Abrogation of the Retinoblastoma Tumor Suppressor Checkpoint During Keratinocyte Immortalization Is Not Sufficient for Induction of Centrosome-mediated Genomic Instability

Siribang-on Piboonniyom, Stefan Duensing, Nathan W. Swilling, Jens Hasskarl, Philip W. Hinds, and Karl Münger

Department of Oral Medicine and Diagnostic Sciences, Harvard School of Dental Medicine, Boston, Massachusetts 02115 [S. P.], and Pathology Department and Harvard Center for Cancer Biology, Harvard Medical School, Boston, Massachusetts 02115 [S. P., S. D., N. W. S., J. H., P. W. H., K. M.]

ABSTRACT

Deregulation of the retinoblastoma (pRB) tumor suppressor pathway and telomerase activity have been identified as rate-limiting steps for immortalization of primary human epithelial cells. However, additional molecular aberrations including p53 inactivation, ras activation, and deregulation of protein phosphatase 2A activity are necessary for full transformation of immortalized epithelial cells. Genomic instability is observed in most human tumors and constitutes an important mechanism to allow emerging tumor cells to acquire additional mutations to efficiently overcome selection barriers during carcinogenic progression. In an attempt to model oral cancer in a human cell-based system, we analyzed normal oral epithelial keratinocytes with the pRB pathway dysregulated by loss of expression of the cyclin-dependent kinase (cdk) 4/cdk6 inhibitor p16INK4A and/or ectopic expression of cdk4 or expression of the human papillomavirus (HPV) type 16 E7 oncoprotein. Ectopic expression of cdk4 and HPV-16 E7 was equally efficient in extending the life span of normal oral keratinocytes, and each was able to cooperate with telomerase (hTERT) to immortalize these cells. HPV-16 E7/hTERT-immortalized normal oral keratinocytes showed centrosome abnormalities, whereas populations of cdk4/hTERT-immortalized cells or hTERT-immortalized cells that had lost expression of p16INK4A showed no such abnormalities. These results demonstrate that disruption of the p16INK4A/pRB checkpoint of epithelial cell immortalization does not necessarily lead to centrosome-associated genomic instability.

INTRODUCTION

Squamous cell carcinomas of the oral cavity and pharynx account for approximately 3% of cancers in the United States, with more than 57,800 new cases estimated in 2002 (1). Although these tumors are generally accessible to early diagnosis and therapy, they are associated with a relatively high rate of mortality due to recurrence and metastasis (2). There is a strong correlation between the development of oral squamous cell carcinomas and tobacco and alcohol abuse, and in addition, infections with high-risk HPVs4 also contribute to a subset of oral cancers (3). As in other cancers, the p53 and pRB tumor suppressor pathways are frequently inactivated in oral cancers; however, the specific molecular pathogenesis of oral cancer is still unclear (reviewed in Ref. 4).

Cancer cells proliferate indeﬁnitely and are therefore considered immortal. Normal cells must circumvent at least two proliferative barriers designated mortality stage 1 (M1) or replicative senescence and M2 or crisis to become immortal (6). The M1 block is regulated by two tumor suppressor pathways, pRB and p53. Inactivation of p53 and pRB by viral oncoproteins such as the SV40 large T antigen or HPV E6 and E7 induces senescence bypass and immortalization of epithelial cells (reviewed in Ref. 7). The M2 stage is triggered by telomere attrition due to the inability of the DNA replication machinery to completely replicate the very ends of the chromosomes that consist of telomeric repeats (reviewed in Ref. 8). Progressive shortening of chromosomes in a multicellular organism can cause telomeric fusions between chromosome arms, leading to dicentric chromosomes and genomic instability (9–11). Ectopic expression of hTERT, the catalytic subunit of telomerase, a ribonucleoprotein with reverse transcriptase activity (12, 13), restores telomerase activity in telomerase-negative cells and extends their replicative life span (14, 15). Telomerase activity is detected in the vast majority of human cancers (reviewed in Ref. 16), including oral squamous cell carcinoma (17, 18).

