jose, CA).

RESULTS

Inhibition of TERT and Telomerase Activity Does Not Alter Dyskerin Expression. Similar to murine cells, it is possible that in normal human cells that exhibit appreciable levels of telomerase activity, dyskerin expression may be intimately linked to TERT and telomerase. Moreover, TERT promotes and sustains epithelial proliferation not only by telomere maintenance, but also by a telomere-independent mechanism through transcriptional modulation of various growth promoting genes.[34,35] A recent study showed that *DKC1* mRNA expression was significantly increased in association with TERT overexpression in normal human mammary epithelial cells.[26] This prompted us to determine whether dyskerin expression was dependent on TERT in immortalized oral keratinocytes.

For these studies, TERT-immortalized human oral keratinocyte (OKF6-TERT2) cells were transfected with either *TERT* antisense DNA oligonucleotide 10 μ*M* or a control, nonspecific oligonucleotide. Twenty-four hours after transfection, the cells were harvested, and telomerase activity levels were assayed by quantitative real-time RT-PCR. Because of technical difficulties, we were unable to reliably assess TERT expression by Western blot. However, although telomerase levels were decreased by almost 50%, and *TERT* mRNA expression reduced by more than 40% relative to the control cells, there was no effect on *DKC1* mRNA expression (Figure 1A, B). Antisense inhibition of *TERT* in telomerase-positive H1299 lung adenocarcinoma cells also did not affect *DKC1* expression (not shown). Thus our findings reinforce those of a previous report, which demonstrated no change in dyskerin expression after antisense inhibition of *TERT* and telomerase activity in oral cancer cells.[2]

