Caveolin-1 Expression Is Down-Regulated in Cells Transformed by the Human Papilloma Virus in a p53-Dependent Manner. Replacement of Caveolin-1 Expression Suppresses HPV-Mediated Cell Transformation†

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ABSTRACT: Squamous cell carcinomas of the lung and cervix arise by neoplastic transformation of their respective tissue epithelia. In the case of cervical carcinomas, an increasing body of evidence implicates the human papillomavirus, HPV (types 16 and 18), as playing a pivotal role in this malignant transformation process. The HPV early genes E6 and E7 are known to inactivate the tumor suppressors p53 and Rb, respectively; this leads to disruption of cell cycle regulation, predisposing cells to a cancerous phenotype. However, the role of caveolin-1 (a putative tumor suppressor) in this process remains unknown. Here, we show that caveolin-1 protein expression is consistently reduced in a panel of lung and cervical cancer derived cell lines and that this reduction is not due to hyperactivation of p42/44 MAP kinase (a known negative regulator of caveolin-1 transcription). Instead, we provide evidence that this down-regulation event is due to expression of the HPV E6 viral oncoprotein, as stable expression of E6 in NIH 3T3 cells is sufficient to dramatically reduce caveolin-1 protein levels. Furthermore, we demonstrate that p53—a tumor suppressor inactivated by E6—is a positive regulator of caveolin-1 gene transcription and protein expression. SiHa cells are derived from a human cervical squamous carcinoma, harbor a fully integrated copy of the HPV 16 genome (including E6), and show dramatically reduced levels of caveolin-1 expression. We show here that adenoviral-mediated gene transfer of the caveolin-1 cDNA to SiHa cells restores caveolin-1 protein expression and abrogates their anchorage-independent growth in soft agar. Taken together, our results suggest that the HPV oncoprotein E6 down-regulates caveolin-1 via inactivation of p53 and that replacement of caveolin-1 expression can partially revert HPV-mediated cell transformation.

Caveolin-1, a 21−24 kDa integral membrane protein, is a principal component of caveolae membranes in vivo (*1*−*3*). Caveolae are ∼50−100 nm vesicular invaginations of the plasma membrane and are thought to form as a result of a local accumulation of cholesterol, glycosphingolipids, and caveolin-1 (*4*−*6*). Two other members of the caveolin gene family have recently been identified and cloned: caveolin-2 and caveolin-3 (*7*, *8*). Caveolin-2 has the same tissue distribution as and co-localizes with caveolin-1, whereas caveolin-3 is expressed only in cardiac and skeletal muscle cells (*9*, *10*).

Although caveolae function in vesicular and cholesterol trafficking (*11*, *12*), they have also been implicated in signal transduction at the plasma membrane (*13*, *14*). Biochemical and morphological experiments have shown that a variety of lipid-modified signaling molecules are concentrated within these plasma membrane microdomains, such as Src family tyrosine kinases, H-Ras, eNOS, and heterotrimeric G-proteins (*15*−*20*).

Several independent lines of evidence suggest that caveolin-1 plays a regulatory role in signaling, i.e., by functioning as an inhibitor of a variety of plasma membrane initiated signaling cascades (reviewed in ref *21*). More specifically, caveolin-1 negatively regulates different mitogenic pathways either by preferentially interacting with signaling molecules in their inactive state or by actively repressing their signaling function. For example, caveolin-1 binding can functionally suppress the kinase activity of both Src family tyrosine kinases and receptor tyrosine kinases (like EGF-R and Neu) (*15*, *22*, *23*). Caveolin-1 interacts preferentially with inactive H-Ras (*16*) and negatively regulates many members of the

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p42/44 MAP kinase cascade in vivo, including Raf, MEK, and ERK (*24*). In accordance with the idea that caveolin-1 functions as a negative regulator of signal transduction, recombinant expression of caveolin-1 in H-Ras- (G12V) and v-Abl-transformed NIH 3T3 cells abrogates their anchorage-independent growth phenotype (*25*).

Caveolin-1 was first identified as a transformation-dependent substrate of v-Src in RSV-transformed fibroblasts (*26*). In addition, caveolin-1 levels are dramatically reduced in H-Ras- (G12V) and v-Abl-transformed NIH 3T3 cells (*27*). Similarly, mutational activation of c-Neu, a receptor tyrosine kinase, is sufficient to down-regulate caveolin-1 expression (*23*). It should also be noted that, in these and other transformed cells, there is a loss of morphologically identifiable caveolae (*25*, *27*), presumably because of the concomitant loss of caveolin-1. Interestingly, despite the down-regulation of caveolin-1 expression in these transformed NIH 3T3 cell lines, caveolin-2 expression remains unaffected by cell transformation (*9*).

In support of the idea that caveolin-1 functions as a possible "tumor suppressor", antisense-mediated ablation of caveolin-1 expression in otherwise normal NIH 3T3 cells promotes their morphological transformation, anchorage-independent growth in soft agar, and tumor formation in nude mice and induces hyperactivation of the p42/44 MAP kinase pathway (*28*).

Despite the documented down-regulation of caveolin-1 expression in NIH 3T3 fibroblasts transformed by activated oncogenes, little is known about the expression of caveolin-1 in human tumor derived cell lines. However, it is known that caveolin-1 expression is down-regulated in human breast cancer cell lines, such as MCF-7 and T47-D, as compared with normal human mammary epithelial cells (*29*). This provides an indication that down-regulation of caveolin-1 expression may be relevant to the development of human tumors.

Here, we examine the expression of caveolin-1 in a panel of squamous cell carcinoma-derived cell lines from the lung and the cervix. We show that caveolin-1 protein expression is consistently reduced in these cell lines, as compared with normal human lung and cervical epithelial cells. In the case of cervical carcinomas, we provide evidence that this down-regulation event is due to expression of the HPV E6 viral oncoprotein, as stable expression of E6 in NIH 3T3 cells is sufficient to dramatically reduce caveolin-1, while p53, the primary E6 target, is able to induce caveolin-1 protein levels. In addition, using SiHa cells, we demonstrate that replacement of caveolin-1 expression can partially revert HPV-mediated cell transformation.

