RbAp48 Is a Critical Mediator Controlling the Transforming Activity of Human Papillomavirus Type 16 in Cervical Cancer*

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Although human papillomavirus (HPV) infections are the primary cause of cervical cancer, the molecular mechanism by which HPV induces cervical cancer remains largely unclear. We used two-dimensional electrophoresis with mass spectrometry to study protein expression profiling between HPV16-positive cervical mucosa epithelial H8 cells and cervical cancer Caski cells to identify 18 differentially expressed proteins. Among them, retinoblastoma-binding protein 4 (RbAp48) was selected, and its differentiation expression was verified with both additional cervical cancer-derived cell lines and human tissues of cervical intraepithelial neoplasia and cervical cancer. Suppression of RbAp48 using small interfering RNA approach in H8 cells significantly stimulated cell proliferation and colony formation and inhibited senescence-like phenotype. Remarkably, H8 cells acquired transformation activity if RbAp48 was suppressed, because H8 cells stably transfected with RbAp48 small interfering RNA led to tumor formation in nude mice. In addition, overexpression of RbAp48 significantly inhibited cell growth and tumor formation. This RbAp48-mediated transformation of HPV16 is probably because of the regulation by RbAp48 of tumor suppressors retinoblastoma and p53, apoptosis-related enzymes caspase-3 and caspase-8, and oncogenic genes, including E6, E7, cyclin D1 (CCND1), and c-MYC. In brief, RbAp48, previously unknown in cervical carcinogenesis, was isolated in a global screen and identified as a critical mediator controlling the transforming activity of HPV16 in cervical cancer.

Cervical cancer is one of the most common neoplastic diseases among women, with a combined worldwide incidence of approximately one-half million new cases annually and rates of morbidity and fatality second only to breast cancer (1). In addition, in recent years the average cervical cancer patient has become progressively younger (2). Over 99% of cervical carcinomas are positive for human papillomavirus (HPV)3 DNA (3), indicating that HPV infection is the most important cause for cervical cancer (4, 5). Thus it is of great importance to elucidate the mechanism by which HPV induces cervical cancer from both the pathophysiological and the therapeutic standpoint.

HPV is a double-stranded DNA virus (6) that affects skin and mucosa epithelia and induces hyperplasia, such as in benign hyperplastic verruca and malignant tumors. Although HPV infection appears to be the prerequisite for causing the great majority of cases of cervical cancer, the molecular events following HPV infection of cervical mucosa epithelial cells remain largely unknown. The transforming genes of HPV include the early genes E6 and E7, whose coding proteins bind tumor suppressor proteins p53 and Rb. These bindings result in an imbalance between proliferation and apoptosis, thereby inducing cervical cancer (7). Growing evidence suggests that in addition to p53 and Rb, various HPV E6- and E7-associated molecules and pathways are involved in cell proliferation, differentiation, transcription regulation, telomerase activation, and apoptosis (8–11). Identification of novel target molecules participating in HPV-induced tumorigenesis will provide the foundation for better understanding the mechanisms of cervical cancer.

In this study we utilized a combination of two-dimensional gel electrophoresis (2-DE), image analysis, mass spectrometry, and bioinformatics to quantify and characterize differentially expressed proteins between the HPV16 immortalized human cervical mucosa epithelial H8 cells and cervical cancer Caski cells and identified retinoblastoma-binding protein 4 (RbAp48, also referred to as RBP4) as one of the 18 differentially expressed proteins.

RbAp48, initially identified as a retinoblastoma-binding protein (12), was characterized as a highly abundant component of various chromatin assembly, remodeling, and distinct nucleo-
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some-modifying complexes, including the nuclear histone deacetylases (13, 14). It was reported that E2F-1 and RbAp48 are physically associated in the presence of Rb and histone deacetylase (15), suggesting that RbAp48 could be involved in the transcriptional repression of E2F-responsive genes. p55, the Drosophila ortholog of RbAp48, has been shown to be required for the repression of dE2F2/RBF-regulated genes (16). Fission yeast centromere protein Mis15, which bears a strong resemblance to human RbAp48, is part of the CENP-A recruitment pathway and forms an evolutionarily conserved complex that includes Mis6 (17). RbAp48 was also isolated as one of three radiosensitive genes in a microarray analysis used for selecting radiosensitivity prediction molecules and RbAp48 overexpression-induced radiosensitization in HS-578T, MDA-MB-231, and MALME-3 M cells, two breast cancer and a melanoma cell line, respectively, when compared with mock-transfected cell lines (18). Our studies provided the first evidence linking RbAP48 to cervical cancer, and both in vitro and in vivo studies demonstrated that RbAp48, previously unknown in cervical cancer, is a novel and critical mediator that controls HPV16 transforming activity in cervical carcinogenesis.

EXPERIMENTAL PROCEDURES

Cell Cultures and Protein Extracts—The HPV16 immortalized human cervical mucosa epithelial H8 cell line was obtained from the Department of Biophysics, Institute of Preclinical Medicine, Peking Union Medical University. This cell line was derived from the squamous cervical epithelia of a normal woman. Briefly, cells were transfected with HPV E6E7 following the procedures previously published (19), and an H8 cell line was generated from the untransformed clones (20). Note that the native promoter drives the expression of the viral E6 and E7 genes in established H8 cells. The H8 cell line, which is anchorage-dependent with otherwise normal structure and function except for the characteristics of transformation by HPV E6E7, does not cause cancer within 6 months after injection into nude mice (21). Both H8 and cervical cancer-derived Caski, SiHa, and HeLa cell lines were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 25 ng/ml amphotericin B, and 10% fetal bovine serum (Invitrogen) at 37 °C with 5% CO2. Upon reaching 80–90% confluency, the cultures were harvested. The cell pellets were dissolved in 600 μl of lysis buffer (9 mM urea, 4% CHAPS, 0.5% sodium dodecyl sulfate, 1% dithiothreitol) containing 35 μg/ml leupeptin, and 0.5 μg/ml leupeptin. After three cycles of quick freezing and thawing in liquid nitrogen, 5 μg/ml RNase and 20 μg/ml DNase were added, followed by 30 min of incubation on ice and centrifugation at 13,000 rpm. The supernatant was collected and used for 2-DE. Protein concentration was determined by a protein assay kit (Bio-Rad). Analytical gels used in image analysis were stained in Brilliant Blue G-250 for 2 h and destained overnight. 2-DE gels were scanned with an ImageScanner in transmission mode. Using H8 cell as a reference, the image from the Caski cell was matched using ImageMaster two-dimensional Elite 3.10. The normalized values were analyzed using Student’s t test.

