p16^{INK4a} Modulates p53 in Primary Human Mammary Epithelial Cells

Jianmin Zhang, Curtis R. Pickering, Charles R. Holst, Mona L. Gauthier, and Thea D. Tlsty

Department of Pathology and UCSF Comprehensive Cancer Center, University of California San Francisco, San Francisco, California

Abstract

***p16^{INK4a}* (*p16*) and *p53* are tumor suppressor genes that are inactivated during carcinogenesis in many tumors. Here we show that *p16* gene activity inversely modulates p53 status and function in primary human mammary epithelial cells. Reduced levels of p16 protein stabilize p53 protein through inhibition of proteolytic degradation, and this increase in p53 protein levels enhances the cellular response to radiation, represses proliferation, and transcriptionally activates downstream targets. Stabilization of p53 is mediated through the retinoblastoma/E2F/p14^{ARF}/murine double minute-2 pathway. However, we have observed that p16 does not modulate p53 in fibroblasts, indicating a possible cell type–specific regulation of this pathway.** (Cancer Res 2006; 66(21): 10325-31)

Introduction

Our understanding of early events in carcinogenesis and how they may influence progression is incomplete. Further understanding of this process is required for the identification of molecular targets for early diagnosis, risk assessment, and preventive therapeutics. Inactivation of tumor suppressor genes is an important step during early carcinogenesis, and *p16^{INK4a}* (*p16*) and *p53* are two examples of genes that are frequently inactivated in many tumor types including breast carcinomas (1, 2). The inactivation of p16 and p53 can occur through a variety of mechanisms including point mutation, deletion, and epigenetic silencing. In this article, we will use the term "inactivation" or "mutational inactivation" to refer to the many ways through which a cell inactivates the function of p16 or p53. The *p16* gene codes for a protein that acts as a cyclin-dependent kinase inhibitor and initiates a G_1-phase cell cycle arrest in response to DNA damage, oncogenic stress, and oxidative stress (1). The *p16* gene is transcriptionally induced in response to these cellular stressors. The *p53* gene codes for a multifunctional protein that is best known for its function as a transcription factor that initiates either cell cycle arrest or apoptosis in response to DNA damage, oncogenic stress, and oxidative stress (2). These cellular stresses induce p53 activity through a variety of posttranslational modifications that stabilize the protein and modulate its binding to other proteins and DNA. Although p16 and p53 respond to many of the same cellular stresses, their mutational inactivation during carcinogenesis may be temporally separated. Whereas very little is known about the sequence of early mutational events during carcinogenesis in most tumor types, there are some premalignant lesions, such as Barrett's esophagus, where this sequence of events has clearly been shown. In this tissue, inactivation of p53 occurs almost exclusively in cells that have already inactivated p16 (3). Although this sequence of events is known, the mechanistic basis for it is not. It has been postulated that lesions containing inactivated p53 may only be able to expand in the absence of p16 function (3), but it is also possible that inactivation of p16 promotes the inactivation of p53. Additionally, it is unknown if there are any relevant functional interactions between p16 and p53.

In breast carcinogenesis, the earliest sequence of mutational events is poorly understood. DNA hypermethylation of the *p16* promoter, which is known to silence gene expression, has been identified in foci of histologically normal breast epithelium (4). *In vitro* experiments have linked this silencing of p16 to both telomeric and centrosomal dysfunction (4–6). Hence, it has been hypothesized that inactivation of p16 may be a very early event in carcinogenesis that precedes histologic changes within the tissue (4, 7). Inactivation of p16 has not been investigated in the ensuing premalignant breast lesions to any great extent. In contrast, inactivation of p53 is first observed in high-grade ductal carcinoma *in situ*, a late-stage premalignant lesion (8). Analysis of premalignant lesions such as atypical ductal hyperplasia or low-grade ductal carcinoma *in situ* has not revealed a significant frequency of p53 mutations (8, 9). In light of these data, we propose that inactivation of p16 precedes inactivation of p53 during early breast carcinogenesis. Mechanistically, we hypothesize that inactivation of p16 increases p53 activity and this increased activity may promote the inactivation of p53. To test this hypothesis, we have used primary human mammary epithelial cells (HMEC) as a model to examine the dynamic interactions between these tumor suppressor pathways.

HMEC can be isolated and propagated in culture from human breast tissue explants from disease-free women (10). These cultures contain two cell populations with distinct proliferative capacities. The majority population, HMEC, cultured under standard conditions, have a life span of 10 to 15 population doublings before reaching a growth plateau characterized by induction of p16 expression and G_1-phase cell cycle arrest (11–13). The minority population, variant HMEC (vHMEC), have an extended life span (30-50 additional population doublings) and become visible when the HMEC population enters the proliferative arrest characterized by elevated p16 levels. The extended proliferative capacity of vHMEC correlates with silenced *p16* gene expression due to promoter DNA hypermethylation and the repression of *p16* promoter activity (11–13). It has been shown that both cell populations have wild-type (wt) p53 protein as shown by sequencing and functional analysis (14). In this study, we investigate if inactivation of p16 in primary human cells has functional consequences on p53 biology.