In addition to extended and increased replicative potential, most cancers are characterized by genomic instability, presumably because the resulting increased genomic plasticity enhances the probability of acquiring advantageous cellular mutations. The most frequent manifestations of genomic instability in human tumors are chromosomal gains and losses resulting in aneuploidy. Such numerical chromosome abnormalities are sequelae of mitotic defects. During a normal mitosis, the duplicated chromosomes are symmetrically distributed between the two daughter cells, a process that requires the formation of a bipolar mitotic spindle. Bipolarity is guaranteed by small cytoplasmic organelles, the centrosomes. Centrosome duplication is tightly synchronized with the cell division cycle. Failure to regulate centrosome duplication results in abnormal centrosome numbers and an increased incidence of multipolar spindles, which can give rise to chromosome mis-segregation and aneuploidy (reviewed in Ref. 19).

Oral cancer cells display a high degree of genomic instability including multipolar mitotic spindles (20). Previous work showed that preinvasive and invasive high-risk HPV-associated genital lesions contain abnormal centrosome numbers that are associated with mitotic abnormalities (21). This process is triggered by the HPV E7 oncoprotein (22). Because HPV E7 can inactivate pRB (reviewed in Ref. 23), we investigated whether inactivation of pRB by other mechanisms such as loss of p16INK4A expression and/or ectopic cdk4 expression could also trigger centrosome duplication errors.

MATERIALS AND METHODS

Cell Culture. Monolayer cultures of human NOKs were prepared from gingival tissues obtained from oral surgeries (24) as described previously (25). HFKs immortalized by hTERT (26) were a gift from Dr. James Rheinwald (Brigham and Women’s Hospital, Boston, MA). Both NOKs and HFKs were grown in keratinocyte serum-free medium (Keratinocyte-SFM; Life Technologies, Inc.) supplemented with epidermal growth factor and bovine pituitary extract. The embryonic kidney-derived cell line 293T was cultured in DMEM (Life Technologies, Inc.) supplemented with 10% fetal bovine serum.
Retroviral Infections. Recombinant retroviruses were produced by transduction of 293T cells according to the method of Yang et al. (27), with some modifications. In brief, 293T cells were trypsinized and plated at 2 × 10^5 cells/6-cm plate at 24 h before infection. Calcium phosphate transfections were performed with 6 µg of PMD.MLV gagpol (helper plasmid), 2 µg of pMD.G (VSVG pseudotype), and 8 µg of the retroviral plasmid of interest [LXSN-wild-type p16INK4A (LXSN-p16wt), LXSN-p16R87P, LXSN-wild-type cdk4 (LXSN-cdk4wt), pBABE (puromycin), pBABE (puromycin) HPV16 E7 (pBABE-E7), pBABE-wild-type cdk4, pBABE (hygromycin), or pBABE (hygromycin) hTERT (hTERT)]. Plasmid DNA was added to 250 µl of 2× BES [50 mM Na2HPO4, 1.5 mM NaH2PO4, and 250 µl of 0.25% (w/v) CaCl2] and mixed vigorously and incubated for 20 min at room temperature. The mixture was evenly dispensed on the 293T cells. After 16 h of incubation, the normal medium was replaced with DMEM supplemented with 10% fetal bovine serum and 100 mM HEPES (pH 7.5). Viral supernatant was collected 48, 72, 96, and 120 h after transfection. A packaging cell line (PA317) producing recombinant LXSN-HPV-16 E7 retrovirus was kindly provided by Dr. D. Galloway (28). These cell lines were maintained in DMEM supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 µg/ml streptomycin, and recombinant retroviruses were prepared using standard methods (29). Infections of 40% confluent NOKs were performed with a mixture of 2 ml of viral supernatant, 8 µg/ml Polybrene, and 2 ml of keratinocyte serum-free medium for 48 h. For sequential infections, cells were reinfected with a second retroviral vector within 1–4 passages after the initial infection and selection.

Determination of Replicative Life Span. Serial cultures of the different NOK cell lines were performed in 6-cm dishes by plating 5 × 10^4 cells, refeeding the cells every 2–3 days, and subculturing every 4–5 days. The number of PDs was calculated at each passage using the formula PD = (n0/nf) where n0 is the initial number of plated cells, and nf is the final number of cells.

Actinomycin D and 5-Aza-2'-deoxycytidine Treatment. Actinomycin D (Sigma) was dissolved in ethanol at a concentration of 2.5 µm. NOKs and all immortalized cells were seeded at a density of 5 × 10^3 cells/6-cm plate at 12–24 h before treatment. Cells were treated for 8, 24, and 48 h with a radiomimetic dose of 0.5 mM actinomycin D or with a corresponding volume of 100% ethanol as a solvent control. Cells were plated at 25–30% confluence and treated 24 h later with 3 µM 5-aza-2'-deoxycytidine for 0.006% DMSO (final concentration) or 0.006% DMSO as the control for 7 days as described previously (30).

