Cancer Research

Rescue of p53 Function by Small-Molecule RITA in Cervical Carcinoma by Blocking E6-Mediated Degradation

Carolyn Ying Zhao, Laszlo Szekely, Wenjie Bao, and Galina Selivanova

Abstract

Proteasomal degradation of p53 by human papilloma virus (HPV) E6 oncoprotein plays a pivotal role in the survival of cervical carcinoma cells. Abrogation of HPV-E6–dependent p53 destruction can therefore be a good strategy to combat cervical carcinomas. Here, we show that a small-molecule reactivation of p53 and induction of tumor cell apoptosis (RITA) is able to induce the accumulation of p53 and rescue its tumor suppressor function in cells containing high-risk HPV16 and HPV18 by inhibiting HPV-E6–mediated proteasomal degradation. RITA blocks p53 ubiquitination by preventing p53 interaction with E6-associated protein, required for HPV-E6–mediated degradation. RITA activates the transcription of proapoptotic p53 targets Noxa, PUMA, and BAX, and repressed the expression of pro-proliferative factors CyclinB1, CDC2, and CDC25C, resulting in p53-dependent apoptosis and cell cycle arrest. Importantly, RITA showed substantial suppression of cervical carcinoma xenografts *in vivo*. These results provide a proof of principle for the treatment of cervical cancer in a p53-dependent manner by using small molecules that target p53. *Cancer Res; 70(8); 3372–81. ©2010 AACR.*

Introduction

Over 90% of cervical carcinomas are causally linked to infections with oncogenic high-risk human papilloma virus (HPV; refs. 1–3). HPV oncoproteins E6 and E7, expressed in cervical carcinoma, facilitate tumor progression by inactivating p53 and pRB, two key regulators of cell proliferation and apoptosis. p53 degradation is an essential requirement for the survival of HPV-infected cervical carcinoma cells; thus, preventing p53 destruction by HPV-E6 might be a potent strategy to combat cervical carcinoma (4).

A strong causal link between the loss of p53 function and tumor development has been established (5). In half of human tumors, mutations render p53 nonfunctional. In the absence of mutations, p53 is inhibited mainly through enhanced degradation by negative regulator Mouse Double Minute 2 (MDM2) or HPV-E6 in cervical carcinoma. In HPV-infected cells in which E6 is present, MDM2 plays a minor role as a p53 destructor (6).

In vivo studies show that established tumors of different types remain vulnerable to p53-mediated suppression, supporting the notion that the restoration of p53 function may be an attractive strategy for treating cancer (7). Several ap-

proaches are currently under development, aiming to inhibit negative regulators of p53, e.g., MDM2, by small molecules (8, 9). In cervical carcinoma, this could be achieved by inhibiting E6 through a number of approaches: by a synthetic peptide ligand (10); by depleting E6-mRNA through ribozymes, anti-sense oligonucleotides, or small interfering RNA (siRNA; refs. 11–13); or by small molecules (14, 15). In addition, inhibiting E6AP by RNAi or dominant-negative mutant has been shown to induce p53 (16, 17). In summary, these studies provide strong evidence that the inhibition of E6 or E6-associated protein (E6AP) can induce p53 accumulation and thus suppress the growth of cervical carcinoma cells *in vitro* and *in vivo* (18). Nonetheless, current methods of ribozyme, anti-sense oligonucleotides, and siRNA delivery have limited efficiency *in vivo*, thus precluding their use in clinic. Therefore, small molecules seem to be an attractive therapeutic approach for developing anticancer drugs due to the easy delivery to and efficient uptake by tumor cells.

Small-molecule reactivation of p53 and induction of tumor cell apoptosis (RITA) has been shown to bind p53 directly, inducing a conformational change that prevents its interaction with several inhibitory proteins including MDM2, Parc, and iASPP (9). The activation of p53 by RITA triggers apoptosis in wild-type p53–exressing colon, breast, lung carcinoma, osteosarcoma, and renal cell carcinoma cells (19).

Here, we address the question whether a small molecule that target p53, rather than inhibiting E6, can restore the tumor suppressor function of p53 and block cervical carcinoma growth. We show that RITA can inhibit the interaction between p53 and E6AP, thus protecting p53 from E6-mediated degradation. RITA restored the transcriptional function of p53, leading to the suppression of cervical carcinoma cells *in vitro* and *in vivo*.

Authors' Affiliation: Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Corresponding Author: Galina Selivanova, Department of Microbiology, Tumor and Cell Biology, Box 280, Karolinska Institutet, Nobels väg 16, SE-17177, Stockholm, Sweden. Phone: 46-8-52486302; Fax: 46-8-330744; E-mail: galina.selivanova@ki.se.

