Published in final edited form as:

Mol Carcinog. 2009 January ; 48(1): 45–55. doi:10.1002/mc.20456.

Activation of the Retinoblastoma Tumor Suppressor Mediates Cell Cycle Inhibition and Cell Death in Specific Cervical Cancer Cell Lines

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Abstract

High-risk human papilloma virus (HPV) encodes two oncoproteins, E6 and E7, which are vital to viral replication and contribute to the development of cervical cancer. HPV16 E7 can target over 20 cellular proteins, but is best known for inactivating the retinoblastoma (RB) tumor suppressor. RB functions by restraining cells from entering S-phase of the cell cycle, thus preventing aberrant proliferation. While it is well established that HPV16 E7 facilitates the degradation of the RB protein, the ability of the RB pathway to overcome E7 action is less well understood. In this study the RB-pathway was activated via the overexpression of the p16ink4a tumor suppressor or ectopic expression of an active allele of RB (PSM-RB). While p16ink4a had no influence on cell cycle progression, PSM-RB expression was sufficient to induce a cell cycle arrest in both SiHa and HeLa cells, HPV positive cervical cancer cell lines. Strikingly, this arrest led to the down-regulation of E2F target gene expression, which was antagonized via enhanced HPV-E7 expression. Since down-modulation of E7 function is associated with chronic growth arrest and senescence, the effect of PSM-RB on proliferation and survival was evaluated. Surprisingly, sustained PSM-RB expression impeded the proliferation of SiHa cells, resulting in both cell cycle inhibition and cell death. From these studies we conclude that active RB expression can sensitize specific cervical cancer cells to cell cycle inhibition and cell death. Thus, targeted therapies involving activation of RB function may be effective in inducing cell death in cervical cancer.

Keywords

HPV; RB; E7; apoptosis; cervical cancer

Introduction

Cervical cancer remains the second most abundant cancer in women worldwide. With over half a million new occurrences each year, accompanied by over 230,000 deaths [1], treatment of cervical carcinogenesis is an area of intense study. Over 90% of cervical cancers are caused by the high-risk serotypes of the human papilloma virus (HPV), HPV16 and HPV18 [2,3]. Specifically, two oncoproteins of the HPV genome, E6 and E7, contribute to the transformation of infected cells [4,5]. HPV initially infects the host through the basal

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cell layer of the epithelium, wherein the virus remains in low copy number in the squamous epithelium. Interestingly, viral replication occurs in the differentiated compartment of the epithelium where cells have exited the cell cycle. Therefore, the virus must reinitiate the host cell cycle machinery to allow for replication to take place. This forced cell cycle re-entry is accomplished by maintaining the expression of the viral oncoprotein E7.

HPV E6 targets the tumor suppressor p53 for proteolytic degradation [6,7], and E7 binds and facilitates the inactivation of the retinoblastoma tumor suppressor (RB) [8]. While enhanced degradation of these two tumor suppressors allows for the virus to replicate and thrive in the cell, the onset of tumorigenesis is due to viral integration that results in the chronic expression of these oncoproteins [9]. E7 has been shown to, not only target RB, but also target the pocket protein family members p107 and p130 for degradation [8,10]. All RB protein family members share a highly conserved C-terminal region known as the “pocket domain” that is responsible for binding E2F transcription factors. However, this same region is exploited by E7, in which tight and preferential binding is believed to compete for association of cellular RB targets [11–14]. E7 has also been implicated in targeting over 20 other cellular proteins, including Brg-1, HDAC-1, p21^{CIP1} and p27^{KIP1} via interactions with the C-terminus of the E7 protein [15–18]. Overall, these interactions serve to disrupt cell cycle control and ultimately lead to the onset of tumorigenesis.