Note: Current address for J. Zhang: Department of Neurobiology and Anatomy, University of Utah, Salt Lake City, UT 84132-3401. Current address for C.R. Holst: Genentech, South San Francisco, CA 94000. J. Zhang and C.R. Pickering contributed equally to this work.

Requests for reprints: Thea D. Tlsty, Department of Pathology and UCSF Comprehensive Cancer Center, University of California San Francisco, 513 Pamassus Avenue, HSW 451, Box 0511, San Francisco, CA 94143. Phone: 415-502-6115; Fax: 415-502-6163; E-mail: thea.tlsty@ucsf.edu.

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Materials and Methods

Cells and cell culture. Primary HMEC and human mammary fibroblasts (HMF) were isolated from reduction mammoplasty samples as previously described (5) and were cultured in modified MCDB 170 (MEGM, Cambrex, Rockland, ME). Specimens from five different individuals, RM9, RM15, RM16, RM21, and RM163, were used in our study. Routine cell culture and the isolation of vHMEC were essentially as described (5). Population doublings were calculated using the following equation: PD = log (*A/B*) / log 2, where *A* is the number of cells collected and *B* is the number of cells plated initially. Human foreskin fibroblasts were isolated from newborn foreskin as previously described (15). All experiments were done two to five times with samples from two to four individuals. HMEC were used at passages 1 to 3, vHMEC were used at passages 2 to 5, normal human fibroblasts were used at passages 2 to 5, and foreskin fibroblasts were used at passages 3 to 6.

Plasmids and retroviral gene transfer. The pBabe-puro retroviral vector construct and human $p16^{INK4a}$ cDNA were a gift from Frank McCormick (UCSF Comprehensive Cancer Center, University of California San Francisco, San Francisco, CA). *p16* cDNA was subcloned into pBabe-puro vector between *Bam*H1 and *Sal*1. The pMSCV retroviral construct encoding the p16-specific short hairpin RNA (sh-p16) under the control of the U6 promoter was generously provided by G. Hannon and S. Lowe (Cold Spring Harbor Laboratories, Cold Spring Harbor, NY). The pBabe-puro retroviral construct encoding dominant-negative p53 (GSE22) was a gift from Andre Gudkov (Department of Molecular Genetics, Lerner Research Institute, Cleveland, OH). The LXSN-E7 construct was provided by Denise Galloway (Cancer Biology, Fred Hutchinson Cancer Research Center, Seattle, WA). Amphotropic retrovirus was produced by transfecting Phoenix-A packaging cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Forty-eight to 72 hours posttransfection, virus-containing culture medium was harvested and filtered through 0.45-μm syringe filters. Cells were infected by exposing them to virus-containing medium in the presence of 4 μg/mL Polybrene (Sigma-Aldrich, Milwaukee, WI) for 6 to 10 hours. Cells were maintained in the appropriate medium for 48 hours postinfection, then transferred to medium containing 2 to 4 μg/mL puromycin (Sigma). Experiments were done 4 to 6 days postinfection to allow selection for cells that exhibit puromycin resistance.

Western blot analysis. Cells were lysed with radioimmunoprecipitation assay buffer or buffer containing 160 mmol/L Tris and 2% SDS supplemented with 1× Complete protease inhibitors (Roche Applied Science, Indianapolis, IN). Protein concentration was determined with bicinchoninic acid (Pierce Biotechnology, Rockford, IL) with bovine serum albumin as the standard (Sigma). Protein from total cell extracts was denatured with 1× loading buffer and then fractionated in gradient (4-20%) polyacrylamide gels (Cambrex) and transferred to Hybond-P (GE Healthcare Bio, Piscataway, NJ) membrane. The following monoclonal antibodies were used to stain the blots: mouse anti-p16 (Ab-4; Lab Vision, Fremont, CA), mouse anti-p53 (DO-1; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-p21 (OP64; EMD Biosciences, San Diego, CA), mouse anti-pRb (BD Biosciences, San Jose, CA), mouse anti-E2F1 (Upstate Biotechnology, Lake Placid, NY), mouse anti–murine double minute-2 (MDM2; SMP14, Santa Cruz Biotechnology), mouse anti-β-actin (AC-15; Sigma), and horseradish peroxidase–conjugated goat anti-mouse antibody (Biomedia Corp., Foster City, CA). β-Actin was used as a loading control for all blots. Proteins (5-20 μg) were analyzed for each sample. Staining was developed with SuperSignal West Pico chemiluminescence detection protocol (Pierce). Images were quantified using ImageQuant software.

Radiation treatment. Cells were plated in 60-mm dishes and irradiated with the indicated dose of γ ionizing radiation from a ^{137}Cs source at 281 rad/min. Culture medium was changed following treatment and protein samples were collected 3 hours post ionizing radiation.

Cell cycle analysis. Cells were metabolically labeled with 10 mmol/L bromodeoxyuridine (BrdUrd) for 4 hours before harvest. Cells were isolated by standard trypsinization, resuspended in PBS, and fixed by addition of ice-cold ethanol to a final concentration of 70%. Nuclei were isolated and stained with propidium iodide and FITC-conjugated anti-BrdUrd antibodies (BD Biosciences; ref. 5). Flow cytometry was done on a FACS-Sorter (BD Biosciences) using CellQuest and Flowjo software for analysis. All analyzed events were gated to remove debris and aggregates. A minimum of 30,000 events were collected for each analysis.