Antibodies and Immunological Methods. The antibodies were purchased from and used according to the protocols provided by the following suppliers: HPV-16 E7 (ED17; Santa Cruz Biotechnology); cdk4 (SC260; Santa Cruz Biotechnology); p16INK4A (SC486; Santa Cruz Biotechnology); p15INK4B (SC611; Santa Cruz Biotechnology); p14ARF (Ab-1; NeoMarkers); p21^{CIP1/WAF1} (Ab1; Oncogene Research Products); p53 (Ab6; Oncogene Research Products); GAPDH (Chemicon); β-actin (Ab1; Oncogene Research Products); hTERT (SC7212; Santa Cruz Biotechnology); p24 (PharMingen); and pRB (S780; Cell Signaling Technology). One hundred µg of protein containing lysates prepared in EBC buffer [50 mM Tris-HCl, 120 mM NaCl, and 1% (v/v) NP40 (pH 8.0)] or 0.5 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin and leupeptin, 2 mM NaF, and 0.5 mM sodium orthovanadate were subjected to SDS-PAGE and immunoblot analysis as described previously (25). Enhanced chemiluminescence signals (Renaissance; NEN Dupont) were digitally acquired using a Bio-Rad Fluor S multimager equipped with a Sony DKC digital camera. More than 100 nuclei were evaluated for each cell population studied. A Leica DNA epifluorescence microscope equipped with a Sony DKC digital camera was used for all studies.

Senescence-associated β-Galactosidase Assay. Detection of the activity of the senescence-associated β-galactosidase (35) was performed as described previously (30). Approximately 500-1000 cells were evaluated for each cell population, and the results are expressed as the percentage of stained cells.

RESULTS

Ectopic Expression of cdk4 and HPV-16 E7 Can Extend the Life Span of Normal Oral Epithelial Cells. The pRBP16INK4A pathway modulates an important checkpoint that limits the proliferative life span of epithelial cells. Hence we investigated whether different molecules that can inactivate the pRB pathway are equally efficient in inducing extension of the life span of NOKs. Ectopic expression of cdk4 would be predicted to interfere with the inhibitory effect of p16INK4A by a stochastic titration mechanism resulting in increased pRB phosphorylation and G1-S-phase progression. In contrast, the HPV-16 E7 oncoprotein directly targets pRB by inducing its proteasome-dependent degradation (36) and hence inactivates pRB by a mechanism independent of phosphorylation (37). Retroviral gene transfer was used to generate populations of NOKs with ectopic expression of cdk4 or HPV-16 E7 (LX-k4 and LX-16E7, respectively) or with empty vector as a negative control (LXSN; Fig. 1A). Cell populations were obtained after selection with appropriate antibiotics and were sequentially passaged to determine their proliferative potential. LXSN-vector-transduced control NOKs (LXSN) proliferated for <10 PDs before they reached replicative senescence. In contrast, the life span of the cdk4- and HPV-16 E7-expressing populations was dramatically extended to 40–60 PDs (>100 days; Fig. 1B). Similar results were obtained in three independent experiments with different donor-derived NOK populations and with either LXSN- or pBABE-based retroviral expression vectors (data not shown). These results support the model that aberration of the pRB/p16INK4A pathway through two different mechanisms can each extend the life span of NOKs with similar efficiency.

Ectopic Expression of cdk4 and HPV-16 E7 in Combination with hTERT Induces NOK Immortalization. A combination of telomerase activation and pRBP16INK4A pathway inactivation is nec-
necessary for efficient immortalization of epithelial cells (15, 26, 38). Hence, we investigated whether ectopic expression of cdk4 and HPV-16 E7 in combination with hTERT was equally efficient in inducing NOK immortalization. NOKs were infected with retroviral vectors expressing hTERT (NOK-hTERT) or sequentially infected with cdk4 and hTERT (NOK-hTERT/cdk4) or HPV16 E7 and hTERT (NOK-hTERT/16E7). Antibodies detecting both hypophosphorylated and hyperphosphorylated pRB (Fig. 2C) or specifically detecting ppRB phosphorylated at serine 780 (bottom panel) were used. A GAPDH blot (bottom panel) is shown to document equal loading.