By inactivating RB and promoting its degradation, E7 circumvents the tumor suppressive function of RB, subsequently promoting uncontrolled cell division. The primary function of RB is to bind the E2F family of transcription factors and prevent entry into S-phase of the cell cycle [19–21]. RB acts as a transcriptional co-repressor, inhibiting expression of multiple genes necessary for cell cycle progression [22,23]. One key target of RB is cyclin A, which exhibits a short half-life in the presence of RB and is required for cell cycle progression [24,25]. The ability of RB to repress target gene transcription is dependent on the interaction with E2Fs and subsequent recruitment of co-repressors (e.g. HDAC, SWI/SNF) that aid in the repression of E2F target genes [23]. Upon RB inactivation (via phosphorylation by CDK/cyclins), the association of RB with E2F is disrupted, allowing for the expression of E2F regulated genes [26]. E2F target genes are implicated in promoting cell cycle (e.g. Cyclin A, Cyclin E) or DNA replication (e.g. MCM2, RNR-II), and the RB pathway plays a critical role in coordinating S-phase entry [26]. These cell cycle promoting events can be reversed by the induction of p16^{ink4a}, another tumor suppressor upstream of RB in the RB-pathway; p16^{ink4a} functions to prevent CDK/cyclin dependent phosphorylation of RB, allowing RB to remain bound to E2F and preventing transcription of S-phase associated genes [27,28].

It is known that HPV E7 inactivates cellular RB by tightly binding the protein and sequestering it for proteolysis [8]. In this study we examined the role of the RB pathway in titrating HPV16 E7 activity. While expression of p16^{ink4a} proved unsuccessful in re-activating endogenous RB in both cervical cancer cell lines, expressing an active allele of RB that cannot be phosphorylated was sufficient to overcome the growth stimulating effects of E7 in SiHa and HeLa cells. Interestingly, we found that under conditions of sustained PSM-RB expression, SiHa and HeLa cells were not viable, while CaSki cells were relatively resistant to RB expression. Combined, these results suggest a role for RB mediating cell death of cervical cancer cells.

Materials and Methods

Cell Culture and Adenoviral Infections

The following cell lines were utilized based on their HPV status. SiHa and CaSki cells harbor HPV16 DNA, HeLa cells contain HPV18 DNA, and all express the E6 and E7

oncoproteins [40]. The HPV negative TSUPR-1 cells are derived from a bladder carcinoma and contain a functional RB pathway. TSUPR-1, SiHa and HeLa cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM), and CaSki cells were cultured in modified Eagle's medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin-streptomycin and 2 mM L-glutamine at 37° C in 5% CO₂. All cell lines were transduced with adenoviruses encoding GFP, PSM-RB, or p16ink4a at a MOI of 10 for 24–96 hours as indicated in each experiment.

Cell Growth Assays

TSUPR-1, SiHa, HeLa, and CaSki cell lines were transduced with adenoviruses as mentioned above. Initial time points were taken prior to transduction, at 0 hours, for each cell line. Cells were washed with PBS and fixed for 5 minutes in ice-cold 100% ethanol. Cells were stained with 1% Crystal Violet and washed three times in dH₂O. At least 5 random fields were counted per plate.

Transfection and Luciferase Assays

SiHa, HeLa and CaSki cells were transfected using FuGene 6 (Roche) with the indicated plasmid constructs: CMV-β-Galactosidase (0.5ug), cyclin A-LUC (0.5ug), empty vector (0.5ug), PSM-RB (0.5ug), HPV16 E7 (0.5ug), or p16ink4a (0.5ug) were transfected into each well of a 6-well dish for 48 h. Cells were harvested and luciferase activity was determined using luciferase assay kit (Promega). β-Galactosidase activity was used as an internal control for transfection efficiency and was measured utilizing the manufacturer's recommended protocol for the Galacto-Star kit (Tropix). Basal activity was set to "1," and relative luciferase activity is shown. Experiments were performed in triplicate with averages and standard deviations shown.

Immunoblotting

Cell lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon-P membrane (Millipore). Membranes were incubated with the following antibodies: Santa Cruz: anti-cyclin A (H432), anti-Lamin B (M20), anti-β-tubulin (D10), anti-p16ink4a (H156), anti-p107 (C-18), and anti-p130 (C-20). Full length RB was detected using BD Pharmingen anti-RB (554136). Anti-PSM-RB antibody was a generous gift from J. Wang. Anti-HPV16-E7 was from Zymed Laboratories (cat#28-0006).