Reverse transcription-PCR. Total RNA samples were isolated with Rneasy Mini Kit (Qiagen, Valencia, CA) as per instructions of the manufacturer. Total RNA (1.5 μg) was used for the first strand cDNA synthesis with Superscript First-Strand Synthesis System (Invitrogen). Following reverse transcription, 2 μL of each sample were subjected to p53-specific PCR with Expand High Fidelity kit (Roche Applied Science). The PCR products were viewed by running on a 2% agarose gel. The primers for p53 were antisense, 5′TCAGTCTGAGTCAGGCCCTTC-3′, and sense, 5′ATGGAGGAGCCGCAGTCAGAT-3′.

Real-time PCR. Total RNA samples were isolated with Rneasy Mini Kit (Qiagen) as per instructions of the manufacturer. cDNA was synthesized from 2 μg of total RNA with TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) as per instructions of the manufacturer. Real-time PCR was done on 5-ng input RNA per reaction containing 1× TaqMan Universal PCR Master Mix (Applied Biosystems) and the appropriate TaqMan probe on the DNA Engine Opticon 2 (MJ Research, Inc., Waltham, MA). $p14^{ARF}$ (Hs00924091_m1) and GUSB (Hs99999908_m1) TaqMan probes were purchased from Applied Biosystems. All samples were analyzed in triplicate on each plate and at least three plates were analyzed. Relative mRNA levels were determined using the relative standard curve method (normalized to GUSB) according to Applied Biosystems User Bulletin #2.[1] The graph represents the average and SD from nine replicates for each sample (three replicates on three plates).

Results

HMEC exhibit an inverse relationship between p16 and p53 protein levels. To investigate the relationship between p16 and p53, we isolated and cultured primary HMEC and did Western blots that examined the protein levels of p16, p53, and the p53 target gene *p21*. The representative growth curve shown in Fig. 1 illustrates the populations from which protein lysates were collected to compare HMEC to vHMEC. Samples isolated from HMEC during their exponential growth (Fig. 1, *sample a*) show elevated levels of p16 and reduced levels of the p53 and p21 proteins when compared with vHMEC isolated during their exponential growth (Fig. 1, *samples b-e*). These data show that elevated levels of p53 and p21 protein correlate with repressed p16 activity due to promoter DNA hypermethylation (11–13).

To determine if p16 activity causally regulates p53 protein levels, we manipulated p16 expression in HMEC. To study the effect of reduced p16 expression, we infected HMEC with a retrovirus containing sh-p16. Expression of sh-p16 in HMEC efficiently repressed p16 expression relative to the control vector (Fig. 2*A*) and resulted in an increase in the protein levels of p53 and the p53 target gene *p21* (Fig. 2*A*). Conversely, to study the effect of increased p16 expression, we infected vHMEC (which lack p16 expression) with a retrovirus containing an expression construct for exogenous wt-p16. Overexpression of exogenous wt-p16 in vHMEC resulted in a decrease in the protein levels of p53 and p21 (Fig. 2*A*). These data show that p16 activity inversely regulates the protein levels of p53 and its downstream target p21.

p16 activity modulates p53-dependent proliferation. In response to diverse cellular stressors, p53 protein participates in the activation of cell cycle checkpoints or the initiation of apoptosis, thereby limiting the expansion of damaged cells. Our finding that reduced expression of p16 mediates up-regulation of p53 protein led us to query if the up-regulated p53 protein was

[1] http://www.appliedbiosystems.com.

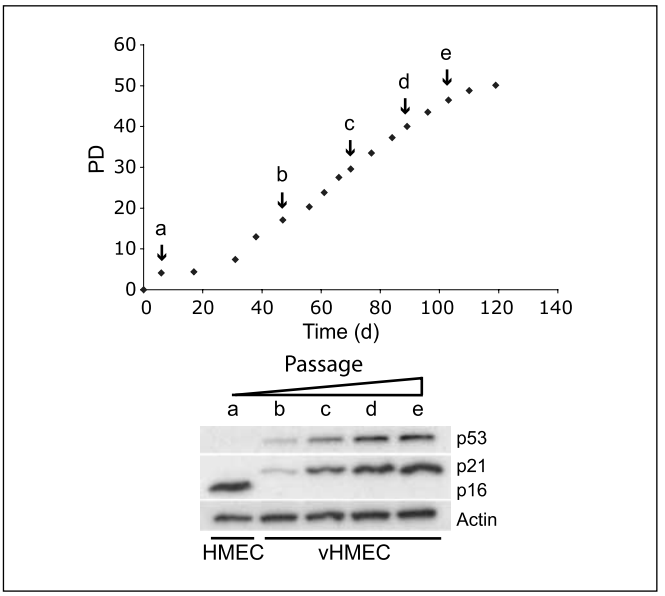

Figure 1. vHMEC, which have silenced p16, exhibit elevated p53 and p21 protein levels. HMEC were cultured and population doublings (*PD*) were calculated at each passage. vHMEC became visible at about day 10. Protein samples were harvested at the indicated points and Western blots were done for p16, p53, p21, and actin as a loading control.