In other epithelial cell types, immortalization by hTERT alone has been associated with the loss of p16INK4A expression, presumably by hypermethylation (26, 38, 39). Therefore we analyzed p16INK4A expression by immunoblot in age-matched early- and late-passage cells (at PD = 32 and PD = 117 for hTERT/cdk4, respectively; see Fig. 2B). At early passage, each of the three cell populations retained p16INK4A expression at levels similar to that of untransfected, early-passage NOKs (Fig. 2C). At late passage, however, p16INK4A levels in the hTERT and hTERT/cdk4 NOK populations were decreased to an extent similar to that seen in a hTERT-immortalized HFK line (HFK-hTERT; Ref. 26; Fig. 2C). As expected, the NOK-hTERT/16E7 population continued to express p16INK4A at high levels (Fig. 2C). In contrast, the p14ARF protein that is encoded by a partially overlapping gene remained expressed at similar levels in early- and late-passage NOKs (Fig. 2C). Moreover, the related CKI p15INK4B also remained expressed in each of the immortalized NOK cell populations (data not shown). In addition, the universal CKIs p21cip/WAF1 and p27kip1 were expressed in the immortalized NOKs (see Fig. 4; data not shown).

We next investigated the phosphorylation status of pRB as readout for cdk4/cdk6 activity in these different cell populations. At low passage number, NOK-hTERT and NOK-hTERT/cdk4 contained low levels of mostly hypophosphorylated pRB. At higher passage number, however, these two cell populations contained mostly hyperphosphorylated pRB. This is consistent with increased pRB phosphorylation by hyperactivity of cdk4/cdk6 through loss of p16INK4A expression and increased cdk4 expression, respectively (Fig. 2D). To further ensure that this apparent pRB hyperphosphorylation reflects increased cdk4/cdk6 activity, the blot was reprobed with an antibody that specifically detects pRB that is phosphorylated at serine 780. The
results reveal increased levels of Ser-780-phosphorylated pRB in immortalized hTERT and hTERT/cdk4NOKs and are thus consistent with increased cdk4 and/or cdk6 activity in these cell populations (Fig. 2D). Levels of pRB were undetectable in both low- and high-passage hTERT/16E7 NOK populations (Fig. 2D), presumably as a consequence of HPV-16 E7-mediated pRB degradation (36, 37).

PCR analysis of exons 1α, 2, and 3 of the INK4A gene using genomic DNA revealed that these sequences are maintained, supporting the model that loss of p16INK4A expression in immortalized hTERT and hTERT/cdk4NOKs was due to gene deletion (Fig. 3A). In addition, analysis of the major CpG island in the INK4A promoter region failed to reveal any evidence for promoter methylation (Fig. 3B).

To determine whether loss of p16INK4A expression in immortalized hTERT and hTERT/cdk4NOKs was due to methylation at other sites, we treated these cells with the demethylation agent 5-aza-2-deoxycytidine for 7 days or with the corresponding concentration of DMSO by hTERT and loss of p16INK4A expression (43, 44). To determine whether p53 was functional in the three different immortalized keratinocyte populations, we analyzed p53 stabilization and induction of the p53 target protein p21WAF1 in response to DNA damage by a radiomimetic dose of actinomycin D. In each of the three cell lines, p53 levels increased in response to this stimulus. The increase of p53 in each of the three cell lines was between 1.5- and 2-fold, similar to what was observed in nonimmortalized oral keratinocytes (1.5-fold; Fig. 4). Moreover, levels of the p53 target protein p21WAF1 also increased in parallel, further suggesting that the p53 tumor suppressor pathway has remained biochemically functional in each of the three immortalized cell lines (Fig. 4).

