Human Papillomavirus Type 16 E7 Up-regulates AKT Activity through the Retinoblastoma Protein

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Abstract

Human papillomaviruses (HPV) are small DNA tumor viruses causally associated with cervical cancer. The early gene product E7 from high-risk HPV is considered the major transforming protein expressed by the virus. Although many functions have been described for E7 in disrupting normal cellular processes, we describe in this study a new cellular target in primary human foreskin keratinocytes (HFK), the serine/threonine kinase AKT. Expression of HPV type 16 E7 in HFK caused inhibition of differentiation, hyperproliferation, and up-regulation of AKT activity in organotypic raft cultures. The ability of E7 to up-regulate AKT activity is dependent on its ability to bind to and inactivate the retinoblastoma (Rb) gene product family of proteins. Furthermore, we show that knocking down Rb alone, with short hairpin RNAs, was sufficient to up-regulate AKT activity in differentiated keratinocytes. Up-regulation of AKT activity and loss of Rb was also observed in HPV-positive cervical high-grade squamous intraepithelial lesions when compared with normal cervical tissue. Together, these data provide evidence linking inactivation of Rb by E7 in the up-regulation of AKT activity during cervical cancer progression. (Cancer Res 2006; 66(11): 5555-9)

Introduction

Human papillomaviruses (HPV) are small dsDNA viruses that contribute to 99.7% of all cervical cancers (1). Of the three oncoproteins expressed by this virus, E7 is thought to be an important contributor to cervical cancer progression through its inhibition of the retinoblastoma (Rb) protein (2). Recent work by our laboratory and others has linked E7 to the serine/threonine kinase AKT. AKT regulates many processes within the cell that, if dysregulated, can contribute to tumor formation and cancer (3, 4). Previous work has provided evidence of E7 deregulating AKT activity during cellular arrest and growth factor stimulation in non-HPV host cell lines (5, 6). The present study shows regulation of AKT by E7 during keratinocyte differentiation and a connection between loss of Rb and up-regulation of AKT activity. The ability of E7 to up-regulate AKT activity requires interaction with Rb protein. Furthermore, activation of AKT is observed in HPV type 16 (HPV-16)–positive high-grade squamous intraepithelial lesions (HSIL), thus providing evidence that this function of E7 may contribute to cervical cancer progression.

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Materials and Methods

Plasmids. The pBabe (puro) and pBabe-E6stopE7 (designated as E7) retroviral constructs were described previously (7). Site-directed mutagenesis (QuickChange kit, Stratagene, La Jolla, CA) was used to introduce a point mutation into the pBabe-E6stopE7 backbone, which changed residue 24 from a cysteine to a glycine [pBabe-E6stopE7.C24G (designated as E7.24)]. pSuper-retro constructs expressing short hairpin RNAs (shRNA) against no known annotated gene (*shScram*) or targeting Rb (shRb1 and shRb2) were cloned as described previously (8). All retroviral plasmid constructs were sequenced before transfection into ΦNYX-GP packaging cells.

Cell culture. Primary human foreskin keratinocytes (HFK) were isolated from neonatal foreskin and transduced with retrovirus produced in ΦNYX-GP packaging cell line (American Type Culture Collection, Rockville, MD) as described previously (7, 9).

Differentiation of HFK cell lines in organotypic raft cultures. pBabe, E7, E7.24, shScram, shRb1, and shRb2 transduced HFK cell lines were differentiated in organotypic raft cultures as described previously (10). The raft cultures were harvested, fixed in 4% paraformaldehyde, and then embedded in paraffin for subsequent sectioning and staining with H&E. To label DNA-synthesizing cells, 20 μmol/L bromodeoxyuridine (BrdUrd) was added to the raft culture 12 hours before harvest.

Reverse transcription-PCR analysis. RNA was harvested, and reverse transcription was done as described previously (7, 11). PCR analysis using primers for E7 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was done as described previously (11).