The differentially expressed protein spots were excised from the gels, cut into 1–2 mm² slices, washed with 25 mM ammonium bicarbonate, 50% acetonitrile, and dried in a vacuum concentrator. After the tryptic peptide mixture was solubilized with 0.5% trifluoroacetic acid, peptide mass analysis was performed using a MALDI-TOF mass spectrometer. Mass spectra were externally calibrated with peptide standard from Bruker or internally calibrated with autodigest peaks of trypsin.

Data Base Search—Proteins were identified by peptide mass fingerprinting using the internet-based program Mascot. The following search parameters were applied: cysteines as S-carboxymethyl-derivative (Cys-CAM), maximum allowed peptide mass error 0.1–1 Da, >4 peptide mass hits for protein match, ≤1–2 enzymatic missed cleavages, methionine in oxidized form, protein mass restricted to range 30%, and species of origin restricted to mammalian.

Real Time PCR—Total RNA was isolated from 1 × 10⁷ Caski and H8 cells with the RNasy easy kit (Qiagen, Alameda, CA), and then reverse-transcribed to cDNA as described in the protocol of the Improm-II Reverse Transcriptase system kit (Promega, Madison, WI). Eighteen genes that are identified using proteomics analysis to be differentially expressed between the Caski and H8 cells were validated via quantitative real time (RT)-PCR. Reactions were performed in a 50-μl SYBR GREEN PCR volume in a 96-well optical reaction plate formatted in the 7300 Sequence Detection System (ABI PRISM, Applied Biosystems) using the following PCR conditions: 40 cycles, 95 °C for 15 s, 60 °C for 1 min. The transcript of 18 S rRNA was employed as an internal control for RNA quality. For each gene, three independent PCRs from the same reverse transcription sample were performed. The presence of a single specific PCR product was verified by melting curve analysis and confirmed on an agarose gel. Other components of the RbAp48 complex like HADC1, HADC3, Rb, and E2F1 were also selected to check expression difference using real time PCR assay. The sequence-specific primers that were used in this assay are listed in supplemental Table 2S.

Western Blotting Assay—Protein samples prepared from cell cultures, cervical intraepithelial neoplasia (CIN), and cervical squamous cell carcinoma tissues were quantitated using the Bradford assay and then subjected to 10% SDS-PAGE and electrotransfered to nitrocellulose membranes for 2 h at 100 V using a standard transfer solution (both CIN II lesion and the cancerous tissue were obtained from six tracked patients, aver-
for an additional 4 h at 37 °C, followed by treatment with 150 μl of Me2SO for 30 min and then measured on a Microplate Reader (model 550, Bio-Rad) at λ540 nm. Experiments were performed in triplicate, and the relative absorbance was determined by setting the absorbance on day 1 to 1.

For colony formation assay in soft agar culture, 5 ml of 0.6% agar in RPMI 1640 growth medium were applied in 60-mm diameter dishes, which was allowed to solidify over 2 h. Above this first layer, 1.6 ml of 0.3% agar in RPMI 1640 growth medium with 1 × 10⁵ cells per plate was added. Plates were then incubated at 37 °C in 5% CO₂ and 100% humidity when the top agar layer had solidified for 4 weeks until the clones were counted.

**In Situ β-Galactosidase Staining**—First 2.0 × 10⁴ cells were seeded in each well of a 96-well plate, and 24 h later 200 μl of the cultures were fixed in solution (2% v/v formaldehyde, 0.2% v/v glutaraldehyde in phosphate-buffered saline) for 10 min. The cells were then stained with 100 μl of freshly prepared staining solution (1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) in 0.05 mM potassium ferricyanide, 0.05 mM potassium ferrocyanide, 0.02 mM magnesium chloride, 0.1% Me₂SO) for 4 h at 37 °C, and the Microplate Reader was used to measure the absorbance at λ540 nm.

**Tumor Growth Assay in Nude Mice**—To determine the function of RbAp48 in controlling tumor formation *in vivo*, we subcutaneously injected nude mice with the stable cells as described above. Briefly, cells were trypsinized and then suspended in Dulbecco’s modified Eagle’s medium. Mice in each group (six mice per group) were injected with 1 × 10⁶ cells. Tumor size and volume were measured every 3 days. At the end of the 4th week after injection, tumors were evaluated by gross and microscopic examination.

**Assays for Cancer-related Genes**—Quantitative RT-PCR was first performed to monitor the expressions of tumor suppressors pRb and p53, apoptosis-related enzymes caspase-3 and caspase-8, and oncogenic genes cyclin D1 and c-MYC. Briefly, total RNA was extracted from RbAp48 knockdown and control stable lines and reverse-transcribed to cDNA. PCR was performed using sequence-specific primers (see supplemental Table 2S). PCRs for all samples were performed in an optical tube with 100 ng of total RNA, 1 μl each of primer and probe pre-mix, and 4 μl of TaqMan 5X buffer (Applied Biosystems) in a 20-μl reaction volume. Gene expression assays were done in triplicate, and 18S rRNA was used to normalize mRNA values.