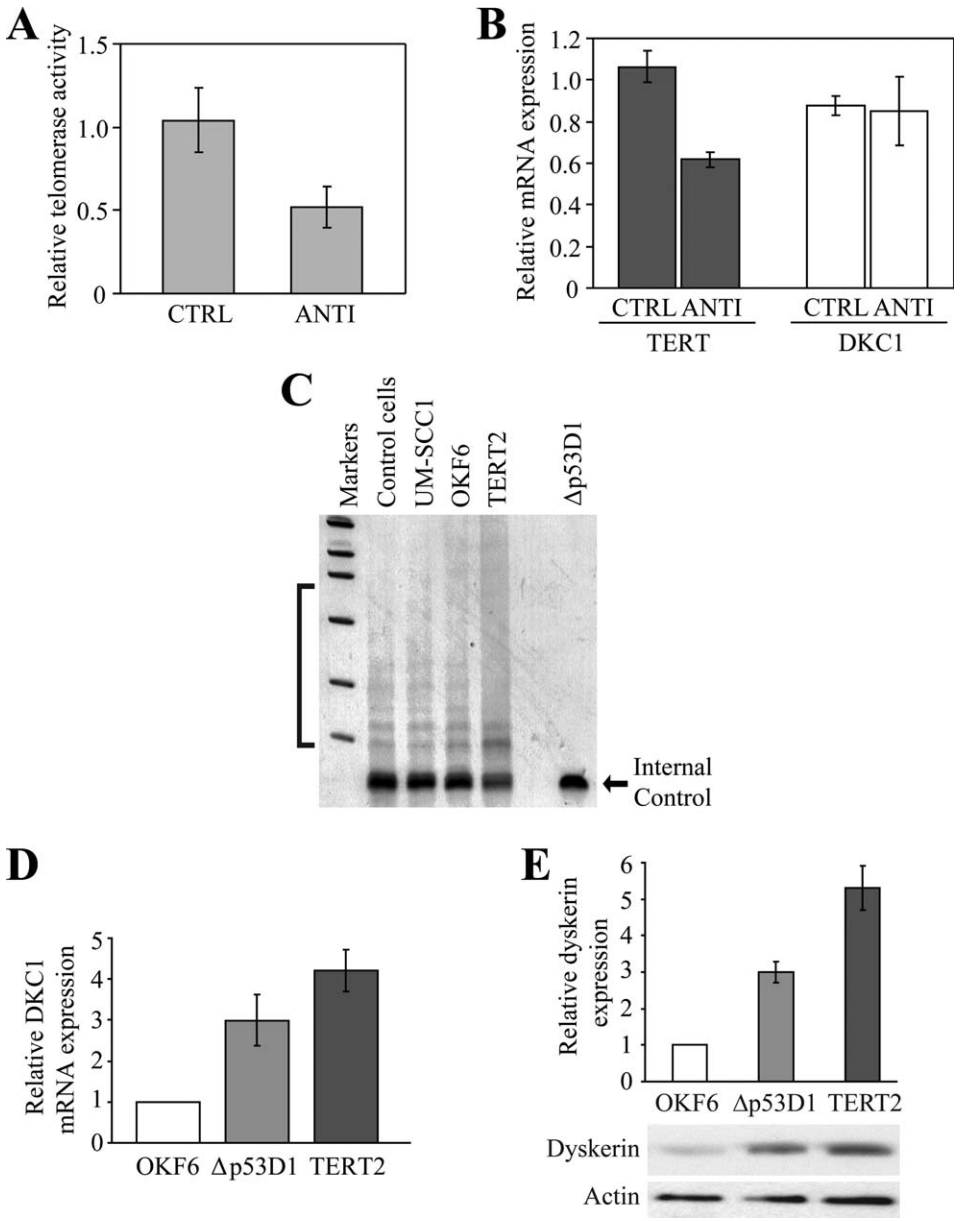

FIGURE 1. Upregulation of dyskerin in immortalized oral keratinocytes is not dependent upon TERT. (**A**) OKF6-TERT2 cells were transfected with either *TERT* antisense DNA oligonucleotide (ANTI) 10 μ*M* or a control, nonspecific oligonucleotide (CTRL). Twenty-four hours later, relative telomerase activity levels were assessed by quantitative RT-PCR and normalized to telomerase levels in the untransfected OKF6-TERT2 cells. (**B**) *TERT*/β-actin and *DKC1*/β-actin expression was normalized to levels of the respective transcripts in the OKF6-TERT2 cells. The *solid bars* represent the mean values of 4 transfections conducted in 1 representative experiment; *error bars* denote the standard deviations. (**C**) A qualitative TRAP assay was used to measure telomerase activity in exponentially-growing OKF6-TERT2 and OKF6-Δp53D1 cells. The characteristic laddering effect was observed in the OKF6-TERT2 and other telomerase-expressing cells, including UM-SCC1 oral cancer cells and a control cell pellet included with the kit, but not in the ALT-immortalized OKF6-Δp53D1 cells. The internal control served as a control for the PCR reaction. (**D**) *DKC1* mRNA expression was assessed by quantitative RT-PCR in OKF6, OKF6-Δp53D1 and OKF6-TERT2 cells. (**E**) Dyskerin protein levels were quantitated relative to β-actin using NIH Image J.

Dyskerin Is Upregulated in Immortalized Keratinocytes Independently of Telomerase Activity. Telomerase-positive OKF6-TERT2 and ALT-immortalized OKF6-Δ53D1 cells are derived from the same parental OKF6 primary oral keratinocyte strain.[28,29] OKF6-Δ53D1 cells do not exhibit telomerase activity and basal *TERT* mRNA levels are decreased relative to the parental OKF6 keratinocytes (Figure 1C, and not shown; see reference 29). However, quantitative RT-PCR revealed *DKC1* mRNA expression to be elevated

at least 3-fold in proliferating OKF6-Δ53D1 and more than 4-fold in OKF6-TERT2 cells relative to OKF6 cells (Figure 1D). Protein expression was also increased approximately 3-fold in the OKF6-Δ53D1 cells and at least 5-fold in OKF6-TERT2 relative to the primary cells (Figure 1E). We cannot exclude the possibility that TERT may at least partially contribute to regulation of *DKC1* expression in human cells. Nonetheless, these data indicate that upregulation of dyskerin in immortalized oral keratinocytes is not dependent on TERT expression or the presence of telomerase activity.

Dyskerin Expression Correlates with the Rate of Active Cell Proliferation. We and others previously demonstrated that human dyskerin expression is increased in experimental conditions that promote cell growth and proliferation.[22–24] Because dyskerin contributes to other important cellular processes that are typically upregulated during active cell growth and proliferation,[9,11] we next asked whether dyskerin expression correlates with the rate of cell proliferation. For this analysis, we examined 2 distinct, exponentially-growing primary oral keratinocyte strains, OKF4 and OKF6, their respective immortalized derivatives, OKF4-TERT-1F, OKF6-TERT2 and OKF6-Δ53D1, and telomerase-positive UM-SCC1 oral cancer cells. To our knowledge, there is no ALT-immortalized derivative of OKF4 cells, and we are not aware of any telomerase-negative oral cancer cell lines. Thus we also included the ALT-immortalized U2OS human osteosarcoma cell line in the assay. U2OS cells do not express either TERT or TERC and do not exhibit any telomerase activity (not shown; see reference 36).