Immunofluorescence and BrdU incorporation

Cells were subjected to the indicated treatment, fixed, and DAPI stained. Cells were treated with BrdU reagent for 4 hours and immunostained for BrdU incorporation as previously described [29].

Flow Cytometry

BrdU reagent was added to cells for 1 hour. Cells were then harvested by trypsinization, fixed with ethanol, and incubated with propidium iodide (PI) and fluorescent anti-BrdU secondary antibody. Histograms represent 10,000 events. Apoptosis assays were performed in the same way, except non-adherent cells were also collected and analyzed using ModFit LT (Verity software Inc).

PCR

Cells were grown in 100mm dishes, harvested by trypsinization. Total genomic DNA was extracted using DNeasy Blood and Tissue Kit from Qiagen (69504). Primers used were as follows, at an annealing temperature of 42°C: 5': GCT CTA GAA TGC ATG GAG ATA

CAC CTA and 3': GCT CTA GAT TAT GGT TTC TGA GAA CAG. Amplified DNA was separated using a 1.5% Agarose gel.

Results

p16ink4a does not arrest cervical cancer cell lines

To interrogate the influence of RB-pathway activation in cervical cancer cells, a bladder cancer cell line (TSUPR-1) that retains a functional RB-pathway [30], and contains no HPV-DNA, was utilized as a positive control, compared against two HPV16-positive cervical cancer cell lines, SiHa and CaSki. As shown in Figure 1A, both SiHa and CaSki cells harbor the HPV16 genomic DNA. However, consistent with the literature, CaSki cells harbor a considerably higher copy number of the HPV16 genome, and correspondingly exhibit higher detectable levels of HPV16-E7 protein (Fig. 1A). To initially define the ability of p16ink4a to modulate cell cycle in these models, the endogenous levels of RB, p107 and p130 were determined (Fig. 1B). In each cell line the complete cohort of pocket proteins could be detected. To determine if activation of the endogenous pocket proteins could act to inhibit cell cycle progression, cells were transduced with adenoviruses encoding p16ink4a and harvested at 48 hours post-infection. As expected, p16ink4a expression strongly arrested TSUPR-1 bladder carcinoma cells as determined by flow cytometry; however, p16ink4a expression had no effect on SiHa or CaSki cells (Fig. 1C and D). Concomitant with the effect on S-phase progression, TSUPR-1 cells exhibited an apparent decrease in RB phosphorylation, (as observed through shift in molecular weight) and p107 protein levels were reduced (Fig. 1B), which is consistent with previous studies [31]. In contrast, SiHa and CaSki cells exhibited no change in pocket protein levels or RB mobility (Fig. 1B). From these experiments we conclude that endogenous RB activation via p16ink4a is compromised, and p16ink4a expression has minimal effects on cell cycle dynamics in these two HPV16 E7 positive cervical cancer cells.

Active RB expression elicits an arrest in specific cervical cancer cell line

To directly address the impact of RB function in cervical cancer cells, a previously constructed phosphorylation site-mutant RB protein (PSM-RB) was utilized [32,33]. PSM-RB acts as a constitutively active RB protein that retains the ability to target native cellular proteins such as E2F, irrespective of phosphorylation status. In order to determine the effect of PSM-RB on cell cycle dynamics, TSUPR-1, SiHa, and CaSki cells were transduced with adenovirus expressing PSM-RB. The expression of PSM-RB was confirmed by western blot (Fig. 2A), and cell cycle stage analyzed by flow-cytometry and *in vitro* BrdU-incorporation. As shown in Fig. 2B and C, TSUPR-1 cell cycle progression was inhibited by PSM-RB expression. Strikingly, SiHa cells exhibited an inhibition of cell cycle with PSM-RB expression, comparable to TSUPR-1. In contrast, CaSki cells remained relatively unaffected by PSM-RB expression (Fig. 2C). This finding suggests that while RB can be overexpressed in all cell lines tested, a fundamental difference exists in the ability of ectopic RB expression to overcome E7 action in the two cervical cancer cells lines. To interrogate this idea, TSUPR-1, SiHa and CaSki cells were infected with increasing amounts of PSM-RB-encoding virus and cell cycle distribution was analyzed by flow cytometry (Fig. 2D). In concordance with previous results, TSUPR-1 and SiHa cells showed an increasing percentage of cells with a 2N DNA content. This effect saturated at an Multiplicity of Infection (MOI) of approximately 100. CaSki cells however, failed to arrest in G₁, exhibiting only a marginal increase in 2N population at an MOI of 300. These results support the concept that active RB can overcome the effects of E7. However, certain cells (i.e. CaSki) are resistant to the effects of RB activation.