We also examined the effects of RbAp48 on the protein level of Rb using immunofluorescence cell staining. Briefly, cultures plated on chamber slides (Nalge Nunc International, Naperville, IL) were fixed with cold 100% methanol and air-dried. After rehydration in phosphate-buffered saline and blocking with 30% goat serum for 30 min, the cells were incubated with primary antibodies against Rb (Santa Cruz Biotechnology; diluted 1:100) and RbAp48 (diluted 1:100) for 1 h. Secondary antibodies against mouse IgG conjugated with rhodamine (Santa Cruz Biotechnology; diluted 1:100) and anti-rabbit IgG conjugated with fluorescein isothiocyanate (Santa Cruz Biotechnology; diluted 1:100) were applied for 45 min, followed by an incubation with 0.5 mg of 4,6-diamidino-2-phenylindole for 5 min. The specimens were observed under a fluorescence microscope.
microscope with appropriate optical filters. Microscopic images were captured using the Image program (Media Cybernetics, Silver Spring, MD) and an Olympus microscope. To visualize the effect of RbAp48 on the protein levels of molecules of interest, including p53, cyclin D1 and c-MYC, Western blotting assays were performed as described above with anti-p53, anti-cyclin D1 and anti-c-MYC antibodies, respectively (Santa Cruz Biotechnology).

RESULTS

Proteomic Analysis of HPV16 Immortalized Cervical Mucosa Epithelial H8 Cells and Cervical Cancer Caski Cells—The immortalized HPV16-positive human cervical mucosa epithelial H8 cell model is particularly useful for studying late stage aspects of HPV16-induced carcinogenesis of cervical mucosa epithelial cells because of its unique properties, i.e. the cells have the structure and function of normal human cervical mucosa epithelial cells except for the transformation by HPV E6E7, so that they fail to result in carcinogenesis. For the purpose of scanning changes in protein levels between HPV16-immortalized cervical mucosa epithelial H8 cells and cervical cancer Caski cells, we used two-dimensional-IPG with wide range, linear IPGs (pH 3–10) in the first dimension. Triplicate protein lysates from H8 and Caski cells were subjected to 2-DE. Fig. 1 shows Coomassie Brilliant Blue-stained two-dimensional IPG standard maps of one representative experiment with these two cell lines. After spot analysis using ImageMaster stained two-dimensional Elite 3.10, 1882 ± 43 spots in H8 cells (Fig. 1, left) and 1757 ± 61 spots in Caski cells were detected (right).

Thirty six differentially expressed proteins between HPV16 immortalized cervical mucosa epithelial H8 cells and cervical cancer Caski cells were excised from 2-DE gels, digested in gel, and applied to a sample template for a MALDI-TOF mass spectrometry. Twenty proteins spots were identified successfully with the internet-based program Mascot using the peptide mass fingerprinting data. The GenBank™ accession numbers, protein names, and theoretical pi and Mr values, as well as the number of peptide matches and probability of wrong assignment, are presented in Table 1. Note that spots 21 and 33 represent the same protein, i.e. chain B of triose-phosphate isomerase (TPI, Tim), and spots 25 and 26 turned out to be uracil DNA glycosylase. These findings suggest that these two proteins might undergo different post-translation modifications in HPV-induced cervical cancer and display distinct mobility in the gel. Of 18 proteins identified, 6 of them, including RbAp48, are down-regulated, whereas 12 proteins are up-regulated in cervical cancer Caski cells.

The 18 “known” proteins identified here were further classified into several categories on the basis of their known functions, including 7 metabolism-related molecules and 2 categorized as cytoskeletal proteins (supplemental Table 1S). These proteins were abundant in the cell and easily detected by 2-DE. Smaller sets of proteins included one transcriptional mediator (RbAp48) and three signaling molecules; other categories, such as oncoprotein (M2-PK) and protein involved in the process of biological oxidation (superoxide dismutase), were also isolated.

Correlation of Gene Expression at the RNA and Protein Levels—We next investigated the relationship between the mRNA and protein levels of these differentially expressed proteins in HPV16 immortalized cervical mucosa epithelial H8 cells and cervical cancer Caski cells. Previous studies in yeast showed weak correlations between average mRNA levels and average protein levels (24–26). These studies focused on the relationship between absolute amounts of mRNA and protein, whereas a much stronger correlation between mRNA and protein levels was observed during human myeloid development (27). To study mRNA expression, we performed a quantitative RT-PCR
using sequence-specific primers (supplemental Table 2S) for all genes identified. As shown in Fig. 2, although the difference at mRNA levels is not the same as that at the protein level, which is probably because of post-transcription regulation, 15 of 17 genes tested had expression trends at the RNA level similar to those at the protein level, with the exception of prolyl 4-hydroxylase and enolase 1, which are down-regulated at the protein level but up-regulated at the mRNA level in cervical cancer Caski cells when compared with HPV16 immortalized cervical mucosa epithelial H8 cells. It is interesting to note that enolase 1 was also shown to be up-regulated at the mRNA level and down-regulated at the protein level during myeloid differentiation (27). Among all genes examined, the gene encoding transthyretin showed the greatest difference at the mRNA level, approximately a 5.5-fold increase in cervical cancer Caski cells, suggesting that transthyretin is a sensitive biomarker for detecting cervical cancer.

### Reduced Expression of RbAp48 in Human Cervical Cancer—Among all proteins identified, RbAp48 is of particular interest to us because this protein is a highly abundant component of various chromatin assembly, remodeling, and distinct nucleosome-modifying complexes, in which it associates with tumor suppressor retinoblastoma protein and recently isolated a radiosensive marker gene in several cancer cell lines (18). To verify the altered expression between HPV16 immortalized cervical mucosa epithelial H8 cells and cervical cancer Caski cells, a Western blotting assay was performed with cell extracts prepared from these two cell lines. As shown in Fig. 3A, there was a robust reduction (1.00 versus 0.21) of RbAp48 protein but a significant increase (1.00 versus 3.60) of M2-PK in cervical cancer Caski cells compared with HPV16 immortalized cervical mucosa epithelial H8 cells. These findings were further verified in human native tissues. Briefly, cervical intraepithelial neoplasia (CIN II) and cervical cancer tissues were collected from six adult patients and total proteins extracted and detected with antibodies against RbAp48 and M2-PK, respectively. As shown in Fig. 3B (one representative experiment) and summarized in Fig. 3C (mean values from six individual experiments), the similar expression pattern for both RbAp48 and M2-PK was demonstrated in native tissues as that in cell lines. In addition, reduced expression of RbAp48 in cervical cancer was further confirmed with additional cervical cancer-derived cell lines, including HeLa and SiHa cells when compared with the primary human cervical keratinocytes cells (Fig. 4A). Taken together, these experiments confirmed our findings from proteomics analysis of cervical cancer cells and suggested that RbAp48 may play an important role in HPV-induced carcinogenesis.