The immortalized cells proliferated more rapidly than their primary counterparts, whereas the transformed cells were the fastest growing (Figure 2A). Relative dyskerin expression strongly correlated with cell proliferation rates ($r^2 = 0.927$, $p < .001$) insofar as there was low basal expression in the slow-growing primary keratinocytes, significantly increased levels in the immortalized cells and even more so in the transformed cell lines (Figure 2B–D). We note that dyskerin expression was greater in OKF6-Δ53D1 cells compared with the faster-growing OKF4-TERT1F cells (Figure 2D). Moreover, the observation that dyskerin expression was significantly decreased after contact inhibition of OKF6 and OKF6-TERT2 cells reinforces the notion the dyskerin expression is closely associated with active cell proliferation and not telomerase status (Figure 2D and E, and not shown).

DKC1 is Upregulated and Not Mutated in Patient-Derived OSCC. Our in vitro studies indicated that dyskerin expression was upregulated in actively proliferating oral keratinocytes, irrespective of the cell immortalization mechanism. However, although *DKC1* mRNA is significantly overexpressed in several cancer types, its status in OSCC had not been previously established. Thus we initially analyzed OSCC microarray data, a portion of which was previously published in a study aimed at identifying gene signatures that are predictive of regionally metastatic OSCC.[31] In culling the dataset, we found that *DKC1* mRNA expression was significantly upregulated ($p < .023$) in OSCC ($n = 49$) relative to normal, patient-matched mucosal control subjects ($n = 16$; Figure 3A).

We then validated *DKC1* expression by quantitative RT-PCR in a subset of the tumors and controls. Of the samples tested, *DKC1* mRNA levels were variably increased in 9/13 tumors (69%, $p < .03$) relative to the patient-matched controls (Figure 3B); the transcript was ubiquitously expressed in all paired samples. Because our sample set was limited, we were unable to reliably correlate *DKC1* expression with any demographic or prognostic variables.

Germline mutations in *DKC1* give rise to the X-linked recessive form of dyskeratosis congenita.[32,37] This is an unusual disorder associated with a wide-ranging phenotype, including cancer susceptibility; OSCC is among the most common cancers that these individuals develop.[38] To our knowledge, none of the tumor samples were derived from individuals with this disorder; most of the patients had classic histories of long-standing smoking and/or alcohol intake. Nonetheless, we proceeded to analyze the 13 tumors for possible *DKC1* mutations. Using genomic DNA extracted from each of the tumors, we bidirectionally sequenced all 15 *DKC1* exons and their associated intron splice sites. In comparing the sequences of the amplicons to those of the known wild-type *DKC1* exon sequences, mutations were not observed in any of the tumors (data not shown). However, a synonymous single nucleotide polymorphism (SNP) was identified in 1 tumor. This SNP, Ex14+93G>A, L477L, was confirmed by sequence analysis of the patient-matched normal tissue sample. Although a matched blood sample was not available for further confirmation, this SNP has been identified in healthy individuals and is not believed to be of any functional or clinical significance.[37,39]

Neither protein nor a sufficient amount of fixed tissues were available from any of the patient samples described above. Thus we used immunohistochemistry to evaluate dyskerin protein expression from a different series of OSCC and preneoplastic oral epithelial lesions obtained from formalin-fixed, paraffin-embedded oral biopsy samples. There was at least some evidence of dyskerin expression in all of the tissue samples tested (benign and malignant), and there was wide intraspecimen and interspecimen variability with respect to the intensity and extent of nucleolar staining (data not shown). Thus differences in dyskerin expression between normal, dysplastic, and transformed epithelium could not be reliably