American Association for **Cancer Research**

Materials and Methods

Plasmids and cell lines. HeLa cells were stably cotransfected with pSUPERp53shRNA-encoding plasmid (Oligo-Engine) and pBabe vector (for puromycin resistance) using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions. Stable clones were selected and maintained in 0.5 μg/mL puromycin.

Growth suppression assays. For the cell viability assay, 3,000 cells per well were plated in a 96-well plate and treated with RITA for 48 h, after which cell viability was assessed with the proliferation reagent WST-1 (Roche) according to the manufacturer's instructions. For colony formation assay, cells were seeded in 12-well plates and treated with RITA for 24 h, after which the medium was replaced and the cells were allowed to grow for 10–14 d. The colonies were stained with crystal violet. For growth curves, 3,000 cells/mL were plated in 12-well plates, treated with RITA, and counted over 5 d.

Detection of apoptosis. Cells were harvested with trypsin and fixed with 70% ethanol, treated with RNaseA (0.25 mg/mL), and stained with propidium iodide (0.02 mg/mL). Samples were analyzed on a Becton Dickinson FACScan. Data were analyzed by the CellQuest software, version 3.2.1. For time-lapse video, microscope pictures were taken at one frame every 2 min over a period of 3 d for control HeLa cells and 7 d for RITA-treated cells.

Quantitative real-time reverse transcription-PCR. Messenger RNA was prepared with QIAGEN miniprep according to the manufacturer's protocol. cDNA was generated from RNA using random primers (Invitrogen) and M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Primers used for real-time reverse transcription-PCR (RT-PCR) were as we previously described (19).

Small interfering RNAs. The sequence of E6AP and HPV18E6 siRNAs were as described in ref. (16) and the sequence for MDM2 siRNA was as describe in ref. (19). All siRNAs were purchased from Dharmacon/ThermoScentific. The siRNAs were transfected into HeLa cells using the HiPerfect Transfection Reagent from QIAGEN according to the manufacturer's instructions.

Pull-down assay. HeLa and HCT116 cells were lysed in 0.5% Nadoxycholate, 0.1% SDS, 1% Triton X-100, 1 mmol/L EDTA in PBS, containing Cocktail protease inhibitors (Roche), and 1 mmol/L phenylmethylsolfonyl fluoride (PMSF). Gluthathion-Sepharose beads with immobilized glutathione S-transferase-E6AP or GST were used to pull down p53 from cell extracts (1 mg per probe). The binding buffer contained 1 mmol/L EDTA, protease inhibitors, and 1 mmol/L PMSF in PBS. RITA was added to the cell extracts in binding buffer for 30 min at room temperature before incubation with immobilized recombinant GST-E6AP or GST. Proteins bound to the beads were collected after incubation for 2 h at 4°C, washed five times in binding buffer, boiled in Laemmli buffer, and separated using 10% SDS-PAGE followed by immunoblotting. Plasmid p4028, encoding GST-E6AP (Addgene), was used to produce recombinant E6AP according to standard protocol.

Antibodies and Western blot. The primary antibodies used were as follows: p53 CM1 was from Novocasta; p53 (DO-1, FL393), MDM2 (SMP14), Gadd45 (C4), Bax, PUMA, and poly ADP ribose polymerase (PARP) were from Santa Cruz; and E6AP (Clone E6AP-330) and β-actin (AC15) were from SIGMA. p21 (Cip1/waf1) was from Nordic Biosite. Noxa antibody and PUMA (Ab-1) were from Calbiochem. Hybridoma cells for the production of mouse monoclonal 2A10 anti-MDM2 antibody were a kind gift from Dr. F. Moretti (Institute of Neurobiology and Molecular Medicine, Rome, Italy). Immunoblotting was performed according to standard procedures.

To detect p53 ubiquitination, HeLa cells were treated with RITA (1 μmol/L) for 8 h and 30 μmol/L proteasome inhibitor MG132 (Sigma) was added 3 h before the harvest to allow the accumulation of ubiquitinated forms of p53, followed by immunoblot.

In vivo experiments. The Northern Stockholm Animal Ethical Committee approved all animal studies and animal care was in accordance with the Karolinska Institutet guidelines. HeLa cells (1×10^6) were injected s.c. on the left and right flanks of 6- to 8-wk-old male severe combined immunodeficient (SCID) mice. Xenografts appeared palpable 6 d after inoculation, at which time the treatment was started. The mice were treated i.p. twice daily with injection of 200 μL solution containing a dose of 10 mg/kg of RITA or PBS without drug for a period of 12 d, followed by 2 treatment-free days, then again 5 d, followed by 2 treatment-free days and 2 more days of treatment. Xenograft volumes were measured every other day.