HPV-E7 levels are critical for effects of PSM-RB

We have shown that PSM-RB expression has differential effects on the ability to arrest SiHa and CaSki cells. To examine more closely the mechanism of this cell cycle arrest, expression of classical RB target gene products were analyzed. Cells were transduced with adenovirus to GFP, p16ink4a, or PSM-RB for 48 hours, and protein levels of cyclin A and ribonucleotide reductase II (RNR-II) were determined (Fig. 3A). As expected, both p16ink4a and PSM-RB expression resulted in attenuated cyclin A and RNR-II protein levels in TSUPR1 cells. Furthermore, in agreement with the observed cell cycle arrest, protein levels of cyclin A and RNR-II were attenuated only upon PSM-RB addition, but not p16ink4a induction in SiHa cells. In contrast, CaSki cells showed no significant decreases in the expression of cyclin A and RNR-II (Fig. 3A) in the presence of PSM-RB. These results are consistent with the finding that SiHa, but not CaSki cells, respond to PSM-RB expression. To determine if the attenuated levels of E2F/RB target proteins was due to restoration of transcriptional repression, reporter assays were utilized to monitor cyclin A and RNR-II promoter activity. As shown in Figure 3B, both cyclin A and RNR-II promoter activity was repressed following PSM-RB expression for 48 hours in SiHa cells. In concordance with protein expression analyses, p16ink4a had no effect on cyclin A or RNR-II promoter activity. Similarly, repression of cyclin A and RNR-II promoters did not occur in CaSki cells with either p16ink4a or PSM-RB (Fig. 3B), thereby indicating that CaSki cells are resistant to the effects of RB on transcriptional repression.

While PSM-RB could presumably titrate the activity of endogenous E7, the relationship between the relative levels of PSM-RB expression and E7 expression had not been examined. To determine the effect of ectopic E7 protein, SiHa and CaSki cells were transfected as above, but with the addition of an HPV16 E7 encoding plasmid. Under this condition, PSM-RB could no longer facilitate repression of the cyclin A or RNR-II promoters in SiHa cells (Fig. 3B), while ectopic E7 expression increased the transcriptional activity of cyclin A and RNR II in both cell lines. These results suggest that the mechanism of PSM-RB transcriptional repression is dependent on levels of E7, and that increased E7 expression levels can antagonize RB functionality in SiHa cells.