EXPERIMENTAL PROCEDURES

Materials. The caveolin-1 mouse mAb 2297 and caveolin-2 mouse mAb 65 [used for immunoblotting (*9*, *30*)] were gifts from Dr. Roberto Campos-Gonzalez, Transduction Laboratories, Inc. The β-tubulin mAb 2.1 was purchased from Sigma. The cervical carcinoma cell lines SW756, Hela-229, and H1-Hela and the lung carcinoma cell lines NCI-H157, NCI-H125, NCI-H2170, and KLN-205 were obtained from ATCC (CRL-10302, CCL-2.1, CRL-1958, CRL-5802, CRL-5801, CRL-5928, and CRL-1453, respectively). The cervical carcinoma cell line, SiHa, was a gift from Dr. Anna Kadish, Albert Einstein College of Medicine. Normal cervical, bronchial, and small airway epithelial cells were obtained from Clonetics, Inc. (CC-2648, CC-2640, and CC-2647, respectively). Balb/c-3T3 cell lines [wild type and stably expressing a p53-valine 135 temperature-sensitive mutant (*31*)] were gifts from Dr. Nissim Hay, University of Illinois, Chicago. A variety of other reagents were purchased commercially as follows: cell culture reagents were from Life Technologies, Inc., and the Effectene transfection reagent was from Qiagen.

Cell Culture. SW756, Hela-229, H1-Hela, NCI-H157, NCI-H125, NCI-H2170, KLN-205, SiHa, normal cervical, bronchial, and small airway epithelium, and the Balb/c-3T3 cell-lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (Life Technologies, Inc.) at 37 °C and 5% CO_2. NIH 3T3 cells were cultured similarly using medium containing 10% donor calf serum (JRH Biosciences).

Expression Vectors. The cDNA encoding the HPV 16 early gene E6 was subcloned into pcDVL, a mammalian expression vector driven by the human β-actin promoter [gift from Dr. Peter M. Howley, Harvard Medical School, Boston, MA (*32*)]. The cDNAs encoding wild-type p53, wild-type Rb, Rb lg (379−928; large pocket—a minimally active form of Rb), and Rb C706F (a loss-of-function mutant) were subcloned into a CMV-based mammalian expression vector, as described previously (*33*, *34*).

Transfection and Selection of Stable Cell Lines. Parental NIH 3T3 cells were cotransfected with the E6-expressing vector and a plasmid containing hygromycin resistance (pCB7) using the calcium phosphate precipitation method. After selection in medium supplemented with hygromycin B (200 μg/mL; Calbiochem, Inc.), resistant colonies were isolated via trypsinization using cloning rings. Individual clones were screened for the expression of E6 via Northern blot analysis using the E6 cDNA as a probe.

Immunoblot Analysis. Cells were cultured in their respective media and allowed to reach 80%−90% confluency. Subsequently, they were washed with PBS and incubated with lysis buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 1% Triton X-100, 60 mM octyl glucoside) containing protease inhibitors (Boehringer Mannheim). Protein concentrations were quantified using the BCA reagent (Pierce), and the volume required for 10 μg of protein was determined. Samples were separated by SDS−PAGE (12.5% acrylamide) and transferred to nitrocellulose. The nitrocellulose membranes were stained with Ponceau S (to visualize protein bands) followed by immunoblot analysis. All subsequent wash buffers contained 10 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% Tween-20, which was supplemented with 1% bovine serum albumin (BSA) and 2% nonfat dry milk (Carnation) for the blocking solution and 1% BSA for the antibody diluent. Primary antibodies were used at a 1:500 dilution. Horseradish peroxidase-conjugated secondary antibodies (1:5000 dilution, Pierce) were used to visualize bound primary antibodies with the Supersignal chemiluminescence substrate (Pierce).

Northern Blot Analysis. Cells were cultured in 10 cm dishes and allowed to reach 80%−90% confluency. Subsequently, they were washed in PBS, and the total RNA was

extracted using the QIA shredder and RNeasy Mini Kits (Qiagen). Ten micrograms of total RNA for each sample was separated by 0.8% agarose gel under RNase-free conditions and transferred to nitrocellulose. The filters were hybridized using the ExpressHyb solution (Clontech). Probes used were the E6 cDNA and the human β-actin cDNA (Clontech, as a control probe for equal RNA loading).

In Vivo Reporter Assay. A 13 kb DNA segment containing the caveolin-1 exons 1 and 2 was identified by screening a mouse genomic DNA library as previously described (*35*). A portion of caveolin-1 exon 1 in addition to intron 1 and the 3 kb upstream promoter was derived from this segment and subcloned into the vector pA3LUC, a promoter-less vector containing the luciferase cDNA as a reporter (*36*, *37*). In this way, the effect of various signal transduction pathways on the regulation of caveolin-1 at the transcriptional level can be assessed. Transient transfections were performed using the Effectene method (Qiagen) as described previously (*38*). Briefly, 150 000 NIH 3T3 cells were seeded in six-well plates 12−24 h prior to transfection. Each cell well was then transfected with 0.5 μg of either the p53 wild type, Rb wild type, Rb lg, Rb C706F, or empty CMV vector, 1.0 μg of the luciferase reporter containing the caveolin-1 promoter, and 0.2 μg of pSV-β-gal (Promega). The pSV-β-gal, a SV40 driven vector expressing β-galactosidase, was used to control for transfection efficiency. Twelve hours post transfection, the cells were rinsed once with PBS and incubated for an additional 12 h. The cells were lysed in 200 μL of extraction buffer, 100 μL of which was used to measure luciferase activity as described (*39*). Another 50 μL of the lysate was used to conduct a β-galactosidase assay, as previously described (*40*). Each experimental value has been normalized using its respective β-galactosidase activity and represents the average of two separate transfections performed in parallel; error bars represent the observed standard deviation. All experiments were performed at least three times independently and yielded virtually identical results.