To determine whether down-regulation of RbAp48 is also true for other human cancers, we next examined the expression of RbAp48 in breast cancer, ovarian cancer, and gastric cancer.
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As revealed in Fig. 4B, no significant differences of RbAp48 expression between cancers and corresponding control tissues were observed, indicating that reduced expression of RbAp48 might be specific for cervical cancer.

Given that RbAp48 is required for the transcriptional repression of E2F activity by Rb, and the repression is mediated, at least in part, by a histone deacetylase complex (13, 14), we next sought to determine whether other components of RbAp48 complex were also altered in Caski cancer cells compared with H8 cells (Fig. 4C). As assayed by real time PCR, the expressions of Rb, HDAC1, and HDAC3 were dramatically reduced in cervical cancer cells, whereas the expression of E2F1 was slightly elevated. These results suggest that reduced Rb-HDACs-RbAp48 repression complex might contribute to the HPV-induced cervical carcinogenesis.

Inhibition of RbAp48 Expression via siRNA-mediated Silencing Results in Significant Stimulation of Cell Proliferation and Colony Formation and a Reduction in Cellular Senescence in Vitro As Well as Leading to Tumor Formation in Nude Mice—To determine whether a specific level of RbAp48 is required for preventing the transformation of HPV-infected cervical epithelial cells to cervical cancer cells, we first suppressed RbAp48 gene expression in H8 cells using an siRNA approach. We identified two 19-nucleotide gene-specific sequences and then generated pSUPER-RbAp48 constructs encoding specific siRNAs targeting these two sequences in the RbAp48 gene. RT-PCR with H8 cells transfected with either pSUPER-RbAp48 constructs or pSUPER vector demonstrated that the siRNAs encoded by these two pSUPER-RbAp48 constructs efficiently knocked down the expression of RbAp48 in the transfected cells (not shown). Next we generated stable lines bearing either pSUPER-RbAp48 (siRbAp48, 1 and 2) or pSUPER vector (CTR) based on H8 cells with a high level of endogenous RbAp48; the level of RbAp48 in each line was examined by a Western blotting analysis. As shown in Fig. 5A, both RbAp48 siRNA (siRbAp48) led to a dramatic inhibition of endogenous RbAp48 when compared with both CTR and parent H8 cells.

Next we investigated the effects of reduced RbAp48 on cell proliferation, colony formation, and cellular senescence. Both an MTT assay (Fig. 5B) and cell number counting (Fig. 5C) revealed that reduction in RbAp48 resulted in enhanced cell proliferation; in addition, reduced RbAp48 significantly

![Image](https://example.com/image1.png)

**FIGURE 3. Reduced expression of RbAp48 and increased expression of M2-PK in human cervical cancer.** A, expression of RbAp48 and M2-PK in immortalized HPV16-positive cervical intraepithelial H8 cells and cervical cancer Caski cells, assayed by Western blotting. Total proteins extracted from H8 and Caski cells were subjected to 10% SDS-polyacrylamide gels and detected with anti-RbAp48, anti-M2-PK, or anti-tubulin (used as a housekeeping gene control). B, expressions of RbAp48 and M2-PK in human CIN and cervical squamous cell carcinoma (cancer) tissues, assayed by Western blotting. Protein extracts prepared from these two tissues were processed as in A. C, RbAp48 is significantly down-regulated in human cervical cancer. CIN and cervical cancer (cancer) tissues were collected from six adult patients, and expression of RbAp48 was assayed as in B. Expression of RbAp48 in each sample was normalized against the β-tubulin endogenous control. The normalized values were then calibrated against the cervical intraepithelial neoplasia value. D, M2PK is significantly up-regulated in human cervical cancer. Samples were processed and data analyzed as in C, except that M2PK antibody was used. The units are arbitrary, and the leftmost bar in each panel indicates a relative level of RbAp48 or M2PK of 1. *** p < 0.001 versus human cervical intraepithelial neoplasia.

![Image](https://example.com/image2.png)

**FIGURE 4. Down-regulation of RbAp48 and other components of RbAp48 complex in cervical cancer cells.** A, reduced expression of RbAp48 in cervical cancer-derived SiHa and HeLa cells. Total proteins extracted from human cervical keratinocytes, SiHa, and HeLa cells were subjected to 10% SDS-polyacrylamide gels and detected with anti-RbAp48 or anti-tubulin (used as an internal control). B, down-regulation of RbAp48 is specific for cervical cancer. Protein extracts prepared from various cancer and control tissues, as indicated, were processed and analyzed as in A. C, expression analyses of HADC1, HADC3, Rb, and E2F1 in H8 and Caski cells, assayed by real time PCR. Expression of genes, as indicated, in each sample was normalized against the 18 S rRNA endogenous control. The normalized values were then calibrated against the H8 cells values that were arbitrarily set as 1. Means from three independent experiments are shown (error bars indicate standard deviations).
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Altered Expression of RbAp48 Affects the Expression of Tumor Suppressors, Oncogenic Genes, and Apoptosis-related Enzymes—We next sought to elucidate the molecular mechanism by which RbAp48 controls the transforming activity of HPV16 in cervical cancer by examining the expression of tumor suppressors pRb and p53, apoptosis-related enzymes caspase-3 and caspase-8, and oncogenic c-MYC gene, molecules critical for carcinogenesis. As shown in Fig. 8, A and C, RbAp48 reduction via the siRNA approach dramatically inhibited the expression of both Rb and p53 tumor suppressors as assayed by real time PCR. Furthermore, immunofluorescent cell staining showed that the Rb protein was barely detectable (Fig. 8B, compare panels b and f) in the H8 cells stably transfected with siRbAp48 that efficiently reduced the level of RbAp48 (Fig. 8B, compare panels a and e); and reduced expression of p53 in siRbAp48 transfected cells was also verified using a Western blotting assay (Fig. 8E). It is interesting to note that RbAp48 also affects the level of apoptosis-related genes, because reduced expression of caspase-3 and caspase-8 was observed in the siRbAp48 stable line, especially caspase-3, whose level was 70% lower in the siRbAp48 line than in the control line (Fig. 8C). On the other hand, the expression of E6 and E7, two oncogenic genes known to play critical roles in HPV-induced cervical cancer, was induced 8- and 28-fold, respectively (Fig. 8D); in addition, both mRNA and protein level of c-MYC, a critical oncoprotein involved in a large number of cancers, were markedly reduced.