Active RB inhibits proliferation and induces cell death

Whilst PSM-RB was able to initiate cell cycle inhibition, the impact on overall cellular proliferation remained unclear. To determine the long-term effects of active RB on cell proliferation, cells were infected with either GFP or PSM-RB encoding adenoviruses and cultured for up to 96 hours. TSUPR-1 (data not shown) and SiHa cells exhibited cessation of proliferation at 48 hours post-infection, and sustained this arrest for up to 96 hours (Fig. 4A and B, data not shown). However, CaSki cells were unresponsive to PSM-RB addition and continued to proliferate normally (Fig. 4A and B). From these results we conclude that active RB expression can inhibit proliferation in SiHa cells, while CaSki cells are resistant to ectopic RB expression. In fact, there was a highly reproducible diminution in SiHa cell number, suggesting these cells were not only arrested, but actively undergoing cell death. To determine if PSM-RB transduced cells were undergoing apoptosis, we examined the nuclear structure of each cell type before and after PSM-RB expression. As shown in figure 5, PSM-RB expression induced the accumulation of cells with pyknotic nuclei, a hallmark of cellular apoptosis. In contrast, CaSki cells did not exhibit any change in nuclear morphology. While the increase in pyknotic nuclei suggests an increase in programmed cell death, it does not explain the decrease in total cell number, thus the DNA content of the three cell types was examined upon PSM-RB expression. As expected, TSUPR-1 cells arrested in G₁, but did not exhibit a dramatic increase in cell death (Fig. 5C, D). In contrast, a marked increase in sub-2N DNA content was present by 72 hours post-PSM-RB expression in SiHa cells (Fig. 5C, D), while CaSki cells did not arrest or confer an increase in cell death upon PSM-RB

addition (Fig. 5C, D). Together, these data support a model in which sustained cell cycle arrest through RB can promote apoptosis in a specific cancer cell line.

HeLa cells respond to PSM-RB with cell cycle inhibition and are not viable

Since SiHa cells, unlike CaSki, are susceptible to PSM-RB mediated cell cycle arrest, our studies suggest that PSM-RB could be effective at inhibiting proliferation of other cervical cancer cell lines. Therefore, the effect of PSM-RB expression was evaluated in another cervical cancer cell line. HeLa cells are an HPV18-containing cell line, with a relatively low copy number (10–50 copies) of HPV DNA in comparison with CaSki cells (60–600 copies) [40]. In order to test the response of HeLa cells, we initially analyzed BrdU incorporation in the presence of ectopic PSM-RB or p16ink4a. HeLa cells were unaffected by ectopic p16 expression (Fig. 6A). In contrast, the response to ectopic PSM-RB expression was specific inhibition of DNA synthesis. In addition, reporter assays were utilized to monitor cyclin A promoter activity. As shown in figure 6B, cyclin A promoter activity was repressed following PSM-RB expression. As with SiHa cells, the repression of the cyclin A promoter was reversed by ectopic E7 expression. These data suggest that HeLa cells are sensitive to PSM-RB expression, with respect to target gene regulation and growth arrest. Finally, the impact of ectopic PSM-RB expression on long-term HeLa cell growth and apoptosis was determined. Cell growth at the 24 hours post-infection was relatively unimpeded by PSM-RB expression. However, at the 48 hours post-infection cell viability was diminished, while the GFP infected culture continued to proliferate throughout the experiment. Under these conditions, induction of senescent arrested cells was not observed (data not shown). Thus, these data support the hypothesis that both HPV16 and HPV18-containing cervical cancer cell lines can respond to active RB expression by undergoing cell cycle inhibition and cell death. In addition, these data bolster the conclusion that cells with a lower copy number of HPV DNA are comparatively more sensitive to overexpression of constitutively active RB.

Discussion

A key component of tumor initiation is uncontrolled cellular proliferation, caused by inappropriate cell cycle control. RB and the pocket protein members, p107 and p130, play an important role in preventing unscheduled S-phase entry and uncontrolled proliferation [34]. These three proteins are highly homologous, sharing the same pocket domain. Among cervical cancers, several studies have shown that HPV16 E7 targets this region to sequester these proteins for proteolytic degradation, allowing for the release of E2Fs and transcription of downstream S-phase promoting genes [10,35,36]. While the exact mechanism by which HPV16 E7 mediates the evasion of growth arrest signals is complex, studies have shown that E7 mutants deficient in RB binding cannot override the G₁ checkpoint [37,38]. This would suggest it is necessary that E7 bind and inactivate RB to promote unscheduled cell cycle entry. In this study we examined whether ectopic activation of the RB-pathway could overcome the growth promoting effects of HPV16 E7 in cervical cancer cell lines. Our data suggests that while active RB can titrate E7 function in defined conditions, this ability is specific to certain cellular contexts.