Abrogation of the pRB/p16INK4A Immortalization Checkpoint Is Not Sufficient for Induction of Centrosome Abnormalities. Genomic instability is a hallmark of the malignant phenotype and is thought to provide the necessary genomic plasticity that is required for a tumor cell to rapidly acquire new mutations that allow such cells to overcome selection barriers in an expanding tumor. We have reported that cells expressing HPV-16 E7 display abnormal centrosome numbers. This gives rise to abnormal, multipolar mitoses that result in
aberrant chromosome segregation and aneuploidy (21, 22). To determine whether other genetic lesions that cause dysregulation of pRB function, including loss of p16INK4A expression and/or cdk4 overexpression, can cause a similar phenotype, we analyzed centrosome numbers by immunofluorescence analysis using the pericentriolar marker γ-tubulin. Centrosome numbers were considered abnormal when a cell contained more than two centrosomes (Fig. 5A). As expected, the proportion of cells with abnormal centrosome numbers was significantly increased in hTERT/16 E7 NOK cultures (18%) relative to primary NOKs (6.5%; Fig. 5B). Expression of hTERT or cellular immortalization does not markedly contribute to this increase because a similar percentage of cells with centrosome abnormalities (19.9%) were detected in a HPV-16 E7-containing NOK population without ectopic hTERT expression that was not immortalized. In contrast, however, the incidence of centrosome abnormalities in the hTERT- and hTERT/cdk4-immortalized NOK populations (5.7% and 7.9%, respectively) was not significantly increased relative to control cells (6.5%; Fig. 5B). To rule out any potential effect caused by the retroviral pBABE vector used for E7 expression in these experiments, populations of empty pBABE-containing NOKs were also analyzed, and the incidence of cells with centrosome abnormalities (3.2%) was not increased relative to control cells (Fig. 4B).

Centrosome abnormalities can drive genomic destabilization or may be markers of genomic instability (reviewed in Ref. 45). If they drive genomic destabilization, centrosome duplication errors will be apparent in mononuclear cells. NOKs contained 1% of mononucleated cells with supernumerary centrosomes, and hTERT- and hTERT/cdk4-immortalized NOKs each contained 2.9% of mononucleated cells with supernumerary centrosomes. In the E7/hTERT-immortalized cell population, the incidence of mononuclear cells with centrosome abnormalities was increased to 10% (Fig. 5C). Hence, as in other cell types, expression of HPV-16 E7 induces primary centrosome duplication errors in NOKs.

Centrosome abnormalities cause abnormal, multipolar mitoses that can lead to chromosome mis-segregation and aneuploidy. To determine the degree of aneuploidy in the different cell populations, we performed FISH analysis using chromosome 11 as a marker. In the hTERT- and hTERT/cdk4-immortalized populations, 89.2% and 77% of all cells showed two chromosome 11 signals and were considered diploid. The remaining cells had 1, 3, or 4 copies of chromosome 11, with the tetraploid population being the most abundant (6.2% and 9.5%, respectively). Importantly, however, we did not detect any cell that contained more than 4 copies of chromosome 11 (Fig. 5D). In contrast, only 58.5% of the hTERT/16E7 population was diploid, and the degree of tetraploidy was increased to 25.7%. In addition, 7.7% of hTERT/16E7-immortalized cells contained >4 copies of the marker chromosome 11 (Fig. 5D), documenting that the degree of aneuploidy in this cell population was greatly increased relative to hTERT- and hTERT/cdk4-immortalized cells. These results are consistent with the increased number of hTERT/16E7-immortalized NOKs that contain centrosome abnormalities (Fig. 4, B and C).

Together, these results demonstrate that dysregulation of the G1-S transition of the cell division cycle by aberration of the pRB pathway through the frequent loss of p16INK4A expression during cellular immortalization and/or overexpression of cdk4 does not necessarily induce centrosome duplication errors and genomic instability.

DISCUSSION

Normal human keratinocytes exhibit a restricted replicative life span in cell culture. After an initial phase of proliferation, they enter a state of permanent growth arrest that is referred to as replicative senescence (46). Senescence is characterized by several phenotypic and molecular changes including an increase in expression of several CKIs as well as telomere shortening (47, 48). It has been suggested that senescence acts as a barrier against tumorigenesis and that acquisition of the immortalized phenotype is an important step in the malignant transformation of normal cells (reviewed in Ref. 41).