To investigate the biological consequence of RbAp48 inactivation in tumorigenesis, we utilized nude mouse xenografts. Stable H8 cells bearing siRbAp48 (1) and control cells were injected into 6-week-old female nude mice. As expected, none of the mice injected with the CTR cells developed tumors within 4 weeks of injection (Fig. 5F, panel a), whereas mice injected with H8 cells stably transfected with pSUPER-RbAp48 developed tumors of considerable size within the same time frame (Fig. 5F, panel b). Pathologic analysis of the resultant tumors revealed that they were poorly differentiated squamous cell carcinoma (not shown). Mean tumor volume, measured at the end of experiment (4 weeks after injection of cells), was 117 ± 26 mm³ for the RbAp48 siRNA group, demonstrating that RbAp48 is a critical modulator of the transforming action of HPV16 in cervical carcinogenesis.

RbAp48 Overexpression Inhibits Cell Proliferation in Vitro and Tumor Growth in Vivo—To further define the function of RbAp48 in cervical carcinogenesis and to determine whether it has the potential for use as a therapeutic target in cervical cancer treatment, we next generated stable cell lines overexpressing RbAp48 in Caski and HeLa cervical cancer cells. As shown in Fig. 6A, approximately a 3–5-fold increase of RbAp48 was observed in the RbAp48 stable lines (RbAp48) when compared with their corresponding CTR cells and parent Caski (left column) or HeLa (right column) cells. We then examined whether overexpression of RbAp48 inhibits cell proliferation and colony formation (Fig. 6, B–D). MTT assay, cell number counting, and soft agar assay indicated this to be the case. In addition, significantly higher β-galactosidase activity was observed in the RbAp48 stable lines than in corresponding control and parent Caski or HeLa cell lines (Fig. 6E). These findings suggest that RbAp48 overexpression suppresses the transformation phenotype and reduces the tumorigenicity of cervical cancer cells in vivo. To test that possibility, we injected nude mice subcutaneously with stable cells overexpressing RbAp48 in Caski cells as well as control cells. As shown in Fig. 7, A and B, tumor growth was substantially inhibited in the RbAp48 group as compared with the CTR group, indicating that the level of RbAp48 is crucial for carcinogenesis in cervical cancer.

FIGURE 5. Inhibition of RbAp48 expression via siRNA-mediated silencing stimulates cell proliferation and colony formation, reduces cellular senescence in vitro, and leads to tumor formation in nude mice. A, RbAp48 is dramatically reduced in the RbAp48 siRNA (1, 5′-CAGGGCATACGCGTACGATG-3′; 2, 5′-CGAGGATACAAAATAGG-3′) stable lines compared with control line. Cell extracts prepared from CTR, siRNA RbAp48 stable cells, as well as H8 parent cells were assayed by Western blotting with anti-RbAp48 antibody. Tubulin was employed as an internal control. B, reduction in RbAp48 increases cell growth rate, measured by MTT assay. Each measurement was made in quadruplicate. The relative absorbance at 540 nm was determined by setting the absorbance on day 1 to 1. C, reduction in RbAp48 increases cell number. The viable cells were counted every other day until day 7. D, reduction in RbAp48 increases colony formation, measured by soft agar assay. Cells were seeded in soft agar, and the colony number was scored at 4 weeks after seeding. **, p < 0.01. E, knockdown of RbAp48 reduces senescence-associated β-galactosidase, assayed by in situ β-galactosidase staining. Cells were stained and the readers measured at λ540 nm. The mean value was from six independent experiments. **, p < 0.005. F, inhibition of RbAp48 leads to cervical carcinogenesis in nude mouse xenografts. Nude mice were injected with either control (a) or siRNA RbAp48 (b) cells and were photographed 4 weeks after injection. One representative mouse from each group is shown; injection site in the control mouse and tumor in the siRNA RbAp48 mouse are indicated by arrows.

increased colony formation, as revealed by a soft agar assay (Fig. 5D). Senescence-associated β-galactosidase is widely used as a biochemical marker for cellular senescence (28), and our staining results showed that knockdown of RbAp48 expression in H8 cells resulted in an ~30% inhibition of the cell senescence-like phenotype (Fig. 5E).

To investigate the biological consequence of RbAp48 inactivation in tumorigenesis, we utilized nude mouse xenografts. Stable H8 cells bearing siRbAp48 (1) and control cells were injected into 6-week-old female nude mice. As expected, none of the mice injected with the CTR cells developed tumors within 4 weeks of injection (Fig. 5F, panel a), whereas mice injected with H8 cells stably transfected with pSUPER-RbAp48
elevated in the siRbAp48 stable line as compared with the control line (Fig. 8, D and E). We also examined whether reduced RbAp48 affects the level of well documented Rb/E2F target genes, including proliferating cell nuclear antigen (PCNA), cyclin E, cyclin A, cyclin D1, and p16 INK4a. Among the molecules tested, cyclin D1 was markedly induced (Fig. 8, D and E), whereas PCNA, cyclin E, cyclin A, and p16 INK4a were not particularly affected by reduced RbAp48 (not shown).