Results

RITA suppressed the growth of cervical carcinoma cells. We have previously shown that small-molecule RITA can induce apoptosis in several types of cancer cells (9, 19). Here, we tested whether RITA can also suppress the growth of HPV-infected cervical carcinoma cells CaSki (HPV-16) and HeLa (HPV-18). Using a short-term cell proliferation assay, we found that RITA induced efficient growth suppression of both CaSki and HeLa cells (Fig. 1A). RITA IC_{50} was calculated to be 1 μmol/L for HeLa cells and 10 μmol/L for CaSki. These doses were used thereafter for these lines in all assays unless indicated otherwise. Measurement of the growth rate showed that RITA efficiently inhibited the growth of both cell lines (Fig. 1B). Long-term colony formation assay, shown in Fig. 1C, showed that treatment with RITA drastically reduced the number of colonies formed by HeLa and CaSki. In contrast, p53-null H1299 cells were not affected, in line with our previous findings (19).

RITA induced apoptosis in cervical carcinoma cells. To examine whether RITA-mediated growth suppression is caused by inhibiting cell proliferation and/or survival, we analyzed the cell cycle profile by fluorescence activated cell sorting (FACS) of propidium iodide–stained cells. RITA induced a substantial accumulation of both HeLa and CaSki cells in the G_2 phase (Fig. 2A and B). Accumulation in G_2 was due to the prevention of entry into the mitotic phase

of cell cycle, as RITA-treated cells displayed reduced amounts of phosphorylated histone H3, an indicator of mitotic entry. Along with the increased G_2 fraction, we observed a prominent induction of cells with fragmented DNA (sub-G1 fraction), a hallmark of cell death. Sub-G_1 fraction gradually increased from day 2. By day 9, almost all cells acquired fragmented DNA. We also observed PARP cleavage, which indicates cell death by apoptosis.

Time-lapse microscopy videos (Supplementary Videos S1 and S2) showed that RITA treatment slowed down the cell proliferation rate considerably: it was extended from 16 to 32 hours (Supplementary Video S1). Control cells overgrew by day 3, after which we stopped the recording, whereas RITA-treated cells on average underwent two cell divisions before they died. We observed clear signs of apoptosis—blebbing of cell membranes, rounding and shrinkage of cells, and formation of apoptotic bodies (Supplementary Video S2;

Fig. 1D). However, we did not detect giant multinucleated cells that are characteristic for mitotic catastrophe (20, 21). The biological response triggered by RITA in CaSki cells (Fig. 2B) was similar to that of HeLa cells (Fig. 2A).

Pan-caspase inhibitor z-VAD-fmk blocked DNA fragmentation and the appearance of apoptotic morphology in HeLa cells, attesting caspase-dependent apoptosis (Fig. 2C). Taken together, our results show that RITA suppresses proliferation and induces apoptosis in cervical carcinoma cells.