Construction of Recombinant Caveolin Adenoviral Vectors. The adenoviral vector (pAd-tet) used consisted of a tetracycline-regulatable expression cassette [a heptamer of *tetO* sequences preceding a CMV immediate early (IE) promoter]. The full-length cDNA for caveolin-1 (canine) was amplified by PCR with a c-Myc epitope tag fused to the C-terminus (*30*) and subcloned into the transfer vector pAd-tet using *Sal*I (5′) and *Not*I (3′) restriction sites. The correct orientation and sequence of the insert were verified by restriction mapping and DNA sequencing. HEK 293 cells containing the E1 early gene region of the adenovirus type 5 genome were subsequently used to package the adenovirus by cotransfection of 293 cells with the *Spn*I restriction fragment of pAD-*tet*-caveolin-1 (pAd-cav-1) and the large right-end fragment of the Ad5/ΔE1ΔE3 genome (adenovirus type 5 genome lacking the E1 and E3 early gene regions). Positive plaques were purified and expanded in 293 cells, and virus titers were determined by OD260 and plaque assay as described previously (*41*). The adenoviral vectors containing tetracycline-regulatable GFP (Ad-GFP) and the CMV-driven *tet*-controlled transactivator (Ad-tTA) were similarly constructed. A cesium chloride banding procedure was used for large-scale virus purification and yielded on the order of 1×10^{12} pfu/mL for each of the three adenoviruses.

Growth in Soft-Agar Assay. Growth in soft agar was assayed as previously described, with minor modifications (*27*). Conditions for adenovirus infection of SiHa cells were optimized by immunofluorescence and immunoblot analysis, so that relatively high protein expression was achieved without toxicity to the cells (unpublished observations). Twenty four hours before infection, ~100 000 SiHa cells were plated in six-well plates. At the time of infection, cells were washed once with PBS and incubated for 1 h with serum-free media containing either Ad-cav-1 alone (20 pfu/cell), Ad-tTA alone (10 pfu/cell), Ad-cav-1 + Ad-tTA (10 pfu/cell for each), or Ad-GFP + Ad-tTA (10 pfu/cell for each). Cells were then washed with PBS and cultured for an additional 12 h in medium containing 10% FBS. Each cell population was then trypsinized and quantitated by counting twice using a hemocytometer. Approximately 1.5×10^4 cells were suspended in 3 mL of DMEM containing 10% FBS and 0.33% SeaPlaque low-melting temperature agarose (FMC Bioproducts). The suspension was plated onto a 60 mm dish containing a 2-mL layer of solidified DMEM, 10% FBS, and 0.5% SeaPlaque agarose. Three 60 mm dishes were used for each cell population. The cells were allowed to settle to the interface between these layers for 30 min at 37 °C, and plates were allowed to harden at room temperature for an additional 30 min before being returned to 37 °C. The cells were fed every 3−4 days by overlaying with 2 mL of medium containing 10% FBS and 0.33% agarose. After 2 weeks, the number of colonies was quantitated manually by counting foci at five predesignated locations on the dish using 10× magnification. Experimental values represent the average number of colonies in the three 60 mm plates for each condition; error bars represent the observed standard deviation between the three plates. Representative regions were also photographed under low magnification (4×).

RESULTS

Caveolin-1 Expression Is Down-Regulated in Squamous Cell Carcinoma-Derived Cell Lines. Using isoform-specific monoclonal antibody probes, we examined the expression of caveolins-1 and -2 in a panel of squamous carcinoma-derived cell lines, as compared with normal squamous cells of the cervix and lung.

Figure 1A shows that caveolin-1 levels are significantly reduced in four widely used human cervical carcinoma cell lines (SiHa, H1-Hela, Hela-229, and SW-756), while normal human cervical squamous epithelia express high levels of caveolin-1. In contrast, caveolin-2 levels are down-regulated in only two of the four carcinoma-derived cell lines. We have also conducted Northern blot analysis on several of these tumorigenic cell lines and discovered a reduction in caveolin-1 mRNA transcripts, as compared with their normal counterparts (data not shown).

Similarly, caveolin-1 levels were dramatically reduced in four lung squamous cell carcinoma-derived lines (NCI-H157, NCI-H125, NCI-2170, and KLN-205), as compared with normal human lung epithelia (bronchial and small airway) and nontransformed NIH 3T3 cells (Figure 1B). Caveolin-2 levels remained relatively constant in three out of four of these lung carcinoma cell lines (Figure 1B). To ensure equal protein loading, the levels of the ubiquitously expressed β-tubulin were also assessed for all cell types (Figure 1).