Given that down-regulation of RbAp48 resulted in the reduced expression of p53 and Rb and elevated expression of E6 and E7 in HPV immortalized cells, thereby leading to a significant stimulation on cell proliferation and tumor formation, we next examined whether overexpression of RbAp48 in cervical cancer-derived cells will also affect the level of E6, E7, p53, and Rb. The data in Fig. 9 revealed that this was the case, because elevated p53 and Rb and reduced E6 and E7 were observed in RbAp48 stable cell lines when compared with their corresponding cervical cancer cells. Taken together, these results indicated that the RbAp48-mediated transforming activity of HPV16 in cervical cancer is probably due, at least in part, to its regulation of the expression of tumor suppressors Rb and p53, apoptosis-related peptidases caspase-3 and caspase-8, oncogene E6, E7, c-MYC, and cyclin D1.

**DISCUSSION**

The transformation from human cervical mucosa epithelial cells to cervical cancer cells has been attributed to abnormal expression of oncogenes, tumor suppressor genes, growth factors, growth factor receptors, cell adhesion factors, and DNA repair genes as detected by Northern blot, differential display PCR, and cDNA chips based on transcription level (29). Because mRNA expression level does not always correlate with protein level as a result of post-translation regulation, and because proteins are the executor of genetic information, proteomics provides a powerful, straightforward tool for studying transformation of cells during carcinogenesis. Previous analyses have found some proteins related to human cervical cancer cell lines or patients (30–32), one of which is the important heat shock protein 27 (HSP27). In addition, several new differentially expressed proteins associated with cervical cancer Caski cells were identified through our comprehensive proteomic analysis comparing them with control H8 cells (Table 1).

Of the 18 known proteins identified herein, the most significant changes related to tumors lies in one protein, RbAp48.
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This protein was identified for the first time to be dramatically changed at the protein and RNA level in cervical cancer cells as compared with human mucosa epithelial cells. RbAp48 is best known as a component of large chromatin-associated complexes recruited by tumor suppressor Rb and most likely acts in concert with other proteins to modify histones and/or remodel nucleosomes (33, 34); furthermore, the Rb/RbAp48-associated histone deacetylase complex has been found to be involved in transcriptional repression of E2F-responsive genes (35–37). Our data indicate that expression of RbAp48 is dramatically reduced in cervical cancer-derived cells and human cervical cancer tissues (Figs. 3 and 4A); it appears that down-regulation of RbAp48 is specific for cervical cancer, because RbAp48 remains unchanged in other cancer tissues tested (Fig. 4B). These findings, together with studies demonstrating that RbAp46 (the protein most closely related to RbAp48) suppresses the growth of breast cancer and even reverts transformed phenotypes of breast cancer in vivo (38–40), prompted us to investigate whether RbAp48 plays a role in preventing the transformation of HPV-positive precancerous cells to cervical cancer cells. Reduced expression of RbAp48 via an siRNA silencing approach in HPV-positive precancerous H8 cells led to a robust increase in cell proliferation, colony formation, and phase-specific genes at the end of G1 (47, 48). One mechanism by which Rb represses transcription is to bind and recruit the histone deacetylase HDAC1, presumably to remodel chromatin structure on a target promoter and thereby limit access of the transcriptional machinery to DNA (35–37). RbAp48 is a critical member of the histone deacetylase complex and is required for transcriptional repression of E2F activity through its interaction with the histone deacetylase HDAC1 and HDAC3 (14, 15). RbAp48, Rb, HDAC1, and HDAC3 were significantly down-regulated in cervical cancer cells (Fig. 4C), and forced reduction of RbAp48 using the siRNA approach led to a marked decrease of Rb (Fig. 8A and B), which in turn results in the release of the transcription factor E2F and activates expression of cell cycle-related genes.

In line with recent findings in studies of exocrine glands that RbAp48 overexpression induces p53-mediated apoptosis caused by estrogen deficiency, whereas reducing the expression of RbAp48 through siRNA inhibits apoptosis (49), we found that the levels of p53 and two other apoptosis-related enzymes caspase-3 and caspase-8 were dramatically reduced in cervical mucosa epithelial cells when RbAp48 was inhibited via the same approach (Fig. 8C). It is conceivable that regulation of tumor suppressor p53 and caspase enzymes by RbAp48 con-

FIGURE 8. Molecular mechanisms by which RbAp48 controls the transforming activity of HPV16 in cervical carcinogenesis. A, reduction in RbAp48 significantly inhibits tumor suppressor Rb mRNA level, assayed by real time PCR. Expression of Rb in H8 and RbAp48 stable cells were normalized against the 18 S rRNA endogenous control. The normalized values were then calibrated against the H8 value. The units are arbitrary, and the left bar (H8) indicates a relative level of Rb mRNA of 1. *** p < 0.001. B, reduction in RbAp48 leads to a dramatic reduction in Rb protein level, assayed by immunofluorescence cell staining. H8 cells (top panel) or RbAp48 siRNA stable cells (bottom panel) were stained with either anti-RbAp48 (panels a and e) or anti-Rb (panels b and f), and the overlapping regions of these two signals are shown as “merge” (panels c and g). The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (panels d and h). C, reduction in RbAp48 significantly inhibits p53, caspase 3, and caspase 8 in mRNA level. Expression of p53, caspase 3 (CASP3) and caspase 8 (CASP8) in H8 and RbAp48 siRNA stable cells were normalized against the 18 S rRNA. The normalized values were then calibrated against the H8 values that were set as 1. D, reduction in RbAp48 markedly induces the expression of oncogenes E6, E7, cyclins D1, and c-MYC, assayed by real time PCR. Expression of E6, E7, cyclin D1 and c-MYC in H8 and RbAp48 siRNA stable cells were normalized against the 18 S rRNA. The normalized values were then calibrated against the H8 values that were set as 1. E, reduction in RbAp48 decreases p53 whereas increase cyclin D1 and c-MYC, assayed by Western blotting. Total proteins extracted from H8 cells and RbAp48 siRNA stable cells were subjected to 10% SDS-polyacrylamide gels and detected with anti-p53, anti-cyclin D1, anti-c-MYC, or anti-tubulin (used as an internal control).