functional. The elevated p53 protein levels observed in many tumors are strongly correlated with p53 mutations; however, Delmolino et al. (14) have previously shown that p53 in vHMEC retains a wt sequence. To investigate the functionality of the elevated p53 protein levels in vHMEC, we abrogated p53 function and determined if the protein levels of the p53 target genes *p21* and *MDM2* were reduced. To abrogate p53 function, we infected vHMEC with a retrovirus containing a genetic suppressor element, GSE22, specific for p53. GSE22 codes for a dominant-negative p53 peptide that increases p53 protein levels (Fig. 2*B*) but inhibits p53-dependent function (16, 17). Expression of GSE22 in vHMEC reduced the levels of the p53 target genes *p21* and *MDM2*, indicating that the elevated p53 protein is necessary for the transcription of p53 target genes (Fig. 2*B*).

Although p53 function is regulated in many ways, up-regulation of wt p53 is known to limit the expansion of populations of cells by inhibiting cell cycle progression or inducing apoptosis. We sought to determine if the elevated levels of p53 protein in vHMEC limit the expansion of this population by comparing the population doublings of vHMEC and vHMEC expressing GSE22. We found that inactivation of p53 resulted in a population with twice as many cells within a specified time (Fig. 2*C*). This increased population expansion could be attributed to an increase in proliferation or a decrease in apoptosis. To measure the amount of proliferation, we analyzed the fraction of cells in the S phase of the cell cycle by flow cytometry with BrdUrd incorporation. We found that vHMEC expressing GSE22 have twice as many cells in S phase of the cell cycle compared with vHMEC controls (Fig. 2*C*, 24% versus 48%). In contrast, the level of apoptosis between vHMEC and vHMEC expressing GSE22 remained unchanged (data not shown). These data indicate that the elevated p53 protein level mediated by the lack of p16 expression reduces proliferation and thereby represses the expansion of the population.

p16 activity modulates a p53-dependent stress response. In response to DNA damage, p53 protein is stabilized and activates cell cycle checkpoints or initiates apoptosis to ensure that damaged cells do not propagate. To determine if inactivation of p16 modulates the response of p53 to DNA damage, we measured the kinetics of p53 protein accumulation in HMEC and HMEC expressing sh-p16 in response to γ ionizing radiation. In vector control HMEC, p53 protein levels increased ~4- and 12-fold following exposure to 4 and 10 Gy of ionizing radiation, respectively (Fig. 2*D*). In contrast, in HMEC expressing sh-p16, p53 protein levels increased ~14- and 20-fold following exposure to 4 and 10 Gy of ionizing radiation, respectively (Fig. 2*D*). These data indicate that reduced levels of p16 protein produce a more robust activation of p53 in response to ionizing radiation.