Western blot analysis. Protein lysate concentrations were either 50 or 100 μg for E7 blots as described previously (6). The following primary antibodies were used in this study: mouse monoclonal anti-Rb (1:1,000; BD PharMingen, San Jose, CA), rabbit polyclonal anti-phosphorylated AKT (Ser^{473}) and anti-total AKT (1:1,000; Cell Signaling, Danvers, MA), mouse monoclonal anti-p120RasGAP, p130, and actin (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-E7 (1:250; Zymed, Carlsbad, CA), rabbit polyclonal anti-keratin-1 (1:5,000; Covance, Princeton, NJ), and goat polyclonal anti-p107 (1:250; Santa Cruz Biotechnology). Secondary antibodies used in this study were goat anti-rabbit horseradish peroxidase (HRP), goat anti-mouse HRP, and donkey anti-goat HRP (1:2,000; Santa Cruz Biotechnology).

Immunofluorescent staining of tissue sections. Paraffin-embedded organotypic raft sections were deparaffinized in Propar reagent (Anatech Ltd., Battle Creek, MI) and then rehydrated with step-down concentration of ethanol. After antigen retrieval with citrate buffer (BioGenex, San Ramon, CA), sections were stained for phosphorylated AKT and Rb. Sections stained with anti-BrdUrd antibody were incubated in 4 N HCl immediately after rehydration for 30 minutes and then treated with 1 mg/mL trypsin in PBS for 10 minutes at 37°C. Sections were mounted with coverslips and ProLong Gold antifade reagent plus 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes, Carlsbad, CA). The following antibodies were used in this study: rabbit monoclonal anti-phosphorylated AKT (Ser^{473}) immunohistochemistry specific (1:25; Cell Signaling), mouse monoclonal anti-Rb (1:25), mouse monoclonal anti-BrdUrd conjugated to fluorophore 594 (1:100; Molecular Probes), rabbit polyclonal anti-keratin-1 (1:2,500), and goat anti-mouse and anti-rabbit conjugated to fluorophores 488 or 594 (1:250; Molecular Probes). Images were captured with a Leica (Allendale, NJ) DM IRB microscope equipped with 20× (0.4 numerical aperture) Plan Fluotar objective and a PCO SensiCam^{QE} CCD camera controlled with Image-Pro Plus 5.0 software.

Results

HPV-16 E7 up-regulates AKT activity in primary HFKs differentiated in organotypic raft cultures. Primary HFKs were transduced with retrovirus that expresses HPV-16 E7 or control virus. Before subsequent experiments, Western blot analysis was carried out to ensure E7 expression (Fig. 1*A*). Interestingly, even in cycling E7 keratinocytes, there seems to be an increase in the amount of active AKT (phosphorylated AKT Ser^{473}), but not total AKT, when compared with control HFK (pBabe; Fig. 1*A*).

After confirming E7 expression in the HFK cell lines, organotypic raft cultures were set up for 14 days. Morphologic analysis of the rafts (H&E; Fig. 1) indicates that the E7-expressing rafts have a thicker basal layer with intact nuclei throughout, indicative of a hyperproliferative state, in contrast to the control raft that shows normal morphology (Fig. 1*B*). Immunofluorescence staining of keratin-1 was carried out to evaluate the differentiation status of the raft cultures. Control rafts (pBabe) exhibited strong keratin-1 staining in the spinous and granular layers, with little or no staining in basal and cornified layers, in agreement with other reports (Fig. 1*B*; ref. 10). In contrast, E7 rafts exhibited low levels of keratin-1 expression in the spinous layers, with weak staining in the granular layer (Fig. 1*B*). To measure proliferative difference between E7 and control rafts, sections were stained for BrdUrd incorporation. Control rafts have BrdUrd-positive cells in the basal layer only, consistent with normal skin biology (Fig. 1*B*), whereas E7-expressing rafts have BrdUrd-positive cells in almost all layers of the skin, indicative of hyperproliferation (Fig. 1*B*).