Reduction in cellular senescence; in addition, H8 cells that failed to induce tumor formation obtained transforming activity and resulted in a tumor with considerable size in nude mice (Fig. 5F), demonstrating that the level of RbAp48 in cervical mucosa epithelial cells is critical for determining the transforming activity of HPV16 in inducing cervical cancer. The crucial role of RbAp48 in regulating cervical carcinogenesis was further demonstrated by overexpression of RbAp48 in cervical cancer cells. Increased levels of RbAp48 significantly inhibited cancer cell proliferation in vitro and tumor growth in vivo (Fig. 6). These findings also suggest that RbAp48 has the potential to be employed as a therapeutic target to treat cervical cancer.

To elucidate the molecular events underlying RbAp48-controlled transforming activity of HPV16 in cervical cancer, we examined the effect of RbAp48 on Rb, a tumor suppressor, and its mutations and loss of its expression have been linked to several kinds of cancers (41–46). Rb protein functions partly through transcriptional repression of E2F-regulated genes, which regulates progression into the S phase of the cell cycle by activating many S

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FIGURE 9. Overexpressing RbAp48 in cervical cancer cells reduces the levels of E6 and E7 but increases the levels of p53 and Rb. A, overexpression of RbAp48 dramatically inhibits the expression of E6 and E7, assayed by real-time PCR. Expression of E6 and E7 in Caski cells and the corresponding RbAp48 stable line were normalized against the 18S rRNA. The normalized values were then calibrated against the Caski values that were set as 1. B, overexpression of RbAp48 increases the expression of Rb and p53, assayed by real-time PCR. Expression of Rb and p53 in Caski cells and RbAp48 stable line were normalized against the 18S rRNA. The normalized values were then calibrated against the Caski values that were set as 1. C and D, overexpressing RbAp48 increases the level of p53 and Rb in cervical cancer cells, assayed by Western blotting. Total proteins extracted from Caski cells (C) and the corresponding RbAp48 stable line as well as HeLa cells (D) and the corresponding RbAp48 stable line were subjected to 10% SDS-polyacrylamide gels and detected with anti-p53, anti-Rb, or anti-tubulin (used as an internal control).

REFERENCES
1. Franco, E. L., Schlecht, N. F., and Saslow, D. (2003) Cancer J. 9, 348–359
2. You, W., Dainty, L. A., Rose, G. S., Krikav, T., McHale, M. T., Olsen, C. H., and Elkas, J. C. (2005) Obstet. Gynecol. 105, 1405–1409
3. Wright, J. D., and Herzog, T. J. (2002) Curr. Womens Health Rep. 2, 259–265
4. Franco, E. L. (1996) Obstet. Gynecol. Clin. North Am. 23, 597–623
5. Walboomers, J. M., Jacobs, M. V., Manos, M. M., Bosch, F. X., Kummer, J. A., Shah, K. V., Snijders, P. J., Peto, J., Meijer, C. J., and Munoz, N. (1999) J. Pathol. 189, 12–19
6. Schwarz, E., Freese, U. K., Gissmann, L., Mayer, W., Roggenbuck, B., Strelau, A., and zur Hausen, H. (1985) Nature 314, 111–114
7. Chen, J. I., Reid, C. E., Band, V., and Androphy, E. J. (1995) Science 269, 529–531
8. Kuhne, C., and Banks, L. (1998) J. Biol. Chem. 273, 34302–34309
9. Filipov, L., Golubovskaya, V., Hurt, J. C., Byrd, L. L., Phillips, J. M., and Kaufmann, W. K. (1998) Oncogene 16, 1825–1838
10. Liu, Y., Hong, Y., Androphy, E. J., and Chen, J. J. (2000) J. Biol. Chem. 275, 30894–30900
11. Gao, Q., Srinivasan, S., Boyer, S. N., Wazer, D. E., and Band, V. (1999) Mol. Cell. Biol. 19, 733–744
12. Qian, Y. W., Wang, Y. C., Hollingsworth, R. E., Jr., Jones, D., Ling, N., and Lee, E. Y. (1993) Nature 364, 648–652
13. Lai, A., Lee, J. M., Yang, W. M., DeCaprio, J. A., Kaelin, W. G., Jr., Seto, E., and Branton, P. E. (1999) Mol. Cell. Biol. 19, 6632–6641
14. Nicholas, E., Ait-Si-Ali, S., and Trouche, D. (2001) Nucleic Acids Res. 29, 3131–3136
15. Nicholas, E., Morales, V., Magagnoli-Jaulin, L., Harel-Bellan, A., Richard-Foy, H., and Trouche, D. (2000) J. Biol. Chem. 275, 9797–9804
16. Taylor-Harding, B., Binne, U. K., Korenjak, M., Brehm, A., and Dyson, N. J. (2004) Mol. Cell. Biol. 24, 9124–9136
17. Hayashi, T., Fujita, Y., Iwasaki, O., Adachi, Y., Takahashi, K., and Yanagida, M. (2004) Cell 118, 715–729
18. Torres-Roca, J. F., Eschrich, S., Zhao, H., Bloom, G., Sung, J., McCarthy, S., Cantor, A. B., Scuto, A., Li, C., Zhang, S., Jove, R., and Yeatman, T. (2005) Cancer Res. 65, 7169–7176
19. Zheng, J., Wahlstrom, T., Paavonen, J., and Vaheri, A. (1994) Int. J. Cancer 58, 713–720
20. Liu, C. Q., Han, G. Z., Li, K., Si, J. Y., Liu, S. D., and Song, G. X. (1999) Zhong Hua Bing Li Xue Za Zhi 28, 24–27

RbAp48 suggest that cyclin D1 may be also involved in RbAp48 activity in cervical carcinogenesis. The role of cyclin D1 in cervical cancer as well as the molecular mechanism underlying its selective up-regulation by reduced RbAp48 remains to be delineated.