RITA stabilized p53 through the inhibition of p53/E6AP interaction. Upon 24 hours of RITA treatment, we observed a substantial accumulation of p53 in HeLa and CaSki cells as evidenced by immunoblotting (Fig. 3A) and immunocytochemistry (Supplementary Fig. S1). We found that RITA treatment significantly prolonged the protein half-life of p53, suggesting that p53 induction was due to increased protein stability (Fig. 3A). In line with these data, RITA
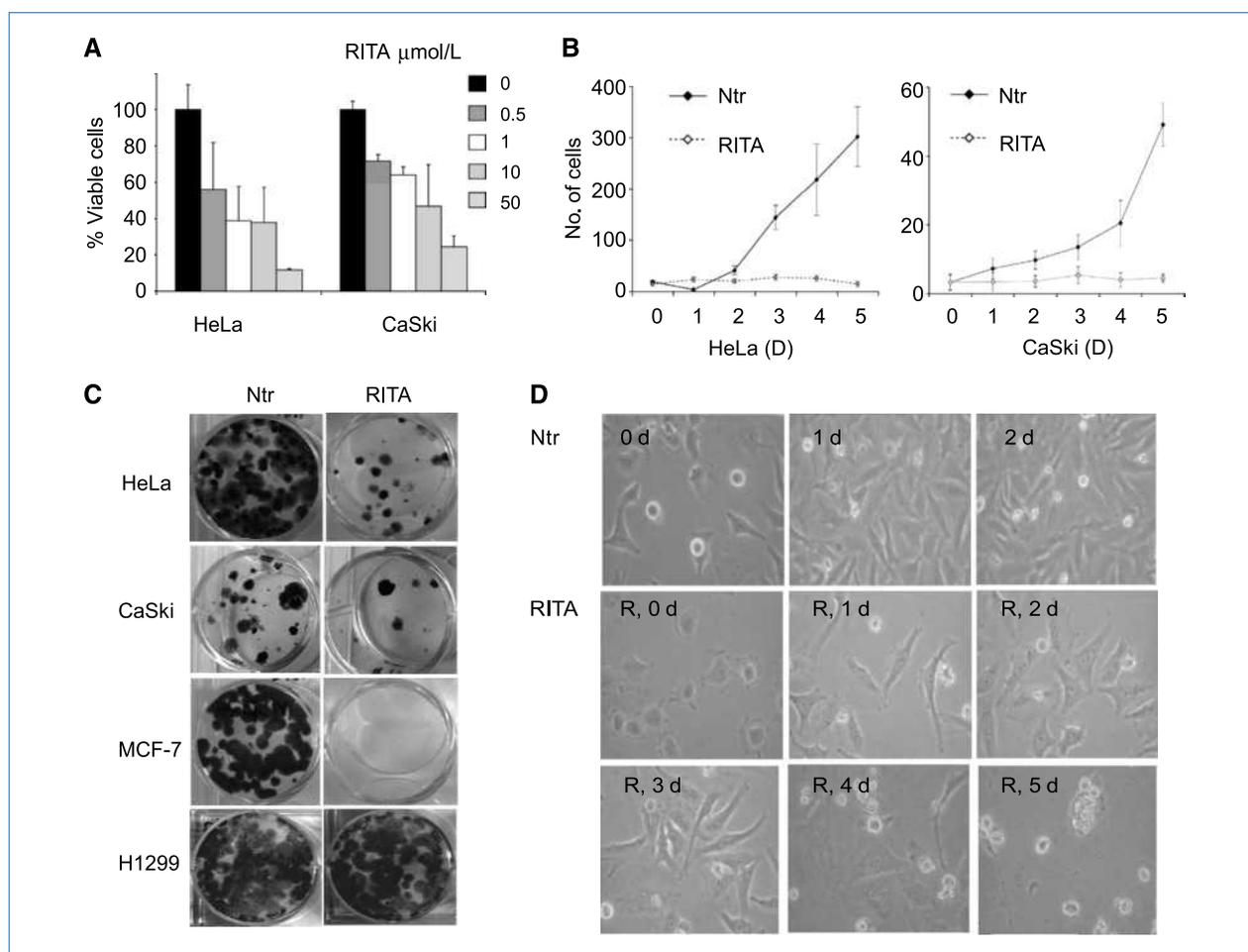
Figure 1. RITA suppresses the growth of cervical cancer cells. A, effects of RITA on the proliferation of HPV16E6 and 18E6-containing HeLa and CaSki cervical carcinoma cells were assessed using the WST-1 cell proliferation assay. B, growth curves show RITA-mediated inhibition of proliferation of HeLa and CaSki cells over 5 d, compared with nontreated (Ntr) cells. C, colony formation assay was used to assess the long-term (10–14 d) effects of RITA on HeLa and CaSki cervical carcinoma cells. Breast carcinoma MCF7 cells (wild-type p53) and lung carcinoma H1299 (p53-null) were used as a positive and negative control, respectively. D, microscopy analysis of RITA effects on HeLa cells. In 2 d, control cells overgrew, whereas RITA-treated cells divided more slowly, once in 32 h (instead of 16 h in control), and start to die after day 2.
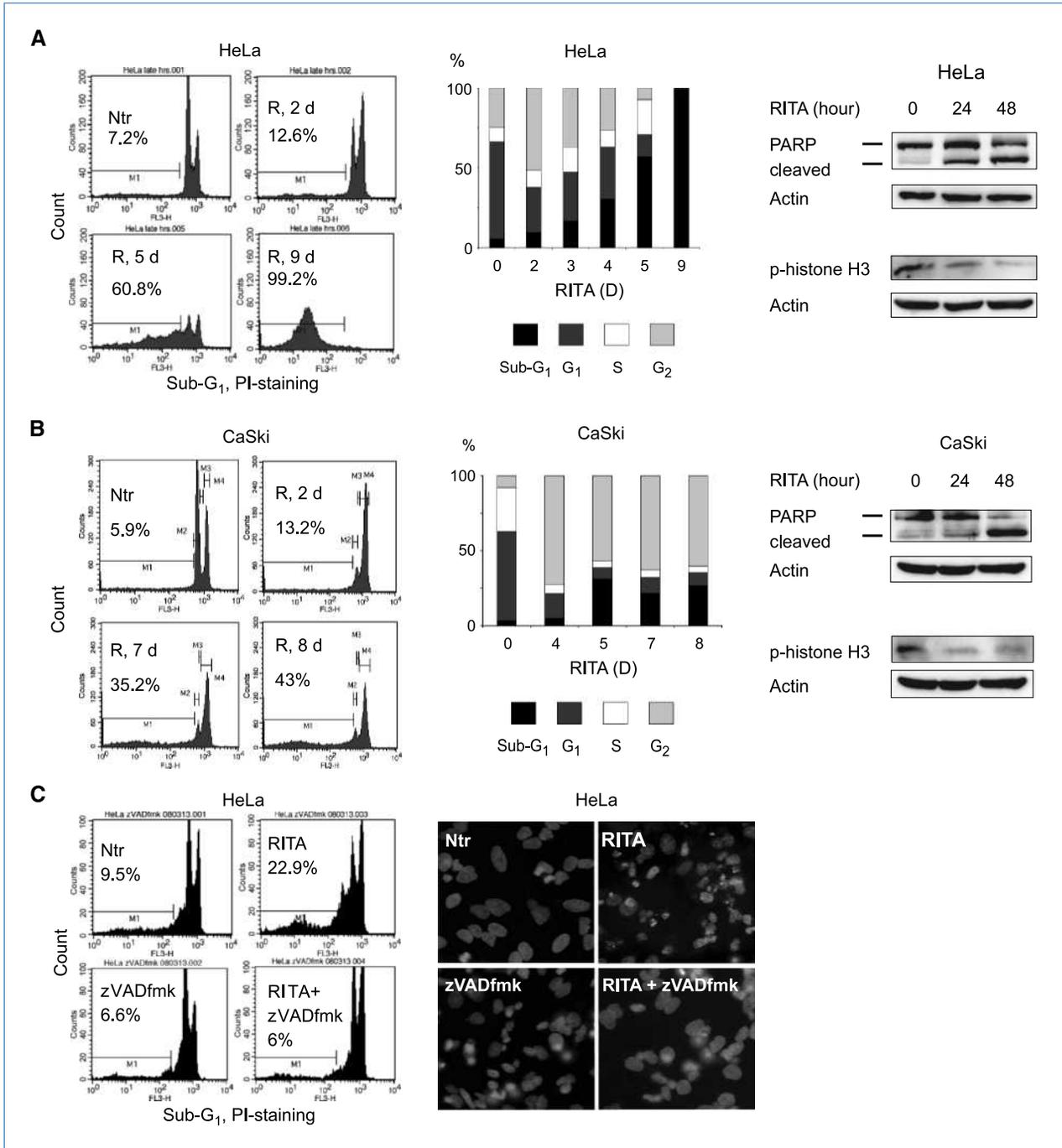