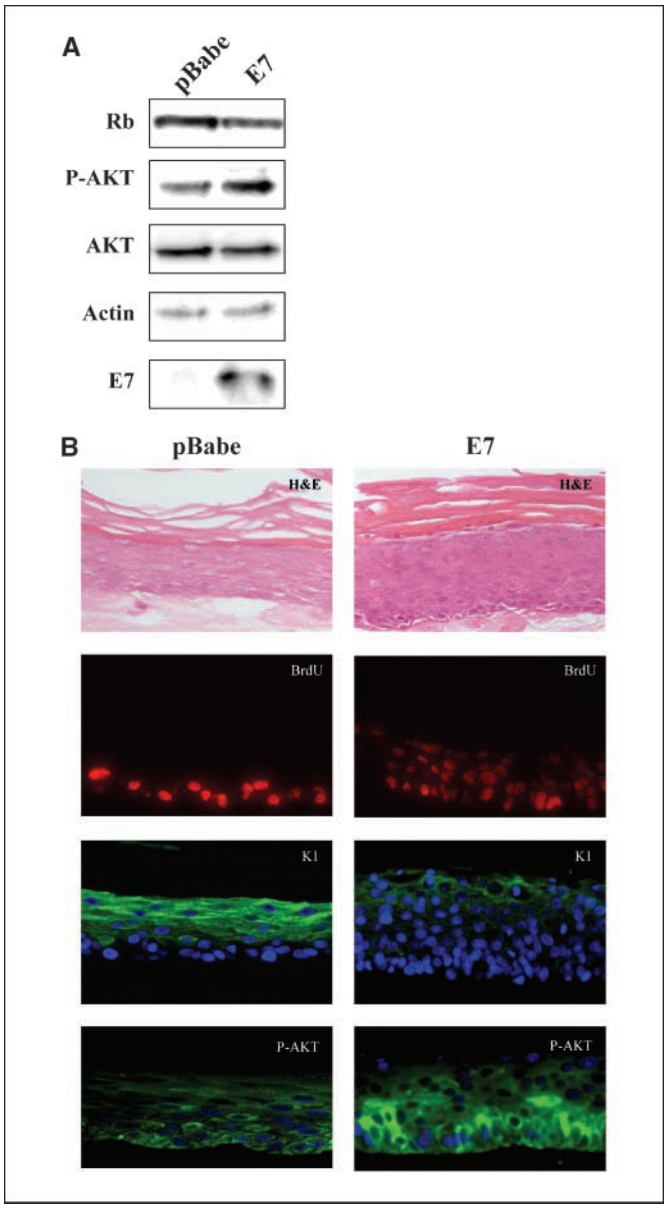


Figure 1. HPV-16 E7 up-regulates AKT activity in primary human keratinocytes (HFK). *A,* primary HFKs were transduced with retrovirus expressing HPV-16 E7 or vector control and Western blots were carried out to determine Rb, phosphorylated AKT (*P-AKT*), AKT (total), and E7 levels. Actin is a loading control. *B,* same cells were differentiated on organotypic raft cultures for 14 days. Cells were pulsed with 20 μmol/L BrdUrd 12 hours before harvesting rafts. The rafts were then fixed, paraffin embedded, sectioned, and stained for BrdUrd (*red*), keratin-1 (*K1*; *green*), phosphorylated AKT (*green*), and DAPI (*blue*) to stain nuclei. H&E-stained sections for both cell lines to examine morphology.

To determine if AKT activity is up-regulated in the E7 rafts, a phosphorylated-specific antibody (Ser^{473}) was used to detect phosphorylated AKT. This antibody has been previously used for immunohistochemistry and immunofluorescent studies evaluating active AKT levels in various cancer tissues (12, 13). Control rafts showed weak phosphorylated AKT staining in the basal and suprabasal layers, whereas E7 rafts exhibited strong staining for phosphorylated AKT in cells within the basal and suprabasal layers (Fig. 1*B*). The up-regulated phosphorylated AKT staining in the E7 rafts was confined to the membrane and cytoplasm of positive cells, which is consistent with other reports (12, 13). Thus, it seems that HPV-16 E7 up-regulates AKT activity in both undifferentiated and differentiated primary HFK.