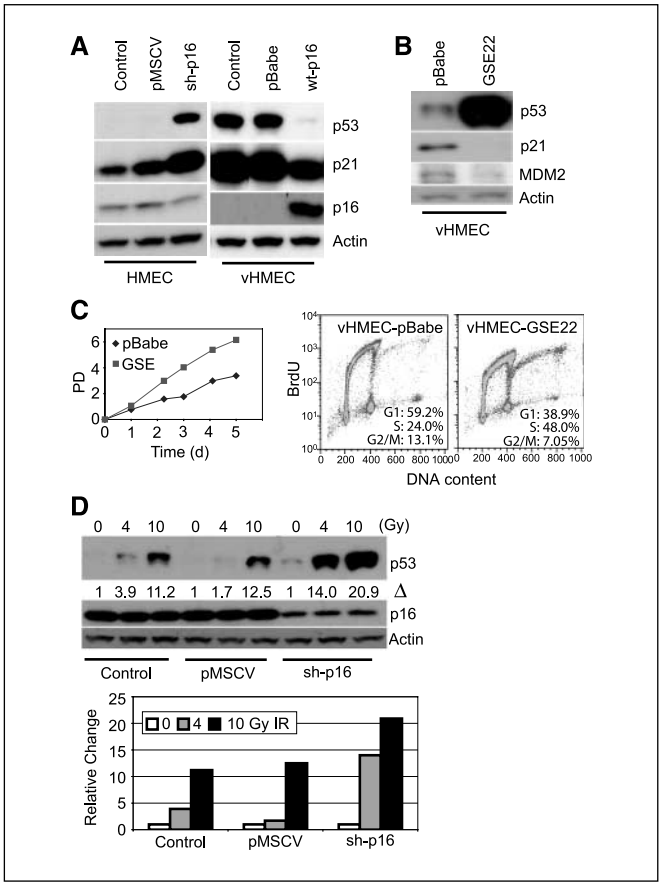

Figure 2. p16 modulates p53 dependent phenotypes. *A,* HMEC and vHMEC, as indicated, were infected with retroviruses containing pMSCV (vector), sh-p16, pBabe (vector), or wt-p16. Protein samples were harvested 4 to 6 days postinfection and Western blots were done for the indicated proteins. Expression of sh-p16 reduced p16 protein levels and increased p53 and p21 protein levels, and wt-p16 increased p16 protein levels and decreased p53 and p21 protein levels. *B,* vHMEC were infected with retrovirus containing either pMSCV (vector) or GSE22 (a dominant-negative p53). Protein samples were harvested 4 to 6 days postinfection and analyzed by Western blot. Expression of GSE22 in vHMEC decreased the protein levels of the p53 target genes *p21* and *MDM2*. *C,* vHMEC containing pBabe (vector) or GSE22 were collected and counted on each of 6 consecutive days and population doublings were determined. To determine cell cycle distribution, BrdUrd (*BrdU*) incorporation and DNA content were analyzed by flow cytometry. Representative cell cycle distributions are listed in the plot. GSE22 increases vHMEC population expansion and increases proliferation. *D,* control uninfected HMEC and either pMSCV (vector)– or sh-p16-infected HMEC were exposed to 0, 4 or 10 of Gy γ-radiation. After 3 hours, protein samples were harvested and p53 and p16 were analyzed by Western blot analysis. Numbers below the p53 blot indicate the relative fold increase in p53 level (Δ) normalized to the 0 Gy sample. The relative changes in p53 level are also graphed below.

Increased p16 activity decreases p53 protein stability. We sought to determine how p16 modulates p53 protein levels. Because the protein level of p53 is primarily regulated through posttranslational mechanisms that modulate protein stability, we first measured p53 protein half-life in HMEC and vHMEC. We found that the half-life of p53 is twice as long in vHMEC compared with isogenic HMEC (a half life of 3 hours versus 1.5 hours, respectively; data not shown; ref. 14). Because the half-life of p53 was reduced in the cells that express p16, we asked whether p16 promotes proteasome-mediated degradation of p53. We found that inhibition of the proteasome by MG132 increased p53 protein levels in vHMEC overexpressing exogenous wt-p16 (Fig. 3*A*). Similarly, proteasome inhibition increased p53 protein levels in HMEC expressing endogenous p16 (Fig. 3*B*). Thus, in both cell populations, the HMEC expressing endogenous p16 and the vHMEC overexpressing exogenous wt-p16, p53 protein levels are regulated by p16 through a proteasome-dependent mechanism. To determine if p53 mRNA expression was also regulated by p16 activity, we measured p53 mRNA levels by reverse transcription-PCR (RT-PCR). In vHMEC overexpressing exogenous wt-p16, p53 mRNA level was minimally altered compared with that in uninfected and vector control cells (Fig. 3*C*). These data show that p16 expression decreases p53 protein stability predominantly via a proteasome-dependent mechanism.

p16 modulates p53 through the retinoblastoma pathway. To determine the mechanism of how p16 promotes the proteasome-dependent degradation of p53 protein, we took a candidate gene approach. The p16 protein regulates the retinoblastoma (Rb) pathway, which, in turn, can modulate p53 protein stability (18). In short, p16 inhibits the phosphorylation of the Rb protein (pRb), thus allowing pRb to inhibit E2F1. E2F1 activates transcription of $p14^{ARF}$ (p14), Rb, and E2F1. p14 binds and inhibits MDM2 (19). Because MDM2 promotes the proteasome-mediated degradation of p53, a functional link may exist between p16 activity and p53 protein levels. This signaling pathway is depicted in Fig. 4*A*. To determine if p16 regulates p53 through the Rb pathway, we analyzed the major proteins in this pathway. We examined the protein levels of pRb, E2F1, and MDM2 by Western blot in four populations of cells: control HMEC and HMEC expressing sh-p16, as well as control vHMEC and vHMEC overexpressing exogenous wt-p16 (Fig. 4*B*). In HMEC expressing sh-p16, we observed an up-regulation of phospho-pRb, E2F1, and MDM2 when compared with the vector control HMEC. Conversely, overexpression of wt p16 in vHMEC, which lack p16, results in a down-regulation of phospho-pRb, E2F1, and MDM2. To determine if p16 directly results in the up-regulation of p14 expression, we probed HMEC and HMEC expressing sh-p16 by quantitative PCR. We found that down-regulation of p16 in HMEC leads to an increase in p14 mRNA levels (Fig. 4*C*).

Because these results suggest that wt p16 down-regulates p53 through the Rb pathway, we sought to determine if Rb signaling is necessary for p16 to modulate p53. To inhibit Rb signaling, we retrovirally infected cells with human papilloma virus E7 (E7), which is known to bind all three members of the Rb family (pRb,