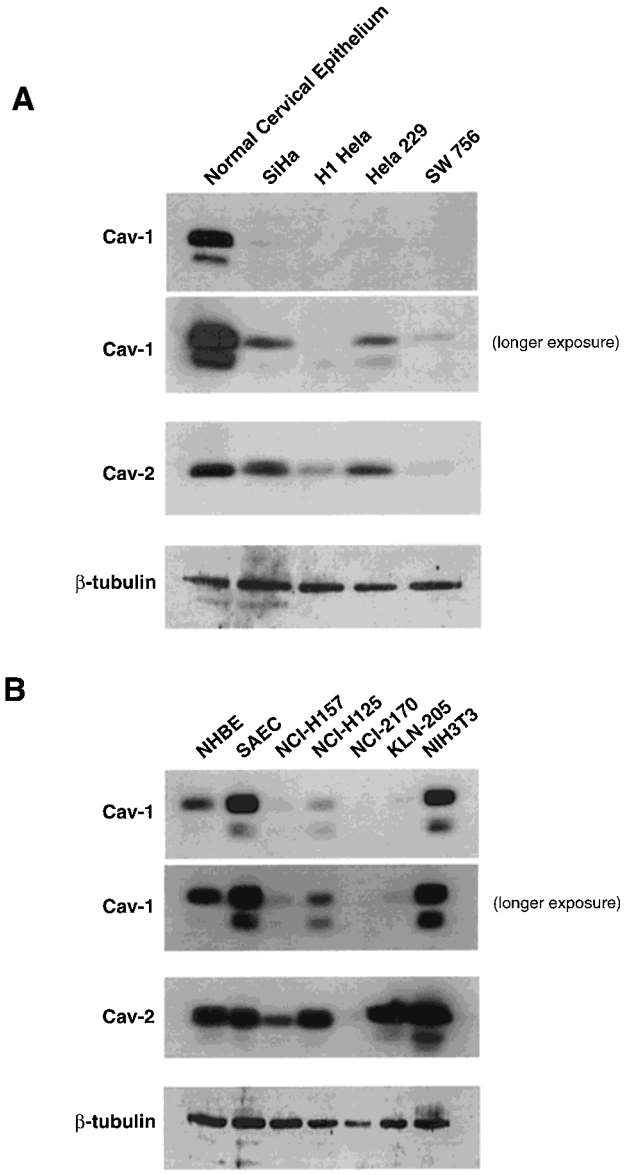

FIGURE 1: Caveolin-1 expression is down-regulated in squamous cell carcinoma-derived cell lines. Using isoform-specific monoclonal antibody probes, we examined the expression of caveolins-1 and -2 in a panel of squamous carcinoma-derived cell lines, as compared with normal squamous cells of the cervix and lung. (A) Cervix. Normal human cervical epithelial cells and four cervical carcinoma-derived cell lines (SiHa, H1-Hela, Hela-229, and SW-756) were cultured to 80%−90% confluency. Lysates were prepared and subjected to immunoblot analysis with anti-caveolin-1 and anti-caveolin-2 IgG. Note that caveolin-1 levels are significantly reduced in the four widely used human cervical carcinoma cell lines, while normal human cervical squamous epithelia express high levels of caveolin-1. Two exposures are shown to better illustrate this point. In contrast, caveolin-2 levels are down-regulated in only two of the four carcinoma-derived cell lines. β-Tubulin protein levels were used as a control for equal loading. (B) Lung. Normal human bronchial epithelial cells (NHBE), normal small airway epithelial cells (SAEC), four lung carcinoma-derived cell lines (NCI-H157, NCI-H125, NCI-2170, and KLN-205), and normal NIH 3T3 cells were subjected to immunoblotting as detailed in panel A. Note that caveolin-1 levels are dramatically reduced in the four lung squamous cell carcinoma-derived lines, as compared with normal human lung epithelia (bronchial and small airway) and nontransformed murine NIH 3T3 cells. Two exposures are shown to better illustrate this point. Caveolin-2 levels remained relatively constant in three out of four of these lung carcinoma cell lines. Again, β-tubulin was used as an equal loading control.

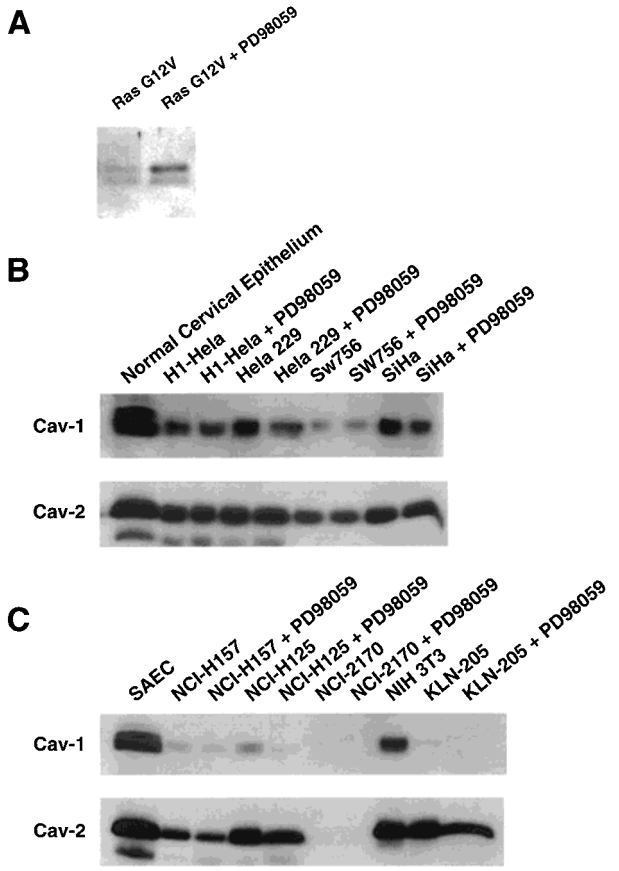

FIGURE 2: Down-regulation of caveolin-1 in squamous carcinoma cell lines is independent of p42/44 MAP kinase activation. The four cervical carcinoma (panel B, H1-Hela, Hela-229, SW-756, and SiHa) and the four lung carcinoma cell lines (panel C, NCI-H157, NCI-H125, NCI-2170, and KLN-205) were treated for 2 days in the presence or absence of PD98059 (50 μM), a well-characterized MEK inhibitor. Lysates were prepared and subjected to immunoblot analysis with anti-caveolin-1 and anti-caveolin-2 IgG. Note that treatment with PD98059 did not up-regulate caveolin-1 levels in these cell lines; caveolin-2 levels also remained unchanged (panels B and C). However, treatment of H-Ras- (G12V) transformed NIH 3T3 cells with PD98059 resulted in up-regulation of caveolin-1 expression, as expected (panel A). Thus, it appears that down-regulation of caveolin-1 in these squamous carcinoma cell lines is clearly independent of p42/44 MAP kinase activation.

Using H-Ras- (G12V) transformed NIH 3T3 cells, we have previously shown that down-regulation of caveolin-1 is mediated by constitutive activation of the p42/44 MAP kinase cascade. For example, treatment of H-Ras- (G12V) transformed NIH 3T3 cells with PD98059 (a potent and specific inhibitor of MEK) restores caveolin-1 mRNA and protein expression to normal levels observed in nontransformed NIH 3T3 cells (*25*, *27*).

To determine if down-regulation of caveolin-1 in squamous carcinoma-derived cells is also dependent on activation of the p42/44 MAP kinase cascade, we next treated these cells in the absence and the presence of PD98059 (50 μM). Figure 2B,C shows that treatment with PD98059 did not up-regulate caveolin-1 levels in these cell lines. However, treatment of H-Ras- (G12V) transformed NIH 3T3 cells with PD98059 resulted in up-regulation of caveolin-1 expression, as expected (Figure 2A). Thus, it appears that down-regulation of caveolin-1 in these squamous carcinoma cell lines is clearly independent of p42/44 MAP kinase activation.

Expression of p53 Up-Regulates Caveolin-1 Promoter Activity and Protein Expression. What is the mechanism by which caveolin-1 levels are down-regulated in squamous carcinoma-derived cells? In the case of cervical squamous carcinoma-derived cells, one possibility is that caveolin-1 levels are down-regulated in response to transformation induced by the human papilloma virus (HPV), such as HPV 16 and 18.