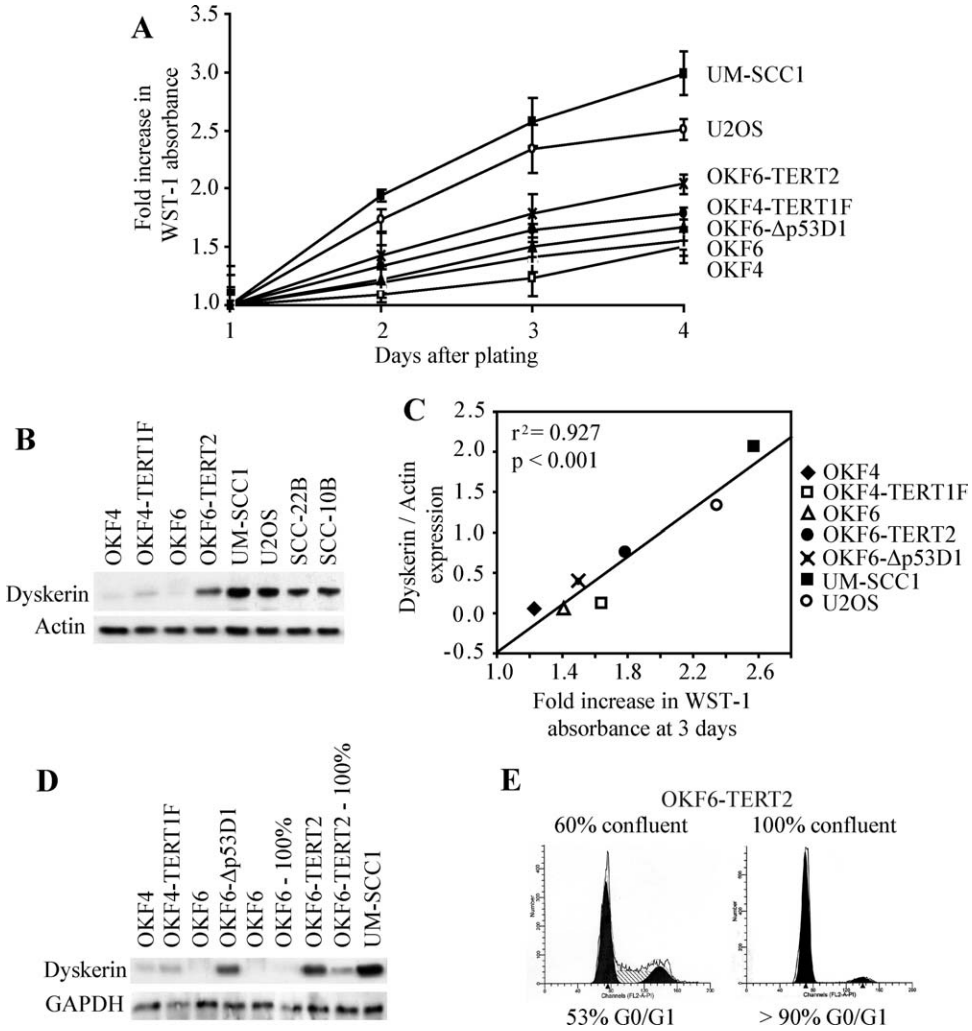

FIGURE 2. Dyskerin expression correlates with the rate of cell proliferation. (**A**) Fold increase in cell proliferation rates were determined by normalizing the relative WST-1 absorbance for each of the respective cell lines to those measured on the first day after plating. Error bars denote standard deviation from triplicate wells for each time point. Experiments were repeated twice with similar results. (**B**) Cells were harvested in log growth phase (3 days after initial plating) and total cell lysates were subjected to Western blot. Dyskerin was increased in the TERT-immortalized cells relative to their parental cells, and even more so in the transformed cells. For comparison purposes, dyskerin expression was also examined in the transformed squamous epithelial cell lines, SCC-10B and SCC-22B. (**C**) Dyskerin expression was normalized to actin, and then compared with the fold increase in WST-1 absorbance 3 days after initial plating. A linear regression analysis showed strong correlation ($r^2 = 0.927$, $p < 0.001$) between relative dyskerin levels and cell proliferation. (**D**) Dyskerin expression was reduced in contact-inhibited OKF6 and OKF6-TERT2 cells (100%) relative to the exponentially growing cells (50%–60% confluent). Except where indicated, all protein lysates were obtained from actively proliferating cells. (**E**) Cell cycle distribution of OKF6-TERT2 cells at 60% and 100% confluency. OKF6 cells showed a similar profile (not shown).

quantitated. Similarly, other investigators were also unable to reliably compare dyskerin expression in tumor tissues relative to controls through use of immunohistochemical methods.[17,20] This suggests that evaluation of dyskerin expression by immunohistochemistry may not be of any practical use.

DKC1 and TERT mRNA Expression Do Not Correlate in OSCC or in Transformed Cell Lines. Telomerase levels are increased in the vast majority of human cancers, including OSCC.[2,3] However, although *TERT* and *DKC1* mRNA are significantly upregulated in many of the same cancer types, the interrelationship between *DKC1* and *TERT* in tumor tissues is not known.