HPV-16 E7 up-regulates AKT activity in primary HFK differentiated in organotypic raft cultures through disruption of Rb. Next, we wanted to address the possible mechanism through which E7 deregulates AKT signaling. The ability of E7 to bind to and inactivate the tumor suppressor Rb is considered to be the main function of this viral oncoprotein (2). To establish if E7 up-regulates AKT activity through Rb, we used a HFK cell line that expressed an E7 mutant (E7.24), which is Rb-binding deficient. The E7 and E7.24 HFK lines were evaluated for E7 expression by reverse transcription-PCR (RT-PCR) analysis because the E7 antibody used previously is against the NH_2 terminal of E7 and thus may not detect the mutant E7.24 (Fig. 2*A*). The cell lines were placed onto organotypic raft cultures for 14 days and processed as described previously.

The H&E staining shown in Fig. 2 shows that the morphology of the E7.24-expressing rafts is more similar to the control pBabe rafts, indicating that the abrogation of the Rb family function is important for the hyperproliferative phenotype observed in the wild-type E7-expressing rafts. In addition, keratin-1 expression and BrdUrd incorporation illustrate identical distribution between control and E7.24 rafts (Fig. 2). Immunofluorescence staining for phosphorylated AKT was again used to evaluate active AKT in the raft cultures. Wild-type E7 contained strong staining phosphorylated AKT cells in the basal and suprabasal layers, whereas control and E7.24-expressing rafts exhibited weaker phosphorylated AKT staining confined to the basal layer (Fig. 2).

To determine if there is a correlation between loss of Rb and up-regulation of AKT activity, we evaluated Rb levels in E7 and control rafts. The results of these experiments are shown in Fig. 2. Rb protein levels are robust within the basal and suprabasal layers of control and E7.24 rafts but are not present in the wild-type E7 rafts. These data together suggest that E7 up-regulates AKT activity,