Several independent lines of evidence implicate the HPV early genes, E6 and E7, in the progression of dysplastic cervical epithelia to the malignant phenotype (*42*, *43*). The E6 and E7 oncoproteins are thought to disrupt cell cycle regulation by binding to and inactivating the p53 and Rb tumor suppressors, respectively (*44*). More specifically, E6 inhibits the function of p53 by enhancing p53 degradation through the ubiquitin−proteasome pathway (*45*), while E7 blocks Rb function by disrupting its direct interaction with E2F, a positive regulator of the cell cycle (*46*, *47*).

Therefore, we first examined the ability of Rb and p53 to regulate caveolin-1 gene expression, as assessed using the caveolin-1 promoter linked to a luciferase reporter. An ~3 kb 5′ upstream region of the murine caveolin-1 gene (which presumably includes the promoter elements for caveolin-1 regulation) and the first exon and intron of caveolin-1 were subcloned into a promoter-less luciferase reporter vector and used in these analyses (Figure 3A). This ~3 kb segment of the murine caveolin-1 gene has been shown to have bona fide promoter activity and undergoes the same regulation as endogenous caveolin-1 transcription in a variety of cell lines (*48*).

We cotransfected this caveolin-1 promoter construct in combination with Rb and p53. Figure 3B shows that wild-type Rb and two Rb mutants (Rb C706F and Rb lg) have little or no effect on caveolin-1 promoter activity, as compared with the empty vector alone. In contrast, expression of wild-type p53 dramatically up-regulated caveolin-1 promoter activity by ~10−20-fold (Figure 3C).

The stimulatory effect of p53 on the caveolin-1 promoter led us to further investigate p53-mediated regulation of caveolin-1 protein expression in vivo. The p53 Val135 mutant is a well-characterized temperature-sensitive version of p53 in which full activity is retained at 32 °C and complete abrogation of function occurs at 39 °C (*31*).

We used a Balb/c-3T3 cell line stably expressing the p53 Val135 mutant to examine caveolin-1 expression by Western blot analysis. Figure 4A shows that after 6 or 12 h of incubation at the permissive temperature (32 °C), caveolin-1 levels are up-regulated by ~2−3-fold. These changes in caveolin-1 levels are not due to the temperature shift alone, as assessed in parallel experiments with wild-type Balb/c-3T3 cells (data not shown). In contrast, expression of caveolin-2 and the control protein β-tubulin remained unchanged (Figure 4A).

The p53-mediated induction of caveolin-1 expression is transcriptional, as we determined by Northern blot analysis of the same Balb/c-3T3 cell line (Figure 4B). Note that caveolin-1 transcripts are up-regulated in a similar 6−12 h time frame; the expression of β-actin mRNA was used as a control for equal loading (Figure 4B). Indeed, this is in agreement with our observation of a robust activation of the caveolin promoter by heterologous p53 expression (Figure 3C).

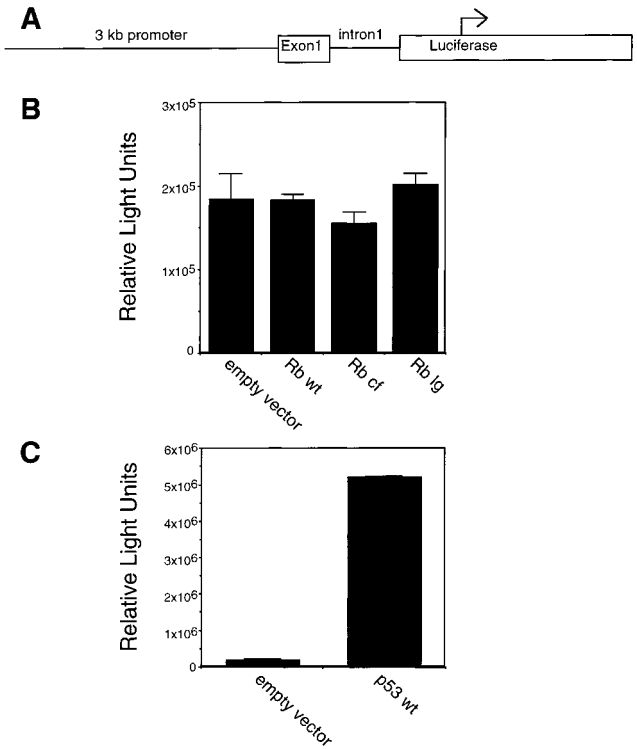

FIGURE 3: Expression of p53 up-regulates caveolin-1 promoter activity. (A) Caveolin-1 reporter construct. This schematic diagram summarizes the organization of the caveolin-1 gene luciferase reporter. The 3 kb upstream promoter, exon-1, and intron-1 of the caveolin-1 gene were used to drive the expression of luciferase. This ~3 kb segment of the murine caveolin-1 gene has been shown to have bona fide promoter activity and undergoes the same regulation as endogenous caveolin-1 transcription in a variety of cell lines (*48*). (B) Effect of Rb. NIH 3T3 cells were cotransfected with the caveolin-1 luciferase reporter and either the empty CMV vector, Rb wt (wild type), Rb C706F (loss-of-function mutant), or Rb lg (379−928; large pocket). Note that wild-type Rb and the two Rb mutants (Rb C706F and Rb Ig) have little or no effect on caveolin-1 promoter activity, as compared with the empty vector alone. The data presented have been normalized to β-galactosidase levels. (C) Effect of p53. NIH 3T3 cells were cotransfected with the caveolin-1 luciferase reporter and either the empty CMV vector or wild-type p53. Note that expression of wild-type p53 dramatically up-regulated caveolin-1 promoter activity by ~10−20-fold. The data presented have been normalized to β-galactosidase levels.

Interestingly, caveolin-1 protein and transcript levels returned to baseline ~24 h after induction (data not shown), indicating that the stimulatory effects of p53 on caveolin-1 expression are transient in nature and occur within hours of p53 activation. This immediate gene induction is commonly seen with other bona fide p53-regulated genes, including regulators of the cell cycle and apoptosis (*49*).

Stable Expression of HPV E6 Is Sufficient To Down-Regulate Caveolin-1 Expression. To test the hypothesis that targeting of p53 plays a definitive role in the mechanism of caveolin-1 down-regulation in cervical carcinoma, we stably transfected NIH 3T3 cells with E6 (from HPV 16), a potent inhibitor of p53 activity (*45*). We chose NIH 3T3 cells for these experiments as they endogenously express both caveolins-1 and -2 and have been used by many investigators as a model to study the tumorigenicity of a variety of oncogenes.