Using the same 13 patient-derived OSCC samples and matched normal mucosal controls described above, we found that *TERT* mRNA was significantly increased in 11/13 tumors relative to their respective controls ($p < .001$; Figure 3C). However, there was no correlation between the relative increase in tumor expression levels of *TERT* and *DKC1* ($r^2 = 0.07$, $p = .18$; Figure 3D). When normalized to the expression of Tata binding

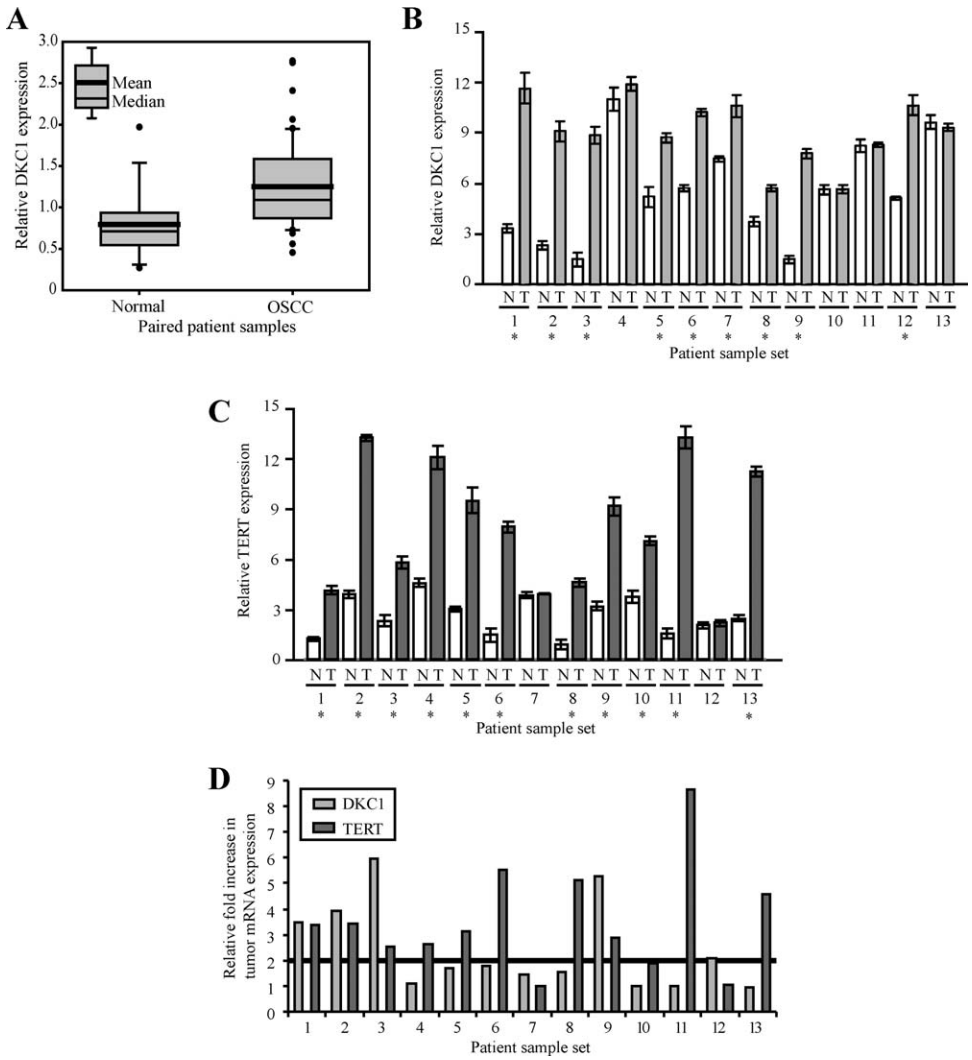

FIGURE 3. *DKC1* and *TERT* mRNA expression do not correlate in patient-derived OSCC. (**A**) Box plot illustrating *DKC1* upregulation in OSCC ($n = 49$) relative to matched normal controls ($n = 16$; $p<0.023$). Data represent the average relative signals for *DKC1* from two independent arrays.[31] (**B**) *DKC1* mRNA levels were significantly ($p < .03$) increased (*) in 9/13 OSCC (light grey bars) relative to patient-matched normal mucosal controls (white bars). (**C**) *TERT* mRNA levels were significantly ($p < .001$) increased (*asterisk*) in 11/13 OSCC (*dark gray bars*) relative to the controls (*white bars*). (**D**) There was no correlation between the relative fold increase in *TERT* and *DKC1* mRNA expression in the tumors. With an arbitrary threshold of a 2-fold increase in tumor mRNA expression relative to the matched normal control (*horizontal line*), 10/11 high *TERT* expressing tumors surpassed the threshold, whereas only 4/9 high DKC1 expressing tumors were increased beyond this threshold. *DKC1* and *TERT* mRNA levels were measured relative to *TBP* mRNA by quantitative RT-PCR with the $2^{-\Delta\Delta Ct}$ method. *N*, Normal; *T*, tumor.

protein (*TBP*) mRNA, there was also no correlation between the expression of *TERT* and *DKC1* in the tumor tissues ($r^2 = 0.0089$, $p > .5$; data not shown). *TBP* mRNA levels remained relatively constant in all the tissue samples tested.