In an effort to create a human cell-based model system to investigate molecular mechanisms underlying oral carcinogenesis, we inactivated the pRB pathway by overexpressing cdk4 or HPV-16 E7 and enhanced telomerase activity by ectopic expression of hTERT in NOKs. Overexpression of cdk4 or HPV-16 E7 was equally efficient in inducing an extension of the life span of NOKs. The cells eventually ceased to proliferate, and no immortal variants were ever recovered in multiple experiments. This result is interesting because a recent report (44) has suggested that ectopic expression of a cdk4 mutant that is not inhibitable by p16INK4A (cdk4R24C) was incapable of increasing the life span of cultured human foreskin and oral keratinocytes. The differences in these two studies may be explained by the fact that the foreskin and oral keratinocyte cell populations (Strain N and OKF4, respectively) used in the Rheinwald study (44) had an increased proliferative potential. The various primary oral keratinocyte populations used in our studies had replicative life spans of approximately 10 PDs (Fig. 1B), whereas the replicative life spans of OKF4 and Strain N are extended to approximately 30 and 50 PDs, respectively (44). Because our HPV-16 E7- and cdk4-expressing NOKs consistently ceased proliferating after approximately 40–60 PDs (Fig. 1B), it is possible that the extension of the replicative life span afforded by ectopic expression of cdk4 or HPV-16 E7 may have been masked in Strain N and OKF4 cells. Alternatively, the cdk4R24C mutant that was used in the Rheinwald study (44) may not only be deficient for p16INK4A binding but may also be defective for other biological activities that contribute to the extension of epithelial life span. Third, p16INK4A titration by cdk4 or its interaction with other cellular proteins may have biological consequences that extend beyond the simple activation of pRB kinase activity achieved by the cdk4R24C mutant. Our data are consistent, however, with a recent report (49) that showed that ectopic expression of cdk4 could extend the proliferative life span of human fibroblasts.

Interestingly, we readily obtained immortalized populations when we overexpressed hTERT in NOKs (Fig. 2B). As expected from other
studies (15, 26, 38), steady-state levels of p16\textsuperscript{INK4A} decreased with increased passage number and were very low in late-passage populations (Fig. 2C). PCR analysis showed no evidence for gross deletions in the p16\textsuperscript{INK4A} gene (Fig. 3A), and somewhat surprisingly, analysis of the major CpG island in the promoter region provided no evidence for methylation in the different cell lines (Fig. 3B). Consistent with this notion, treatment of these NOK lines with the demethylation agent 5-aza-2'-deoxycytidine did not yield increased expression of p16\textsuperscript{INK4A}, and the cells did not acquire morphological alterations characteristic of senescent cells (data not shown). These results suggest that promoter methylation at known CpG islands or gross deletions are not major factors that contribute to silencing of p16\textsuperscript{INK4A} expression. In addition, expression of Id1, a potential repressor of p16\textsuperscript{INK4A} transcription (50), was not increased in late-passage cells (data not shown), ruling out Id1-mediated transcriptional repression of p16\textsuperscript{INK4A} expression as a contributing mechanism. Additional experiments to determine the mechanism of p16\textsuperscript{INK4A} silencing in these cell lines during immortalization are currently under way.

Fig. 5. Centrosome abnormalities and aneuploidy in immortalized NOKs. A, representative cells with normal (top panel, NOK) and abnormal (bottom panel, NOK hTERT/16E7) centrosomes. Centrosomes were visualized by immunofluorescence staining using an antibody against the centrosomal protein γ-tubulin and counterstained with Hoechst 33342 DNA dye. B, quantification of centrosome abnormalities in immortalized NOK populations. NOKs and NOKs infected with empty vector (pBABE) were used as negative controls, and NOKs expressing E7 (16E7) were used as a positive control. Bar graphs show averages ± SD of three independent experiments. C, quantitation of centrosome abnormalities specifically in mononucleated cells. The results of a representative experiment are shown. D, quantitation of chromosome 11 copy numbers in NOKs immortalized by hTERT, hTERT/cdk4, or hTERT/16E7 as determined by FISH analysis.
Ectopic expression of p16INK4A in hTERT-immortalized cells induced a senescence-like phenotype (Fig. 3C), further supporting the notion that the observed loss of p16INK4A expression during continued passaging of hTERT-expressing NOKs contributed to immortalization. It is interesting to note that in our experiments, spontaneous loss of p16INK4A expression was a much more frequent event than spontaneous telomerase activation, supporting the contention that in human epithelial cells, telomere erosion represents a more obdurate roadblock to cellular immortality than p16INK4A expression. Coexpression of cdk4 markedly increased the growth rate of hTERT-expressing NOKs, and during the 1-year culture period, hTERT/cdk4 cells had undergone approximately 250 PDs compared with the approximately 150 PDs of hTERT/immortalized cells. Somewhat unexpectedly, the levels of p16INK4A were similarly decreased in late-passage hTERT/cdk4 cells as in the hTERT cells (Fig. 3C). These results suggest that increased cdk4 expression may not suffice to fully protect cells from the growth-inhibitory effects of p16INK4A or, alternatively, that cdk4 and/or p16INK4A may have additional functions and/or targets that contribute to cellular immortalization. We have previously observed that loss of p16INK4A expression in oral cancer cell lines is associated with a more prominent increase in activity of cdk6 than of cdk4 (25, 51). It is thus tempting to speculate that cdk4 and/or cdk6 may contribute to cellular immortalization through mechanisms that might be independent of pRB pathway inactivation.