A vector containing E6 was used to stably express the protein in NIH 3T3 cells (*32*). E6-positive clones were identified by Northern blot analysis; the expression of β-actin

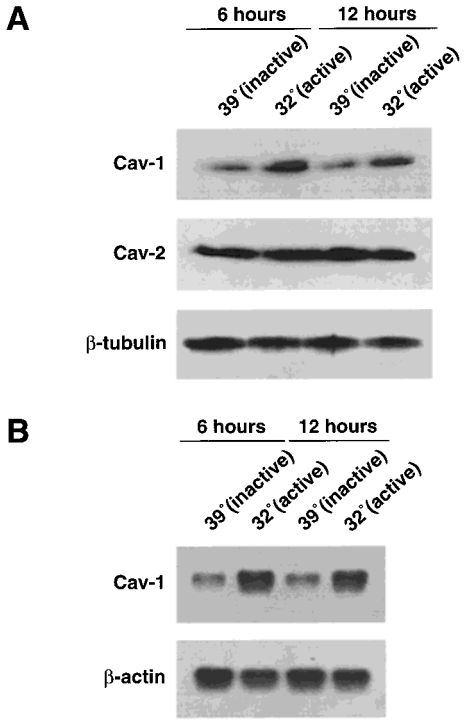

FIGURE 4: Expression of active p53 up-regulates caveolin-1 protein expression. (A) The p53 Val135 mutant is a well-characterized temperature-sensitive version of p53 in which full activity is retained at 32 °C and complete abrogation of function occurs at 39 °C (*31*). Thus, we used Balb/c-3T3 cells stably transfected with the p53 Val135 mutant to assess the effect of p53 activity on caveolin-1 and caveolin-2 expression. Cells were plated on 60 mm dishes and allowed to adhere at 37 °C. They were shifted to either 32 °C (permissive) or 39 °C (nonpermissive) temperatures for 6 and 12 h. Lysates were prepared and subjected to immunoblot analysis with anti-caveolin-1 and anti-caveolin-2 IgG. Note that there is a clear increase in caveolin-1 protein expression (~2−3-fold), but not caveolin-2, under conditions of active p53 (32 °C). *β*-Tubulin was used as an equal loading control. (B) Total RNA was extracted from 100 mm dishes of the p53-inducible Balb/c-3T3 cell line and subjected to Northern blot analysis over a 6 and 12 h time course. In agreement with the increase in protein levels, there is a notable up-regulation of caveolin-1 transcripts. *β*-Actin mRNA was used as a control for equal loading.

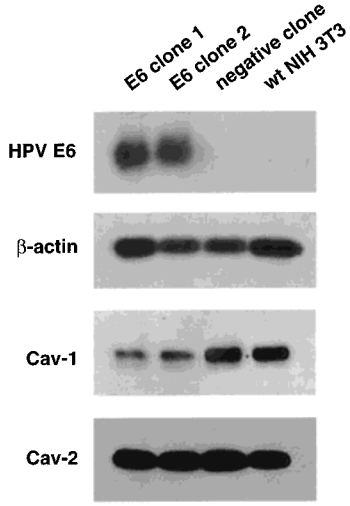

FIGURE 5: Stable expression of HPV-E6 is sufficient to down-regulate caveolin-1 expression. A vector containing E6 was used to stably express the protein in NIH 3T3 cells. E6-positive clones were identified by Northern blot analysis; the expression of *β*-actin mRNA was used as a control for equal loading (upper panels). Note that caveolin-1 protein levels are dramatically reduced in both E6-positive clones, as compared with parental NIH 3T3 cells and an E6-negative NIH 3T3 clone; however, caveolin-2 levels remain unchanged (lower panels).

mRNA was used as a control for equal loading (Figure 5, upper panels).

Note that caveolin-1 protein levels are dramatically reduced in both E6-positive clones, as compared with parental NIH 3T3 cells and the E6-negative NIH 3T3 clone; however, caveolin-2 levels remain unchanged (Figure 5, lower panels). Similarly, NIH 3T3 cells transformed by the expression of a single activated oncogene [e.g., H-Ras (G12V), v-Src, v-Abl] show down-regulation of caveolin-1 expression, but caveolin-2 levels remain unaffected (*9*, *25*, *27*).

Recombinant Expression of Caveolin-1 in a HPV-Transformed Cervical Carcinoma Cell Line Abrogates Their Anchorage-Independent Growth Phenotype. We and others have shown that reexpression of caveolin-1 in (i) oncogenically transformed NIH 3T3 cells [either H-Ras (G12V) or v-Abl] and (ii) T47-D cells (a human mammary carcinoma-derived cell line) can partially revert their transformed phenotype, as assessed by suppression of anchorage-independent growth in soft agar (*25*, *50*).

To determine if caveolin-1 also has transformation suppressor activity in HPV-transformed cells, we chose to express caveolin-1 in the SiHa cell line. SiHa cells are derived from a human cervical squamous carcinoma, contain a fully integrated HPV 16 genome (including E6) (*51*), and undergo anchorage-independent growth in soft agar (our unpublished observations).

To efficiently express caveolin-1 in SiHa cells, we developed an adenoviral approach for efficient gene transfer of the caveolin-1 cDNA (*52*). The C-terminally Myc-tagged canine caveolin-1 cDNA was placed under the control of a CMV immediate early (IE) minimal promoter preceded by a heptamer of *tetO* sequences. Protein expression could then be induced by co-infection of the caveolin-1 adenovirus with a second adenovirus containing a constitutive CMV-driven *tet*-controlled transactivator (Ad-tTA). The tTA is a fusion between the bacterial *tet* repressor and the activation domain of the herpes simplex virus protein VP16 that transcriptionally activates promoters containing *tetO* elements in the absence of *tet* (*53*, *54*).

Figure 6A shows robust expression of caveolin-1 in SiHa cells co-infected with Ad-cav-1 and Ad-tTA. Note the presence of a dose response for caveolin-1 expression when varying multiplicities of infection are used (moi) (lanes 1 and 2) and that, in the absence of Ad-tTA, caveolin-1 expression is prevented (lane 3). As a control for these experiments, we utilized a green fluorescent protein adenovirus (Ad-GFP) engineered in the same manner as for caveolin-1. The expression pattern for GFP qualitatively mimicked that of caveolin-1 (data not shown).