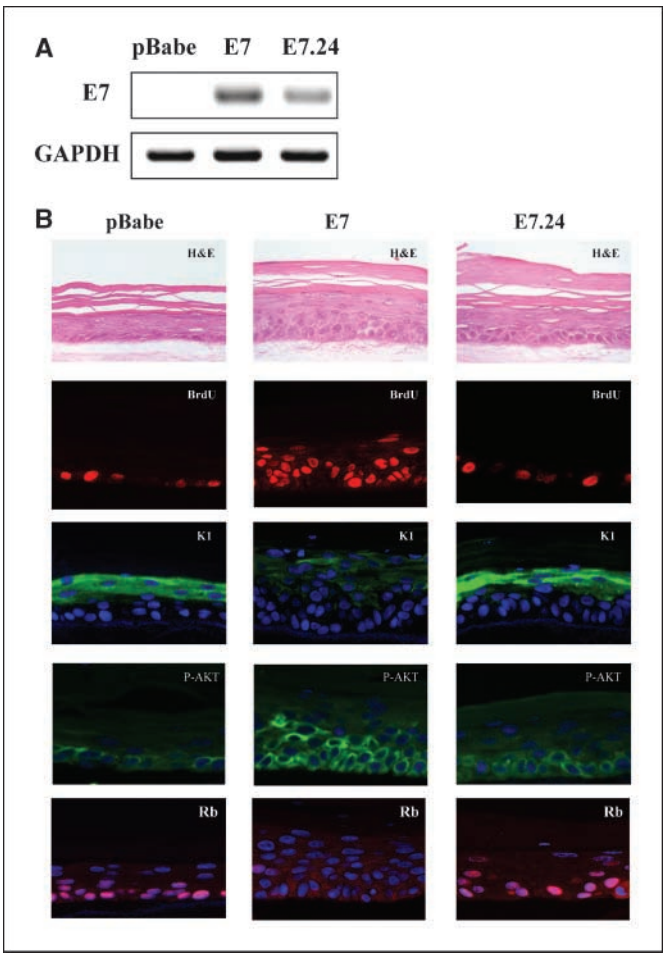


Figure 2. HPV-16 E7 up-regulates AKT activity in HFK through its interaction with Rb. Primary HFKs were transduced with retrovirus expressing HPV-16 E7 or the Rb-binding–deficient point mutant E7.C24G. *A,* RT-PCR analysis was done to evaluate E7 and E7.24 expression. GAPDH is a loading control. Ratio of densitometry of E7 to GAPDH levels. *B,* cells were then differentiated on organotypic raft cultures for 10 to 14 days and pulsed with 20 μmol/L BrdUrd 12 hours before harvesting the rafts. The rafts were then fixed, paraffin embedded, sectioned, and stained for BrdUrd (*red*), keratin-1 (*green*), phosphorylated AKT (*green*), Rb (*red*), and DAPI (*blue*) to stain nuclei. Rb-positive nuclei appear pink due to Rb (*red*) and the DAPI (*blue*) staining. H&E-stained sections for all cell lines to examine morphology.

disrupts differentiation, and causes hyperproliferation within the skin through its interaction with the Rb family members.

To further implicate Rb in the up-regulation of AKT activity, RNA interference, using two different shRNA molecules against Rb, was used to address this issue. These retroviruses expressing shRNAs to Rb (shRb1 and shRb2) were transduced into HFKs along with a nonspecific shRNA control (shScram). Western blot analysis showed that both shRNA molecules, shRb1 and shRb2, designed were able to knock down Rb protein levels efficiently, with a knockdown of 70% and 80%, respectively (Fig. 3*A*). Both Rb family members p107 and p130 protein levels were shown not to change, showing the specificity of the shRNA molecules (Fig. 3*A*).

The shRNA HFK cell lines were placed on organotypic raft cultures for 14 days and processed as described previously. Parakeratosis, an indication of inhibition of terminal differentiation, was observed in the H&E stains of the two shRb rafts when compared with the control (Fig. 3*B, arrows*). A reduction in keratin-1 staining was observed in the Rb knockdown rafts in the suprabasal layers when compared with the control raft, consistent with the E7 raft (Fig. 3*B*). BrdUrd incorporation was evaluated using immunofluorescence to indicate proliferating cells. Interestingly, BrdUrd-positive cells were only found in the basal layer of all three raft sections, suggesting that knocking down Rb levels was sufficient to abrogate keratin-1 expression but not sufficient to cause hyperproliferation. This is presumably due to the presence of the other two Rb family members, p107 and p130.

AKT activity was evaluated in the Rb knockdown rafts as before and was consistent staining in the E7-expressing rafts (Fig. 3). Strong staining for phosphorylated AKT in the basal and suprabasal layers was observed in shRb rafts when compared with the control raft (Fig. 3*B*). The fact that neither one of the Rb knockdown rafts were hyperproliferative, yet both had stronger staining for active AKT, suggests that up-regulation of AKT activity is independent of hyperproliferation in the skin. Therefore, up-regulation of AKT activity through disruption of Rb is a specific function of E7 and not a subsequent consequence of hyperproliferation.

An up-regulation of phosphorylated AKT is observed in HPV-16-positive cervical HSILs and correlates with loss of Rb. The data presented thus far describe the ability of HPV-16 E7 to up-regulate AKT activity, through disruption of Rb, in an *in vitro* system of epithelial differentiation. To examine if up-regulation of active AKT occurs *in vivo* as a result of HPV infection, we determined phosphorylated AKT levels in 10 cervical biopsies from patient with HSILs. Up-regulation of phosphorylated AKT was observed in all cases of HSIL when compared with normal tissue within the biopsies (Fig. 4*A* and *B*). In normal tissue, active AKT was found in the basal and suprabasal layers of the skin, whereas strong phosphorylated AKT-stained cells were found throughout all epithelial layers of the HSIL sections (Fig. 4*A*).

To correlate the up-regulation of AKT activity in the biopsies with the presence of HPV-16 E7, DNA was isolated from each sample and PCR analysis was used to confirmed the presence of HPV-16 genome using primers to E7 (Fig. 4*B*). HPV-16 E7 was present in 70% of the biopsies tested.