Expression of HPV-16 E7 in hTERT-expressing cells did not lead to a similarly marked increase in cellular proliferation (Fig. 2B) as expression of cdk4. Because HPV-16 E7 inactivates pRB by direct binding, downstream of phosphorylation, hTERT/HPV-16 E7-expressing NOKs continued to express p16INK4A. This is consistent with earlier reports (52, 53), and in fact, high-level p16INK4A expression is a sensitive marker for high-risk HPV-positive cervical lesions (54).

It has been reported that cells immortalized by ectopic expression of hTERT in combination with loss of p16INK4A expression have defects in the p53 tumor suppressor pathway (43) and that there may be a p53-dependent, telomere-independent pathway that restricts the growth of cell populations that have lost p16INK4A expression (44). Thus, we compared the p53 response in our immortalized cell populations and NOKs by treating the cells with a radiomimetic dose of actinomycin D. We observed an increase in the steady-state levels of p53 and its target protein p21cip1/WAF1, suggesting that the p53-mediated DNA damage response remains at least biochemically functional in these cells (Fig. 4). In addition, expression of the tumor suppressor p14ARF, a negative regulator of mdm2-mediated p53 degradation, was also retained in all of the immortalized cell lines (Fig. 2C).

Most importantly, however, the different immortalized cell populations allowed us to study whether cells with disruption of the pRB pathway due to loss of p16INK4A expression as a consequence of cellular immortalization and/or high-level expression of cdk4 had a higher incidence of centrosome-mediated mitotic abnormalities that can lead to aneuploidy. We had previously shown that expression of HPV-16 E7 in normal HFKs could rapidly induce centrosome duplication errors leading to supernumerary centrosomes, multipolar mitoses, and aneuploidy (21, 22). The ability of E7 to induce centrosome duplication errors is restricted to the cancer-associated high-risk HPVs (21), which can efficiently interact with pRB (55) and induce its proteolytic degradation (56). Moreover, the ability of HPV-16 E7 to induce centrosome abnormalities is dependent on a functional pRB-binding site (21). As expected, the hTERT/HPV-16 E7-immortalized NOK population showed a dramatically increased incidence in centrosome abnormalities (Fig. 5B). Importantly, supernumerary centrosomes were detected in mononucleated cells, supporting the notion that they represent primary centrosome duplication errors that are directly triggered by E7 expression (Fig. 5C). Interestingly, however, even though expression of cdk4 in hTERT-expressing NOKs increased cell proliferation more effectively than expression of HPV-16 E7 (Fig. 2B), the hTERT/cdk4-immortalized NOKs did not show a significantly increased incidence of centrosome abnormalities relative to hTERT-immortalized NOKs or early-passage primary NOKs (Fig. 5, B and C). Hence, disruption of the pRB pathway through loss of p16INK4A expression (and/or increased cdk4 levels) as a consequence of keratinocyte immortalization does not lead to uncoupling of the process of centrosome duplication from the cell division cycle and generation of supernumerary centrosomes. Moreover, generation of centrosome abnormalities does not simply represent a generic manifestation of hyperproliferation. Oral squamous cell carcinoma cells display a multitude of chromosomal segregation defects, including multipolar mitoses (20). Future studies using this human cell-based model of oral carcinogenesis will be focused on the identification of molecular events other than HPV-16 E7 expression that can induce this type of genomic instability. Moreover, the generation of immortalized NOK lines that have different degrees of genomic instability will allow us to directly test whether centrosome-associated mitotic abnormalities and aneuploidy can expedite the process of malignant progression.

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