Because our cohort was limited, we proceeded to evaluate *TERT* and *DKC1* expression levels in a panel of 93 unique human cell lines derived from a variety of tumor types. To do this, we analyzed 1 of the gene microarray datasets (NCI60 on U133A, gcRMA) available from the open access Gene Expression Atlas.[33] To our knowledge, at least 5 of the cell lines in this dataset are ALT-immortalized (Supporting Information Table 2). These include U2OS, SAOS2 (osteosarcoma), Hs578T (breast adenocarcinoma), NCI H226 (non-small cell lung carcinoma), and IOSE80 (ovarian adenocarcinoma). Most of the remaining lines are known to be telomerase-positive.

A linear regression analysis of the log-transformed expression values revealed no correlation ($r^2 = 0.0048$, $p > .05$) between *TERT* and *DKC1* mRNA expression (Figure 4A). A similar result was obtained using the Human Gene Atlas GNF1H, gcRMA dataset, which is derived from a collection of 78 normal

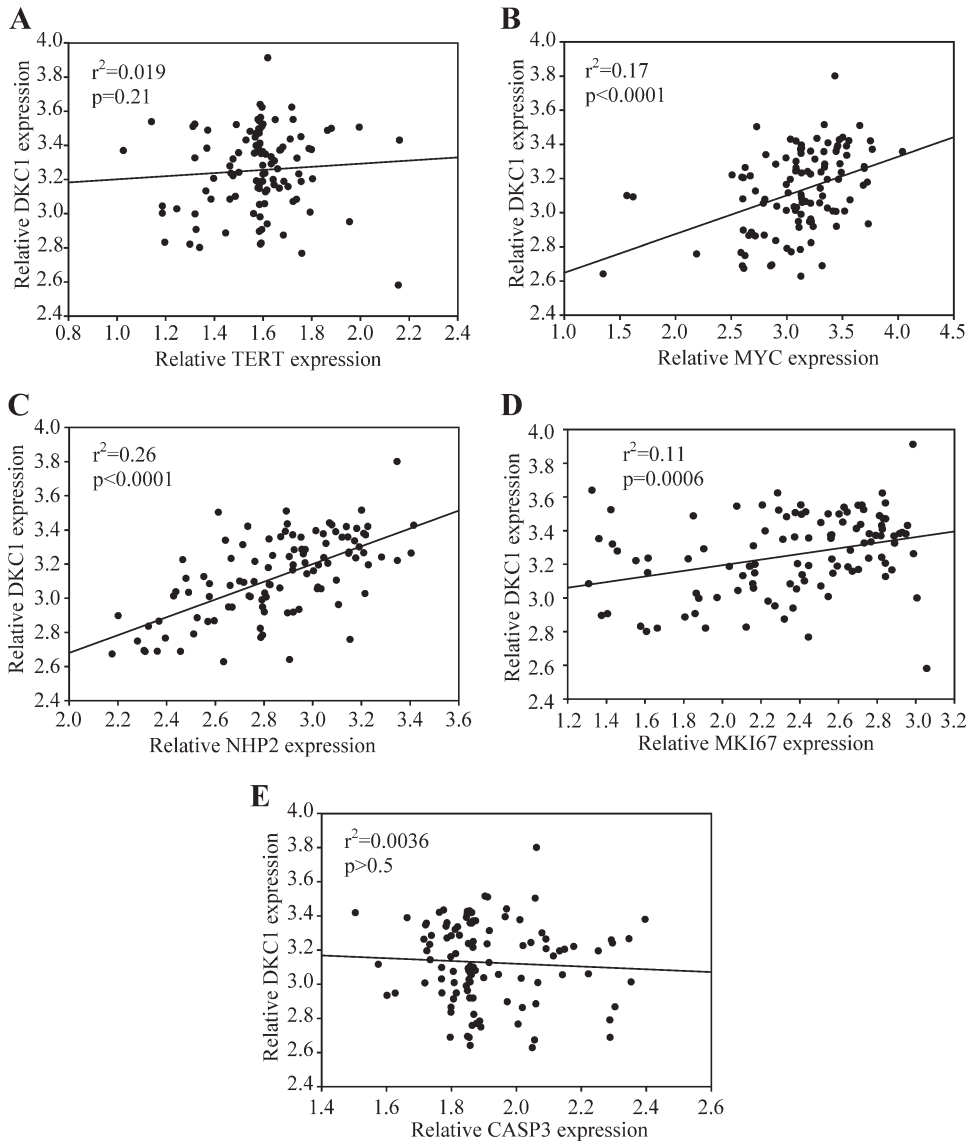

FIGURE 4. *DKC1* and *TERT* mRNA expression do not correlate in transformed human cell lines. (**A**) A linear regression analysis of the log-transformed expression values revealed no correlation ($r^2 = 0.019$, $p = .21$) between *TERT* and *DKC1* mRNA expression. (**B**) *DKC1* and *MYC* expression showed significant correlation ($r^2 = 0.17$, $p < .0001$). (**C**) Relative levels of *DKC1* and its H/ACA snoRNP binding partner, *NHP2*, also strongly correlated ($r^2 = 0.26$, $p < .0001$). (**D**) *DKC1* and the cell proliferation marker *MKI67* also showed correlation ($r^2 = 0.11$ $p = .0006$). (**E**) There was no correlation between *DKC1* and *CASP3* expression ($r^2 = 0.0036$, $p > .5$); *CASP3* was randomly chosen as a factor that does not have any known relationship to *DKC1*. The raw data and corresponding log transformations for each of the genes are listed in Supporting Information Table 2.

and transformed human tissues and cell lines (not shown). As a positive control for the in silico analysis, we also examined the relationship between *DKC1* and *MYC* expression. MYC promotes cell proliferation and oncogenesis, and its amplification is common to many different tumor types.[22,23] As we have previously shown,[22] *DKC1* and *MYC* expression showed significant correlation ($r^2 = 0.17$, $p < .0001$; Figure 4B). Similarly, the relative levels of *DKC1* and its H/ACA RNP binding partner, *NHP2*/*NOLA2*, also strongly correlated ($r^2 = 0.26$, $p < .0001$; Figure 4C). There was also significant correlation between *DKC1* and *MKI67* (Ki-67) expression ($r^2 = 0.11$, $p = .0006$; Figure 4D). Ki-67 is required for normal ribosomal RNA synthesis and is widely used as a marker of cell proliferation; high tumor levels of Ki-67 may be associated with poor prognosis in some cancer types.[40] There was no correlation between *DKC1* and *CASP3* expression ($r^2 = 0.0036$, $p > .5$; Figure 4E); *CASP3* was randomly chosen as a factor that does not have any known relationship to *DKC1*. Together, these cumulative findings suggest that there is no overt