To correlate the loss of Rb with overactivation of AKT *in vivo*, a HPV-16 E7-positive cervical specimen, containing both normal and HSIL areas, was costained for Rb (Fig. 4*C, red*) and phosphorylated AKT (Fig. 4*C, green*). In normal cervical tissue, Rb is found in the nucleus of basal and suprabasal cells of the skin (Fig. 4*C*), and in striking contrast, Rb appears almost totally absent in the HSIL tissue (Fig. 4*C*). Taken together, these data correlate with the *in vitro* observations, which suggest that HPV-16 E7 up-regulates AKT activity through its disruption of Rb during cervical cancer progression.

Discussion

The activity of the serine/threonine kinase AKT is up-regulated in many human cancers, including various carcinomas (3). The data presented in this study show that the serine/threonine AKT is a bonafide target of HPV-16 E7 in primary human keratinocytes. Previous studies in our laboratory linked E7 to altered AKT activity during cellular arrest through Rb (6). A recent report showed that E7 could increase AKT activity during growth factor stimulation in a Rb-independent manner (5). Both of these studies linking E7 to altered AKT signaling were done in immortalized cell lines, which are not the natural epithelial host cell of HPV. The data in this study provide the first evidence that HPV-16 E7 up-regulates AKT activity in primary HFKs. Furthermore, we show that E7 activates AKT during epithelial differentiation. The ability of E7 to up-regulate

AKT activity in keratinocytes was dependent on Rb, and knocking down Rb with shRNAs was sufficient to increase phosphorylated AKT in raft cultures. Together, these data provide evidence that inhibition of Rb by E7 causes up-regulation of AKT activity in keratinocytes.

Because HPV is causally associated with cervical cancer, we also evaluated if AKT activity was altered in cervical biopsies. We show that AKT is indeed up-regulated in neoplastic tissue sections when compared with normal tissue and strongly correlates with HPV-16 E7 expression. Consistent with the *in vitro* data, we also observed a loss of Rb expression in the HSIL sections. Others have also described loss of Rb in HSIL sections positive for high levels of HPV-16 E7 protein (14).

Although we have provided data that HPV-16 E7 up-regulates AKT activity in the skin, we have not yet shown the biological consequence of this high AKT activity. AKT can regulate many important cellular functions, such as cell cycle progression, survival, differentiation, migration, and other processes, which, if dysregulated, could contribute to cancer. In our *in vitro* raft system of keratinocyte differentiation, up-regulation of AKT activity in HPV-16 E7 and shRb rafts seemed to correlate more with inhibition of keratin-1 rather than hyperproliferation. There is evidence in the literature showing that AKT regulates various aspects of keratinocyte differentiation, including survival during stratification, as well as early differentiation and late differentiation marker expression (15, 16). What specific function of differentiation AKT is disrupting in the context of E7 is still unclear but is of future interest.

The mechanism by which E7 up-regulates AKT activity is still unclear. Previous work established that E2F transcription factors regulated AKT activity through Gab2 (17). Gab2 is a scaffold protein that binds to phosphatidylinositol 3-kinase (PI3K) and regulates its activity as well as downstream effectors, such as AKT. E2F transcription factors have been shown previously to regulate AKT activity during the cell cycle through transcriptional regulation of Gab2 (17). Transcriptional up-regulation of Gab2 mRNA is mediated by E2F transcription factors, and disruption of Rb by adenovirus E1A increased levels of Gab2 mRNA (17). We have preliminary evidence that expressing Gab2 in primary human keratinocytes increases AKT activity, whereas a mutant Gab2, unable to bind PI3K, does not. Therefore, it is plausible that disrupting Rb in human keratinocytes may lead to up-regulation of Gab2 levels, which in turn increases AKT activity. Evaluating the role of Gab2 in Rb-mediated AKT activation is of future interest.

There is a large body of literature also linking up-regulation of AKT activity to human cancer progression from various tissue origins (3). AKT activity is found to be up-regulated in carcinomas, glioblastomas, and hematologic cancers (3). In fact, there have been previous studies showing high phosphorylated AKT staining in HSIL cervical tissue samples specifically (18). Therefore, up-regulation of AKT activity by E7 may contribute to the progression of cervical cancer and thus may represent a new chemotherapeutic target for treatment of cervical neoplasia.