In conclusion, RbAp48 was isolated in a global analysis of protein expression profiling based on two-dimensional gel electrophoresis with mass spectrometry and identified as critical modulator of the transforming activity of HPV16 in cervical cancer in both in vitro and in vivo experiments. Identification of RbAp48 as a novel critical mediator in cervical cancer as well as the elucidation of the molecular events involved provide insights into the processes regulating HPV16-induced carcinogenesis and may highlight a new research direction in understanding pathological mechanisms for cervical cancer. Considering its potent inhibition of tumor growth, RbAp48 also has great potential to be employed as a therapeutic target for treating HPV-related disorders, including cervical cancer.

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21. Naghashfar, Z., DiPaolo, J. A., Woodworth, C. D., and Passaniti, A. (1996) Cancer Lett. 100, 47–54
22. Zor, T., and Selinger, Z. (1996) Anal. Biochem. 236, 302–308
23. Gorg, A., Obermaier, C., Boguth, G., Harder, A., Scheibe, B., Wildgruber, R., and Weiss, W. (2000) Electrophoresis 21, 1037–1053
24. Greenbaum, D., Jansen, R., and Gerstein, M. (2002) Bioinformatics (Oxf.) 18, 585–596
25. Gygi, S. P., Rochon, Y., Franza, B. R., and Aebersold, R. (1999) Mol. Cell. Biol. 19, 1720–1730
26. Futcher, B., Latter, G. I., Monardo, P., McLaughlin, C. S., and Garrels, J. I. (1999) Mol. Cell. Biol. 19, 7357–7368
27. Lian, Z., Kluger, Y., Greenbaum, D. S., Tuck, D., Gerstein, M., Berliner, N., Weissman, S. M., and Newburger, P. E. (2002) Blood 100, 3209–3220
28. Xin, H., Curry, J., Johnstone, R. W., Nickoloff, B. J., and Choubey, D. (2003) Oncogene 22, 4831–4840
29. Cheng, Q., Lau, W. M., Tay, S. K., Chew, S. H., Ho, T. H., and Hui, K. M. (2002) Int. J. Cancer 98, 419–426
30. Bae, S. M., Lee, C. H., Cho, Y. L., Nam, K. H., Kim, Y. W., Kim, C. K., Han, B. D., Lee, Y. J., Chun, H. J., and Ahn, W. S. (2005) Gynecol. Oncol. 99, 26–35
31. Yim, E. K., Meoyng, J., Namakoong, S. E., Um, S. J., and Park, J. S. (2004) DNA Cell Biol. 23, 826–835
32. Castagna, A., Antonioli, P., Astner, H., Hamdan, M., Righetti, S. C., Perego, P., Zunino, F., and Righetti, P. G. (2004) Proteomics 4, 3246–3267
33. Verreault, A., Kaufman, P. D., Kobayashi, R., and Stillman, B. (1996) Cell 87, 95–104
34. Lee, E. Y., To, H., Shew, J. Y., Bookstein, R., Scully, P., and Lee, W. H. (1988) Science 241, 218–221
35. Bookstein, R., Shew, J. Y., Chen, P. L., Scully, P., and Lee, W. H. (1990) Science 247, 712–715
36. Ferres-Marco, D., Gutierrez-Garcia, L., Vallejo, D. M., Bolivar, J., Gutierrez-Avino, F. J., and Dominguez, M. (2006) Nature 439, 430–436
37. Lee, J. O., Russo, A. A., and Pavletich, N. P. (1998) Nature 391, 859–865
38. Li, G. C., Guan, L. S., and Wang, Z. Y. (2003) Int. J. Cancer 105, 762–768
39. Zhang, T. F., Yu, S. Q., Loggie, B. W., and Wang, Z. Y. (2003) Anticancer Res. 23, 4621–4627
40. Zhang, T. F., Yu, S. Q., Deuel, T. F., and Wang, Z. Y. (2003) Anticancer Res. 23, 3735–3740
41. Bosco, E. E., Wang, Y., Xu, H., Zilfou, J. T., Knudsen, K. E., Aronow, B. J., Lowe, S. W., and Knudsen, E. S. (2007) J. Clin. Investig. 117, 218–228
42. Lee, E. Y., To, H., Shew, J. Y., Bookstein, R., Scully, P., and Lee, W. H. (1988) Science 241, 218–221
43. Bookstein, R., Shew, J. Y., Chen, P. L., Scully, P., and Lee, W. H. (1990) Science 247, 712–715
44. Ferres-Marco, D., Gutierrez-Garcia, L., Vallejo, D. M., Bolivar, J., Gutierrez-Avino, F. J., and Dominguez, M. (2006) Nature 439, 430–436
45. Lee, J. O., Russo, A. A., and Pavletich, N. P. (1998) Nature 391, 859–865
46. Morgenbesser, S. D., Williams, B. O., Jacks, T., and DePinho, R. A. (1994) Nature 371, 72–74
47. Black, A. R., and Azizkhan-Clifford, J. (1999) Gene (Amst.) 237, 281–302
48. Muller, H., and Helin, K. (2000) Biochim. Biophys. Acta 1470, M1–M12
49. Ishimaru, N., Arakaki, R., Omotehara, F., Yamada, K., Mishima, K., Saito, I., and Hayashi, Y. (2006) Mol. Cell. Biol. 26, 2924–2935
50. Govan, V. A. (2005) Ann. N. Y. Acad. Sci. 1056, 328–343
51. Jones, E. E., and Wells, S. I. (2006) Curr. Mol. Med. 6, 795–808
52. Au, W. W., Abdou-Salama, S., and Al-Hendy, A. (2007) Gynecol. Oncol. 104, 276–280
53. Jia, X., Liu, B., Shi, X., Gao, A., You, B., Ye, M., Shen, F., and Du, H. (2006) Cell. Biol. Int. 30, 183–189
54. Foster, J. S., Henley, D. C., Bukovsky, A., Seth, P., and Wimalasena, J. (2001) Mol. Cell. Biol. 21, 794–810
55. Wang, A., Schneider-Broussard, R., Kumar, A. P., MacLeod, M. C., and Johnson, D. G. (2000) J. Biol. Chem. 275, 4532–4536
56. Duman-Scheel, M., Weng, L., Xin, S., and Du, W. (2002) Nature 417, 299–304