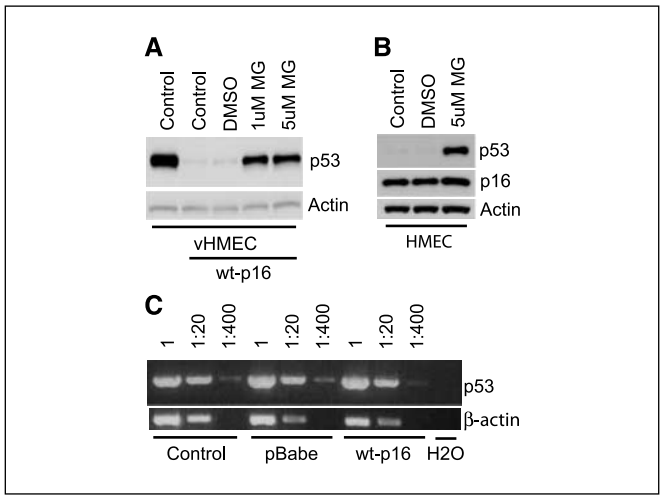

Figure 3. p16 decreases p53 protein stability. *A* and *B,* the indicated cells were exposed to the proteasome inhibitor MG132. Proteasome inhibition increases p53 level in both vHMEC expressing exogenous wt-p16 and HMEC expressing endogenous p16. *C,* RT-PCR analysis of control uninfected vHMEC or vHMEC infected with either pBabe (vector) or wt-p16 shows that wt-p16 does not dramatically alter p53 mRNA level. The PCR templates were diluted as indicated above the lanes. H_2O was used as a negative control.

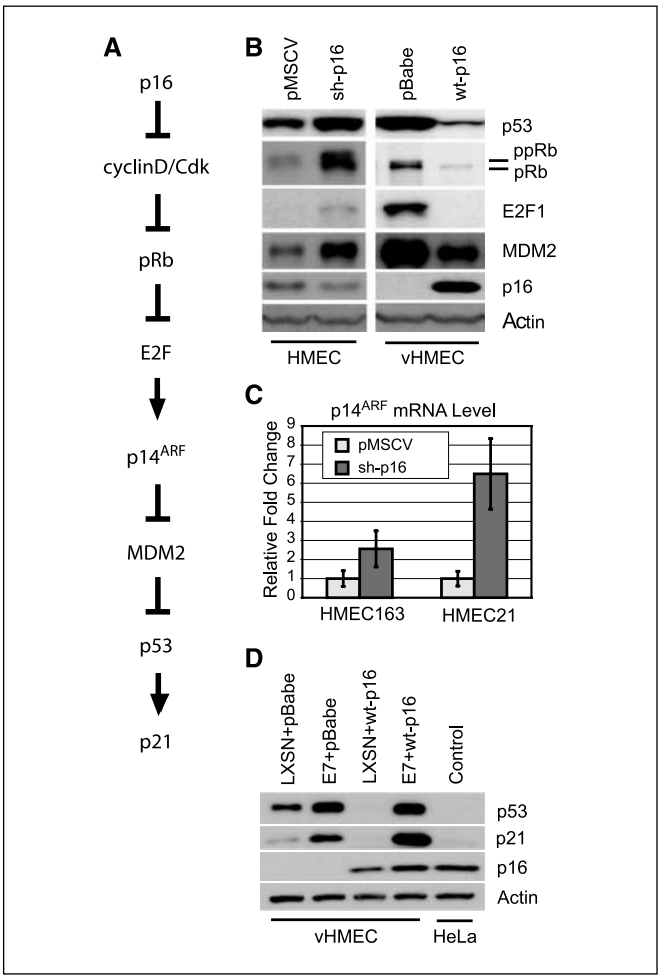

Figure 4. p16 modulates p53 through the Rb pathway. *A,* simplified diagram of the proposed signaling pathway between p16 and p53. *B,* HMEC and vHMEC were infected with the retroviruses as indicated. Protein samples were harvested 4 to 6 days postinfection and analyzed by Western blot for the indicated proteins. p16 modulates members of the Rb pathway as predicted. MDM2 is also a p53 target. *C,* TaqMan real-time PCR was done on the indicated samples to determine p14 mRNA level. Expression of sh-p16 increases p14 mRNA level. HMEC163 and HMEC21 are samples derived from two different individuals. *D,* pBabe (vector)– and wt-p16–expressing vHMEC were infected with retrovirus containing either LXSN (vector) or E7 to inhibit Rb function. E7 expression prevents wt-p16 from decreasing p53 level. HeLa is used as a control.

p107, and p130) and promote their degradation, thus allowing E2F transactivation of target genes (20). Figure 4*D* shows that expression of E7 in vHMEC increases p53 and p21 levels above that observed in vector control cells. To determine if p16 down-regulation of p53 and p21 is Rb dependent, we coinfected vHMEC with both wt-p16 and E7. Inactivation of Rb signaling by E7 prevented p16 from down-regulating p53 and p21. We conclude that functional Rb signaling is necessary for p16 to modulate p53 protein stability.

p16 down-regulation of p53 is cell type specific. We have shown that in HMEC, p16 activity regulates p53 protein stability. To determine if this regulation occurs in other cell types, we modulated p16 levels in isogenic HMF. Whereas expression of sh-p16 in HMF decreased p16 protein levels (up to 90%), the p53 and p21 levels were not dramatically altered (Fig. 5*A*). Similarly, when wt p16 was overexpressed in HMF, p53 and p21 levels did not change significantly (Fig. 5*B*). To determine if these observations are unique to HMF, we examined human foreskin fibroblasts. Similar to our observations with HMF, p53 levels are not modulated by down-regulation of p16 using sh-p16 (Fig. 5*C*). We next investigated if inactivation of p16 in HMF sensitized the cells to DNA damage as was observed in epithelial cells. The kinetics of p53 and p21 protein accumulation were examined in HMF and HMF expressing sh-p16 following exposure to 1, 4, 8, and 10 Gy of ionizing radiation. In contrast to our observations in epithelial cells, the kinetics of p53 stabilization are similar in both control and sh-p16 HMF (Fig. 5*D*). We conclude that p16 does not modulate basal levels of p53 or induced levels of p53 in response to DNA damage in fibroblasts as observed in epithelial cells. Thus, we conclude that regulation of p53 by p16 is cell type specific.