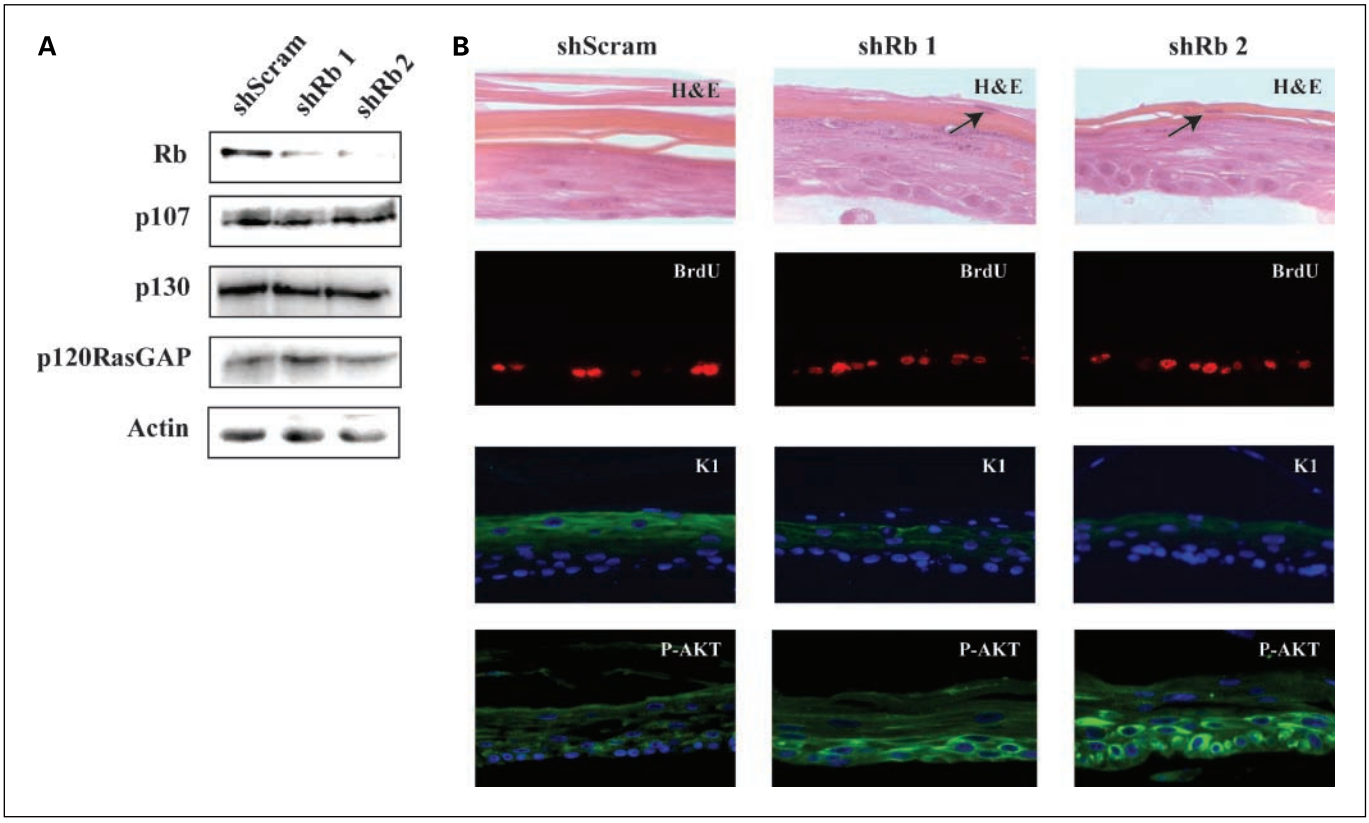
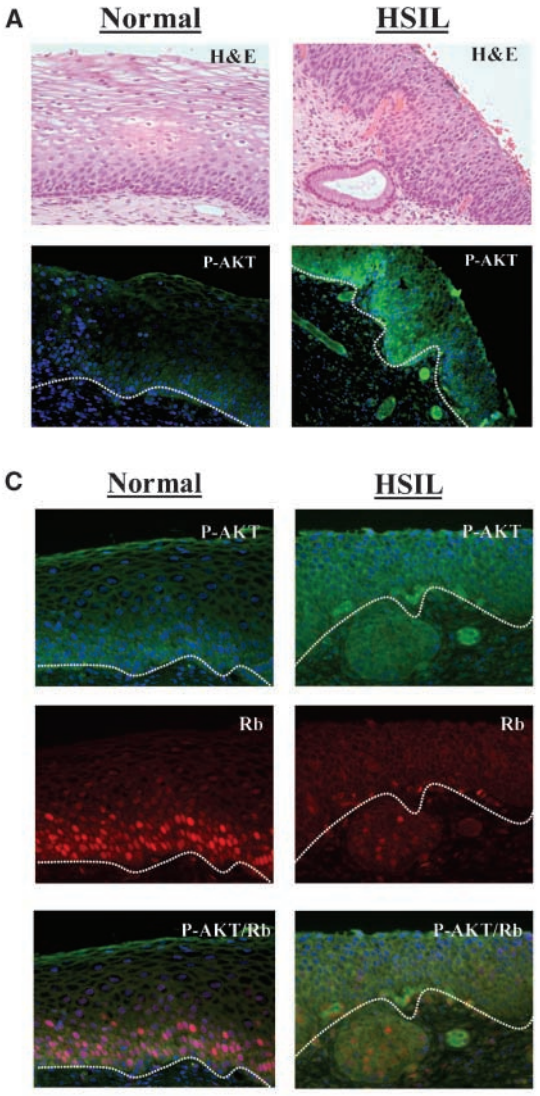