relationship between *DKC1* and *TERT* gene expression in human cancer. Instead, as suggested by our in vitro data, *DKC1* expression correlates with other factors that are known to be upregulated in actively proliferating cells irrespective of telomerase status.

DISCUSSION

The mechanisms by which *DKC1* mRNA levels are increased in neoplasia, as well as during active cell proliferation remain to be clarified. Dyskerin binds to NHP2 in H/ACA RNPs and to TERT within the telomerase complex.[9,14] However, although *DKC1* and *NHP2* expression showed strong correlation in the NCI60 dataset, there was no relationship between *DKC1* and *TERT*. We have previously shown that *DKC1* is a direct and conserved transcriptional target of the MYC oncoprotein.[22] MYC has also been shown to directly regulate the transcription of *TERT*[41] and *NHP2*.[43] Yet, although *MYC* and *DKC1* expression strongly correlated, there was only weak correlation between *MYC* and *TERT* and *MYC* and *NHP2*, respectively (not shown). We note that the in silico analysis does not account for posttranscriptional or posttranslational modifications that may influence the expression and/or function of either dyskerin or TERT. Nonetheless, our findings suggest that *DKC1* and *TERT* mRNA may be differentially regulated in tumor cells and likely by different factors. To that end, we have observed overexpression of *DKC1* in the absence of any appreciable changes in *MYC* expression (unpublished data), and *TERT* is also known to be regulated by a variety of different factors.[44]

TERT expression is regarded as a reliable marker for telomerase. Although recent reports suggest TERC levels may be a rate-limiting determinant of telomerase activity under certain conditions, amplification of TERT is the primary limiting factor for telomerase activity.[26,27,45] To that end, similar to the findings described in our current report, Cao et al[26] demonstrated that *DKC1* mRNA expression was upregulated 2- to 4-fold after TERT-immortalization of mammary epithelial cells, but there was no consistent correlation between *DKC1* mRNA expression and either *TERT* expression or telomerase activity levels. Although it remains possible that TERT may exert at least a partial influence on dyskerin expression in telomerase-positive cells, telomerase activity is not sufficient for immortalization of human oral keratinocytes or mammary epithelial cells; these cells also require loss of pRB/p16^{INK4a} cell cycle regulatory control.[28,46]