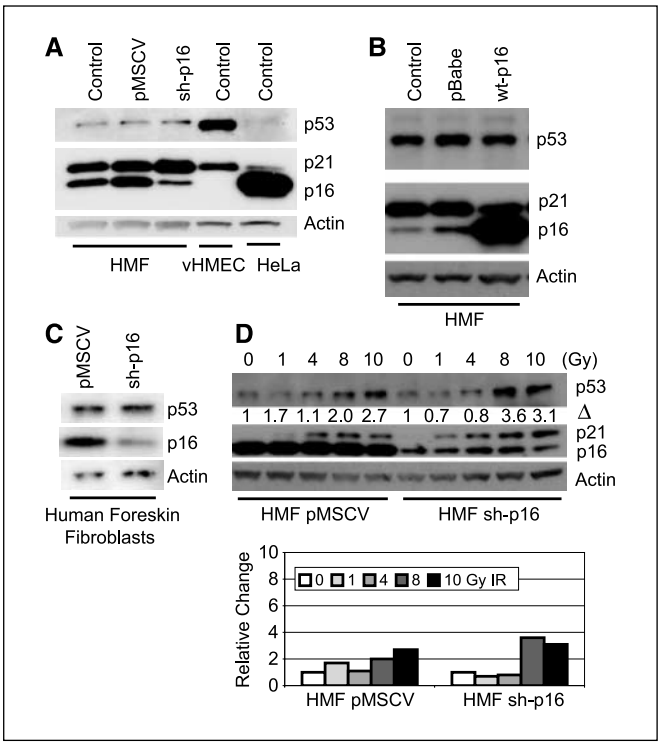

Figure 5. HMF and HMEC differ in their ability for p16 to modulate p53. *A* and *B,* HMF were infected with retroviruses containing pMSCV (vector), sh-p16, pBabe (vector), or wt-p16 as indicated. Protein samples were harvested 4 to 6 days postinfection and analyzed by Western blot. vHMEC and HeLa were used as controls. sh-p16 did not alter p53 levels in HMF to a comparable extent as observed in epithelial cells. Likewise, expression of wt-p16 did not alter p53 levels in HMF to a comparable extent as observed in epithelial cells. *C,* human foreskin fibroblasts were infected with retrovirus containing either pMSCV (vector) or sh-p16. Expression of sh-p16 minimally altered p53 levels in human foreskin fibroblasts. *D,* HMF infected with either pMSCV (vector) or sh-p16 were exposed either to sham, 1, 4, 8, or 10 Gy of γ-radiation. After 3 hours, protein samples were harvested and p53, p21, and p16 were analyzed by Western blot analysis. Numbers below the p53 blot indicate the relative fold increase in p53 level (Δ) normalized to the 0 Gy sample. The relative changes in p53 level are also graphed below.

Discussion

We have used primary human cells to investigate the consequences of inactivation of the tumor suppressor gene *p16* as an early event in carcinogenesis. Our results are of broad interest because they shed new light on the process of carcinogenesis and may provide a mechanism for a sequence of genetic changes that have been observed in many tumors. Our results show that p16 activity inversely modulates p53 protein levels in primary HMEC. Reduced levels of p16 protein increase p53 function, whereas p16 expression reduces p53 protein stability through the Rb pathway. Despite the extensive number of studies on the p16 and p53 pathways, we know of no other study, to date, that has analyzed these two pathways in primary epithelial cells or observed this antagonistic relationship between these two critical tumor suppressors. In fact, this regulation of p53 by p16 has not been observed in other systems. For example, mouse embryonic fibroblasts isolated from p16 knockout mice (21) and human skin fibroblasts isolated from individuals with germ-line mutations in p16 do not exhibit elevated p53 protein levels when compared with controls (22). In concordance, we have shown that reduced p16 expression in human mammary or skin fibroblasts does not increase p53 protein levels. It is not known why this regulation of p53 by p16 does not occur in some cell types, but because it has been observed in HMEC, it may have important implications for the early steps in carcinogenesis in some tumor types. In addition, by analyzing multiple infections of HMEC with retroviruses containing sh-p16, we have observed various levels of p16 inhibition, ranging from >90% to ~50% inhibition, depending on the specific infection, and the p53 level is always dramatically increased (data not shown). In mouse models, p16 has been shown to be haploinsufficient for tumor suppression and we believe that our modest decrease in p16 protein level may mimic haploinsufficiency and show that p16 is haploinsufficient for suppressing p53.