Figure 6B shows the number of colonies for SiHa cells infected with Ad-cav-1 or Ad-GFP, with or without Ad-tTA. Note that caveolin-1 expression (Ad-cav-1 + Ad-tTA) produced a dramatic reduction (~4−5-fold) in the number of colonies formed, as compared with the GFP control (Ad-GFP + Ad-tTA). Furthermore, in the absence of the corequisite Ad-tTA (Ad-cav-1 alone), the Ad-cav-1 infected cells formed colonies as readily as the Ad-GFP control. These

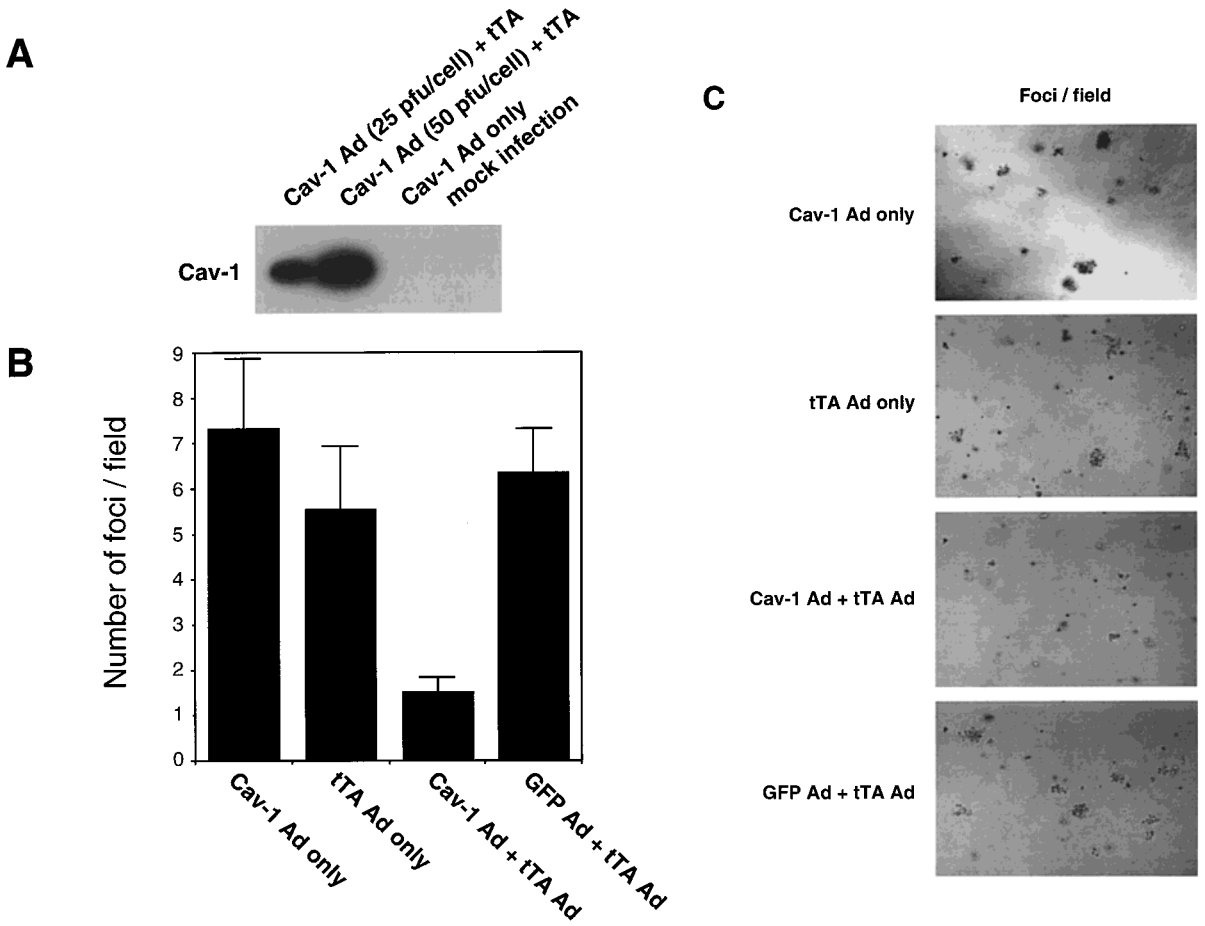

FIGURE 6: Recombinant expression of caveolin-1 in a HPV-transformed cervical carcinoma cell line abrogates their anchorage-independent growth phenotype. (A) Western blot analysis. To assess the fidelity of the tetracycline-inducible caveolin-1 adenovirus (Ad-cav-1) expression system, six-well dishes were plated with 100 000 SiHa cells/well and infected with either (i) Ad-cav-1 (25 pfu/cell) + Ad-tTA (25 pfu/cell), (ii) Ad-cav-1 (50 pfu/cell) + Ad-tTA (25 pfu/cell), or (iii) Ad-cav-1 (50 pfu/cell) alone or (iv) mock-infected. Note the robust and dose-responsive expression of caveolin-1 in cells co-infected with Ad-cav-1 and Ad-tTA. Also note that, in the absence of Ad-tTA, caveolin-1 expression is prevented. (B) Quantitation of colony formation. Twenty four hours prior to infection, ~100 000 SiHa cells were plated in six-well plates. At the time of infection, cells were washed once with PBS and incubated for 1 h with serum-free media containing either (i) Ad-cav-1 alone (20 pfu/cell), (ii) Ad-tTA alone (10 pfu/cell), (iii) Ad-cav-1 + Ad-tTA (10 pfu/cell for each), or (iv) Ad-GFP + Ad-tTA (10 pfu/cell for each). After allowing for a period of protein expression, each group of cells was seeded in triplicate at a density of ~1.5 $\times 10^4$ cells per dish in prepared 60 mm soft-agar plates and incubated for 2 weeks with periodic feeding. Experimental values represent the average number of colonies in the three 60 mm plates for each condition; error bars represent the observed standard deviation between the three plates. Note that caveolin-1 expression (Ad-cav-1 + Ad-tTA) produced a dramatic reduction (~4−5-fold) in the number of colonies formed, as compared with the GFP control (Ad-GFP + Ad-tTA). Furthermore, in the absence of the corequisite Ad-tTA (Ad-cav-1 alone), the Ad-cav-1-infected cells formed colonies as readily as the Ad-GFP control. (C) Colony morphology. Representative fields of SiHa colonies for each experimental condition are shown. Note that caveolin-1-expressing cells (Ad-cav-1 + Ad-tTA) not only show that the number of colonies are reduced but also show that the size of these colonies is dramatically reduced.

studies were performed in triplicate for each experimental condition.