Defects in the pRB/p16^{INK4a} molecular pathway are common in oral and breast carcinogenesis,[28,46] and presumably are relevant in the pathogenesis of both telomerase-positive and telomerase-negative tumors. Under normal conditions, pRB binds to and sequesters the transcription factor E2F1 thereby preventing G_1 to S phase transition. However, loss of pRB/p16^{INK4a} regulatory control results in dysregulation of E2F1 leading to its transcriptional overactivity[47]; this could be a contributing factor in dyskerin upregulation in cancer. In support of this notion, a recent high-throughput microarray study suggests that E2F1 may directly regulate *DKC1* expression.[48] Moreover, analysis of the Gene Expression Atlas NCI60 on U133A dataset[33] indicates that *E2F1* and *DKC1* expression do weakly correlate in transformed cells (not shown). Thus future studies will be needed to determine whether E2F1 or other components of the pRB/p16^{INK4a} pathway regulate dyskerin.

As an individual variable, high *DKC1* mRNA levels are associated with increased tumor aggressivity and poor prognosis in various cancer types.[14–19] In addition, *DKC1* expression is upregulated in high-grade lymphomas but downregulated relative to normal controls in indolent lymphomas.[14,49,50] Moreover, a recent high-throughput study identified *DKC1* as 1 of only 70 genes that, collectively, constitute a gene expression profile that strongly correlates with the development of aneuploidy and poor clinical prognosis.[51] Although our sample cohort was too limited to assess statistical significance, further study is needed to determine if the same is true for OSCC. Moreover, more detailed studies will be needed to determine the exact mechanisms by which dyskerin contributes to tumorigenesis. Nonetheless, our cumulative findings indicate that dyskerin expression is not dependent upon TERT in either immortalized or transformed cells, or in patient-derived OSCCs. However, it is apparent that mutations, targeted inhibition, or low endogenous levels of dyskerin, respectively, do have negative impacts on normal function of the telomerase RNP, including in tumors.[2,7,8] Yet, the effect is one of only partial functional abrogation. Conversely, Wong et al[44] demonstrated that re-introduction of wild-type dyskerin into dyskerin-mutant cells did not revert telomerase activity to normal levels. Moreover, we have also observed that ectopic expression of dyskerin into cells with wild-type dyskerin does not confer additional increases in telomerase activity (unpublished data). Gu et al[25] showed that murine dyskerin could contribute to telomere maintenance through a mechanism that was independent of telomere shortening. Thus we cannot exclude the possibility that human dyskerin may also play a role in telomere homeostasis in TERT- and ALT-immortalized cells that can be uncoupled from the role of the protein within the telomerase complex. Alternatively, it is possible that dysregulation of dyskerin may also perturb other vital cellular functions.

Emerging evidence implicates aberrant ribosome biogenesis and function in neoplastic transformation and progression.[13] Whether upregulation of dyskerin contributes to alterations in ribosome synthesis or activity is currently unknown. It has also been suggested that high levels of dyskerin may be broadly disruptive to the

assembly of H/ACA snoRNPs.[45] To that end, 1 of dyskerin's obligate binding partners, NHP2, is also upregulated in neoplasia; high levels of this factor are associated with poor prognosis in lung squamous cell carcinoma and colorectal adenocarcinoma.[19,43] Like dyskerin, NHP2 is also essential for the assembly and maturation of H/ACA RNPs and is needed for normal rRNA processing and telomere maintenance; NHP2 is also a telomerase component.[9] We have shown that *DKC1* and *NHP2* mRNA expression strongly correlate in transformed cell lines (Figure 4C). Therefore it is possible that dysregulation of H/ACA RNP biogenesis, irrespective of the mechanism, may be broadly disruptive to normal cellular homeostasis and that such alterations may contribute to tumorigenesis. This will require more detailed investigation.

Finally, it is possible that dyskerin may contribute to the regulation of other important molecular processes beyond those for which the protein has already been implicated in. To that end, we have recently shown that acute loss of dyskerin function impairs the accumulation of a specific subset of microRNAs.[52] MicroRNAs are a class of small non-coding RNAs that directly regulate post-transcriptional gene expression.[53] MicroRNAs regulate a wide array of cellular functions including cell proliferation; a number of microRNAs have been implicated in tumorigenesis.[53] Thus future studies will be needed to determine whether dyskerin contributes to normal and tumor cell growth through regulation of miRNA-mediated post-transcriptional gene expression.

Acknowledgments. We are very grateful to Drs. James Rheinwald, Anil Rustgi, and Thomas Carey for providing us with the cell lines described here. This work was supported in part by NIH grants DE018416 (FA) and DE015856 (BZ), University of Pennsylvania Research Foundation, University of Pennsylvania's Abramson Cancer Center, and by the Pennsylvania Department of Health. The Department specifically disclaims responsibility for any analysis, interpretations or conclusions.

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