Endogenous RB protein that has been inactivated via CDK phosphorylation can be reactivated by induction of p16ink4a activity. p16ink4a functions by inhibiting cyclin D-cdk4/6 complexes, preventing RB phosphorylation and allowing RB to remain active and bound to E2F transcription factors, thus eliciting a cell cycle arrest at the G₁/S boundary. However, in cervical cancer cells that have been transformed by HPV, RB is bound by E7, eliminating the necessity of CDK4 phosphorylation, and subsequent inactivation of RB. As such, p16ink4a expression had no effect on cell cycle dynamics (Fig. 1C and D), or endogenous pocket protein expression (Fig. 1B). These results confirm that E7 facilitates the inactivation of the RB-pathway downstream of the cdk4/p16ink4a axis. Previous studies

have shown that expression of constitutively active alleles of RB potently suppress cellular proliferation by impeding cell cycle progression [24]. In stark contrast to the lack of p16ink4a action in HPV-positive cervical cancer cells, the ectopic expression of PSM-RB caused decreased cellular proliferation in SiHa, and HeLa but not CaSki cells. These data indicate there is a differential response in how cervical cancer cells react to RB reconstitution. While it is not clear why SiHa and HeLa cells respond differently to RB-pathway reconstitution, in comparison with CaSki, a possible explanation is E7 activity is simply much more abundant in CaSki cells. This model is supported by the elevated expression of E7 protein observed in CaSki cells, and the finding that SiHa and HeLa cells can be converted to a resistant phenotype via the ectopic expression of E7. However, it is also possible that CaSki cells contain additional lesions preventing cell cycle arrest or apoptosis in response to PSM-RB. Lesions, such as loss of the SWI/SNF complex, have been identified in cancer cells, and are associated with a deficiency in RB-mediated transcriptional repression, similar to that observed in CaSki cells [45].

The relative involvement of E7 function in the maintenance of cervical carcinogenesis has been the subject of intense study. Utilizing multiple approaches, down-regulation of E7 expression has been achieved in cervical cancer cell lines. In this context, akin to our expression of PSM-RB, there is the cessation of cellular proliferation and increased apoptosis. In both SiHa and CaSki cells it has been shown that interfering with E7 by RNA interference approaches causes a massive apoptotic response—this is in contrast to non-HPV containing cell lines [42–43]. This phenomenon is also largely associated with the re-activation of endogenous cell cycle control pathways in the absence of E7 expression, and demonstrates that HPV-containing cell lines are dependent upon E7 function for cell survival. Surprisingly, while PSM-RB expression could mediate cell cycle inhibition, it did not lead to chronic cell cycle arrest as characterized by the establishment of senescence. Rather, the PSM-RB expressing SiHa and HeLa cells underwent cell death. The mechanism through which these cells die as a consequence of PSM-RB remains under investigation. Traditionally, while RB functions to suppress apoptosis, these cell lines thus represent two of the few models wherein re-establishment of RB activity leads to cell death. Intriguingly, the other cell line where this has occurred is the HPV-negative cervical cancer line, C33A [44]. More detailed analyses of this phenomenon in the context of primary mouse models or organotypic cultures would be required to determine whether these cell culture findings have implication for cervical cancer treatment. The results of our study, however, do suggest that in the context of cervical cancer cells, restoration of RB function is more likely to generate an apoptotic response that could be highly significant in the treatment of cervical cancer.

ABBREVIATIONS

RB	retinoblastoma
HPV	human papilloma virus
PSM-RB	phosphorylation site mutant RB
CDK	cyclin-dependent kinase
RNR-II	ribonucleotide reductase II

Acknowledgments

The authors thank members of the Erik and Karen Knudsen laboratories for critical review of the manuscript and insightful discussion. ESK is supported by grants from the National Cancer Institute (CA 104213). SIW is supported by a grant from the National Cancer Institute (CA 102357).