Figure 6C displays representative fields of SiHa colonies observed in soft agar for each experimental condition. Note that caveolin-1-expressing cells (Ad-cav-1 + Ad-tTA) not only show a reduced number of colonies but also show a dramatically reduced colony size.

DISCUSSION

Caveolin-1 is a potent negative regulator of a variety of mitogenic signaling pathways, and down-regulation of caveolin-1 expression occurs in NIH 3T3 fibroblasts transformed by a variety of activated oncogenes. However, little is known about the expression of caveolin-1 in human tumor derived cell lines. Here, we have examined the status of caveolin expression in cervical and lung squamous carcinoma-derived cell lines and HPV-transformed cells.

Although much evidence indicates a role for the HPV early genes in the progression and malignant transformation of cervical epithelium, there are currently no reports on the effects of these viral oncogenes on the regulation of caveolin-1 expression. In this study, we show that caveolin-1 expression is dramatically down-regulated in both cervical and lung carcinoma-derived cell lines, that expression of the HPV early gene E6 in NIH 3T3 cells is sufficient to reduce caveolin-1 expression, and that HPV-induced down-regulation of caveolin-1 may be due to inactivation of p53, as this tumor suppressor is able to up-regulate caveolin-1 gene transcription and protein expression. Furthermore, we demonstrate that recombinant expression of caveolin-1 in a human cervical carcinoma/HPV-transformed cell line (known as SiHa cells) abrogates their anchorage-independent growth phenotype. These observations are consistent with the idea that down-regulation of caveolin-1 expression may be a

critical event in the development of HPV-related squamous cell carcinomas of the cervix.

Among the most common cancers in the world, cervical carcinoma is intriguing in its high incidence of concomitant infection with the human papilloma viruses (HPV), most frequently HPV types 16 and 18 (*55*). In fact, in a recent multinational study, Walboomers and colleagues raise the possibility that HPV infection is a precursor for all invasive cervical carcinomas and that previously diagnosed HPV-negative cancers are probably due a lack of detection sensitivity (*56*). Our understanding of the signaling pathways that regulate caveolin-1 expression in normal and transformed cells is still rudimentary; thus, we focused on the role of viral oncogenes in the development of cervical cancer as a approach to dissect the pathways that regulate caveolin-1 gene expression. We observed that, in cell lines derived from cervical cancers and which contain the HPV genome (i.e., Hela, SW 756, and SiHa) (*44*, *51*, *57*), caveolin-1 expression is consistently down-regulated or undetectable.

Of the viral genes expressed in HPV-infected cervical cells, the early genes E6 and E7 have long been associated with tumor formation. The E6 and E7 proteins of the "high risk" HPV types 16, 18, 31, and 33 have an ability to immortalize and transform cells in culture (*44*). The well-characterized targets of these oncogenes could provide a basis for our elucidation of caveolin-1 gene regulation. Classically, the E6 protein forms a complex with the tumor suppressor p53 and promotes its degradation via a ubiquitination-dependent pathway (*45*), while the E7 protein binds to Rb1 (p105), thereby disrupting its direct inactivation of E2F, a transcription factor involved in cell cycle progression (*46*, *58*).

Our results showed that, of these two commonly inactivated proteins (p53 and Rb), only p53 was capable of inducing caveolin-1 levels. Furthermore, stable expression of E6 was sufficient to down-regulate caveolin-1 levels in NIH 3T3 cells. These observations provide a possible mechanism for E6-mediated caveolin-1 reduction. The involvement of p53 in up-regulating caveolin-1 expression is consistent with our initial observation of reduced caveolin-1 levels in cervical carcinoma-derived cell lines, as many of these cells have extremely low p53 levels (*44*). p53-mediated induction of caveolin-1 gene expression is intriguing, as this would indicate that caveolin-1 is a previously unrecognized p53-regulated gene in cancers. As the observed induction was on the order of 6−12 h, caveolin-1 is likely to be an immediate target. Interestingly, MEF cultures derived from p53-deficient mice also display a distinct lack of caveolin-1 expression (*50*). Furthermore, p53 has also been directly implicated in the up-regulation of caveolin-1 during free cholesterol-mediated cellular growth (*59*). However, it is also possible that p53 could additionally act on its many bona fide target genes (e.g., p21, mdm-2, GADD45, cyclin G, etc.) (*49*), activating signaling cascades, thereby indirectly leading to caveolin-1 induction.

An increase in p53 has been shown to have potent yet diverse effects on cells, ranging from apoptosis to cell cycle arrest at G1 or the G2/M boundaries (reviewed in refs *49* and *60*). Akin to p53 function, proapoptotic activity of caveolin-1 has been previously observed. Transient overexpression of caveolin-1 in cell culture causes apoptosis as assessed by TUNEL assay (*25*). Although, the ability of caveolin-1 to regulate the cell cycle has not been directly addressed, there are indications to that effect. Caveolin-1 inhibits or inactivates a variety of mitogenic signaling pathways (*15*, *22*−*24*). Caveolin-1 expression is down-regulated in rapidly dividing cells and dramatically up-regulated at confluency (*28*). The caveolins are induced during differentiation and are most abundantly expressed in terminally differentiated cell types (*13*). The possible role of caveolin-1 as a p53-regulated inhibitor of cell cycle progression and/or apoptosis is indeed curious and one that will require further experimentation.

We also showed that the ability of a cervical carcinoma cell line to form colonies in soft agar is abrogated upon recombinant expression of caveolin-1. This observation, together with E6's and p53's opposing regulation of caveolin-1 expression, supports an emerging role for caveolin-1 as a tumor suppressor. Indeed, genes with general growth inhibitory capabilities are commonly targeted in carcinogenesis and during the progression of dysplastic lesions to a malignant phenotype.

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