The p16 and p53 pathways are important for the activation of senescence. Senescence is a permanent cell cycle arrest that acts as a barrier to immortalization and tumorigenesis and can be induced by a variety of stimuli, including oxidative stress, DNA damage, oncogene activation, and aging. Although senescence has been studied primarily *in vitro*, it has recently been shown that senescence is also a barrier to tumorigenesis *in vivo* (23–26). Senescence markers were identified in premalignant lesions, which supports the hypothesis that bypass of senescence may be an early event in carcinogenesis (26). Whereas p16 and p53 are both involved in the activation of senescence, these proteins seem to function in separate pathways. For example, oncogenic Ras induces a p16-dependent senescence (27) whereas oncogenic phosphatase and tensin homologue (PTEN) induces a p53-dependent senescence (25). Additionally, during the senescence of human skin fibroblasts induced by extended proliferation in culture, two distinct populations of cells are identifiable (28). One cell population has elevated p53 and p21 protein levels, exhibits markers of DNA

damage and shortened telomeres, and does not have elevated p16 protein levels. The other population has elevated p16 protein levels, does not exhibit markers of DNA damage or shortened telomeres, and does not have elevated p53 or p21. The ability of p16 activity to modulate p53 activity adds another layer of complexity to the initiation of senescence and the ability to bypass senescence. Our results suggest that activation of p16-dependent senescence may inhibit p53-dependent senescence. Conversely, bypass of p16-dependent senescence, through inactivation of p16, may promote the activation of a p53-dependent arrest.

The results described here also provide new insight into how inactivation of p16 may be a critical early event that influences subsequent oncogenic events. This study has shown that reduced levels of p16 can increase p53 protein level and functions. This elevated p53 function may allow the cell to compensate for loss of p16-dependent checkpoints; however, sustained up-regulation of p53 activity may also increase the selective pressure to inactivate p53. We have shown that inactivation of p53 in a cell that has already reduced p16 expression provides a proliferative advantage, and inactivation of p16 has previously been shown to promote genomic instability (5, 29). Therefore, inactivation of p16 provides both a selective pressure (elevated p53) and a mechanism (genomic instability) to inactivate p53, and this may be a driving force during carcinogenesis.

We hypothesize that inactivation of p16 may initiate a sequence of events during early carcinogenesis where inactivation of p53 follows inactivation of p16. Although very little is known about the early events in breast carcinogenesis, other organ systems do provide support for this hypothesis. An elegant set of experiments by Maley et al. (3) examined tissue from Barrett's esophagus and determined that inactivation of p53 is found almost exclusively (14 of 15 cases) in cells that have already inactivated p16 and that this subsequent inactivation of p53 predicts progression to esophageal adenocarcinoma (30). These experiments show a sequence of events in Barrett's esophagus where inactivation of p16 precedes inactivation of p53 and subsequent tumor progression. To our knowledge, modulation of p53 by p16 has not been investigated in esophageal epithelial cells, and thus our results may help explain why inactivation of p16 precedes inactivation of p53 in Barrett's esophagus. We suggest that this sequence of events may occur in breast carcinomas and other tumor types (e.g., pancreas and head and neck) because the frequently observed inactivation of p16 may promote the inactivation of p53.

We have shown that reduced levels of p16 protein increase the p53 response to radiation. An implication of these findings is that p16 expression alters the cellular response to genotoxic damage and may modify radiation toxicity. Because foci of cells with silenced p16 have been found in normal human tissues (4) and loss of p16 activity via deletion, mutation, or methylation often occurs during tumor progression (1), these observations could have implications for the response to genomic damage in normal, premalignant, and tumor tissue.

Finally, we have observed that p16 modulates p53 in HMEC but not in fibroblasts. Our data indicate that the signaling pathway between p16 and p53 is context dependent and is regulated by either extrinsic microenvironmental conditions or intrinsic cell type specific factors. If this pathway is regulated extrinsically by microenvironmental conditions, then the conditions that modulate this important pathway need to be identified. The conditions that are known to induce p16 expression include DNA damage, oncogenic stress, and oxidative stress. There are arguments that HMEC are cultured under "stressed" conditions (31, 32), and if the interaction we have observed occurs during stress-induced conditions, it is very relevant to p53 function and the carcinogenic process. It is appreciated that tumors frequently form under conditions that could be equated with "stress" (i.e., chronic injury; ref. 31). It will be interesting to determine if we have identified "stressful" conditions that can dramatically alter the interaction between p16 and p53 signaling and if similar conditions occur *in vivo*.

If the signaling pathway between p16 and p53 is regulated intrinsically in a cell type specific manner, then results obtained in one cell type may not be directly applicable to others. Because epithelial cells and fibroblasts have very different roles in response to damage and stress, it should not be surprising that key damage and stress response pathways would be regulated in a cell type specific manner. Cell type specific signaling adds an additional layer of complexity to our understanding of carcinogenesis but may provide new directions for therapeutic targets. Additionally, our results in HMEC indicate that inactivation of p16 may increase the selective pressure to inactivate p53. If this effect occurs in epithelial cells but not in fibroblasts, this could partially explain the preponderance of carcinomas (epithelial origin) that occur in humans compared with sarcomas (fibroblast origin).

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Jianmin Zhang, Curtis R. Pickering, Charles R. Holst, et al.

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