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Figure 1. HPV16 E7 action on RB is resistant to p16ink4a expression

(A) Cells were cultured in 100mm plates: (PCR) cells harvested, total genomic DNA extracted and PCR performed for total E7-encoding DNA, amplified product resolved on agarose gel in equal amounts or (WB) cells harvested, equal total protein was resolved by SDS-PAGE, and the indicated protein was detected by immunoblotting. (B) Cells were cultured in 100mm plates and transduced with adenoviruses encoding GFP or p16ink4a for 48 h. Cells were harvested, equal total protein was resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting. (C) Cells were transduced with adenovirus encoding GFP or p16ink4a for 48 h, incubated with BrdU reagent, fixed, and stained for BrdU and PI. Percent S-phase was analyzed by flow cytometry. (D) Cells were plated on coverslips, transduced with adenovirus to GFP or p16ink4a, then fixed, and immunostained for anti-BrdU and DAPI. Data represent three independent experiments with at least 200 cells scored per assay.

Figure 2. Active RB overcomes HPV16 E7 action in SiHa but not CaSki cervical carcinoma cell lines

(A) Cells were cultured in 100mm plates and transduced with adenoviruses encoding GFP or PSM-RB for 48 h. Cells were harvested, equal total protein was resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting. (B) Cells were transduced with adenovirus encoding GFP or PSM-RB for 48 h, incubated with BrdU reagent, fixed, and stained for BrdU and PI incorporation. Percent S-phase was analyzed by flow cytometry. (C) Cells were plated on coverslips, transduced with adenovirus to GFP or PSM-RB, then fixed, and immunostained for anti-BrdU and DAPI. Data represent three independent experiments with at least 200 cells scored per assay. (D) Cells were cultured in 100mm plates and

transduced with adenoviruses encoding GFP, or PSM-RB at an MOI of 10, 100 or 300 for 48 hours. Cells were then fixed, and stained for PI incorporation and analyzed by flow cytometry.

Figure 3. RB target gene deregulation is dependent on RB-pathway status

(A) Cells were cultured in 100mm plates and transduced with adenoviruses encoding GFP, p16ink4a or PSM-RB for 48 h. Cells were harvested, equal total protein was resolved by SDS-PAGE, and the indicated proteins were detected by immunoblotting. (B) Cells were cultured in 6-well plates and transfected with the indicated plasmids for 48 h. Cells were then lysed and analyzed for luciferase activity.

Figure 4. SiHa and CaSki cells elicit differential growth responses following RB expression
 (A) 3×10^5 cells were seeded and transduced with adenovirus to GFP or PSM-RB for the indicated time-points. Cells were stained with 1% crystal violet and images taken from infected plates. (B) Cell number from each plate was scored blindly (4 independent fields per 100mm dish). Data represent 3 independent experiments.

Figure 5. PSM-RB expression induces apoptosis in SiHa cells

[A, B] Cells were seeded at 3×10^5 and transduced with adenovirus to GFP or PSM-RB for the indicated time-points. (A) Cells were DAPI stained and fluorescent images taken. (B) Cells were scored for total pyknotic nuclei. Data represent 3 independent experiments. [C, D] Cells were transduced with adenoviruses expressing either GFP or PSM-RB for the indicated time-points and harvested for flow cytometry. (C) Adherent and non-adherent cells were harvested for the analysis and sub- G_1 populations were analyzed using ModFit LT software. (D) Modfit LT analysis from A scored as fold increase in sub-2N populations. Data represent 3 independent experiments.

Figure 6. Ectopic PSM-RB expression causes cell cycle arrest and apoptosis in HeLa cells

(A) Cells were plated on coverslips, transduced with adenovirus to GFP or PSM-RB, then fixed, and immunostained for anti-BrdU and DAPI. Data represent three independent experiments with at least 200 cells scored per assay. (B) Cells were cultured in 6-well plates and transfected with the indicated plasmid for 48 h. Cells were then lysed and analyzed for luciferase activity. (C) 3×10^6 cells were seeded and transduced with adenovirus to GFP or PSM-RB for the indicated time-points. Cells were stained with 1% crystal violet. Cell number from each plate was scored blindly (4 independent fields per 100mm dish).