Figure 3. Knockdown of Rb is sufficient to up-regulate AKT activity in HFK. *A,* primary HFKs were transduced with retrovirus expressing two different shRNAs targeting Rb (shRb1 and shRb2) as well as a shRNA targeting no known gene (*shScram*) as control. Western blot analysis was done to measure the amount of knockdown of Rb. The relative knockdown of Rb, determined by densitometry analysis, was ~70% and 80% for shRb1 and shRb2, respectively. The levels of Rb family members p107 and p130 are unaltered. p120RasGAP and actin are loading controls. *B,* cells were then differentiated on organotypic raft cultures for 14 days. Cells were pulsed with 20 μmol/L BrdUrd 12 hours before harvesting rafts. The rafts were then fixed, paraffin embedded, sectioned, and stained for BrdUrd (*red*), keratin-1 (*green*), phosphorylated AKT (*green*), and DAPI (*blue*) to stain nuclei. H&E-stained sections for all cell lines to examine morphology.



B

Biopsy Sample	Normal or HSIL	P-AKT (IFA)	HPV-16 E7 PCR Analysis
1	Normal HSIL	+ +++	+
2	HSIL	+++	+
3	Normal HSIL	+ +++	-
4	Normal HSIL	+ +++	+
5	HSIL	+++	+
6	HSIL	+++	+
7	Normal HSIL	+ +++	-
8	HSIL	+++	+
9	HSIL	+++	+
10	Normal HSIL	+ +++	-

Figure 4. HPV-positive cervical biopsies with HSILs have up-regulated AKT activity and Rb loss. *A,* cervical biopsy #4 section exhibiting normal or HSIL morphology based on H&E staining were immunostained for active AKT. Phosphorylated AKT is stained with FITC (*green*), whereas nuclei are stained with DAPI (*blue*). *B,* phosphorylated AKT staining in HSIL was compared with normal areas within the biopsies. Each biopsy was given a sample number (1-10) and scored for high (+++) or low (+) phosphorylated AKT immunostaining within normal and/or HSIL areas within the samples. DNA was also isolated from each biopsy, and PCR analysis was carried out to look for the presence (+) or absence (−) of HPV-16 E7. *C,* normal and HSIL tissue from cervical biopsy #4 were stained for Rb (*red*), phosphorylated AKT (*green*), and DAPI (*blue*) to stain nuclei. Overlay of Rb (*red*), phosphorylated AKT (*green*), and DAPI (*blue*) staining.

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