Figure 2. Induction of caspase-dependent apoptosis by RITA. A and B, cell cycle profiles and sub-G$_1$ apoptotic fraction of HeLa (A) and CaSki (B) were detected with propidium iodide (PI) staining using FACS analysis. Histograms show the quantification of cell cycle distribution changes in HeLa cells (A) and in CaSki (B) after RITA treatment. PARP cleavage, an indicator of cell death, was detected by Western blot in HeLa cells (A) and in CaSki (B) upon RITA treatment. Inhibition of mitotic entry by RITA manifested as a decrease of phosphorylated histone-H3 detected in HeLa cells (A) and in CaSki (B). Ntr, nontreated. C, pan-caspase inhibitor z-VAD-fmk blocked the enhancement of sub-G$_1$ fraction by RITA treatment (72 h) in HeLa cells, detected by FACS. Hoechst staining shows that apoptotic morphology induced by RITA treatment (72 h) in HeLa cells was inhibited by zVADfmk.

treatment decreased the amount of polyubiquitinated p53 (Fig. 3A). Taken together, our results suggest that RITA induces the accumulation of p53 in cervical carcinoma through blocking its proteasomal degradation.

We addressed whether the stabilization of p53 by RITA could be linked to the induction of DNA damage. As shown in Fig. 3B, although induction of p53 by neocarzinostatin correlated with the strong induction of γH2AX, the same level of p53

accumulation after RITA was followed by a barely detectable increase of γH2AX. These results suggest that the induction of p53 by RITA is not due to the nonspecific genotoxic effects.

Next, we tested whether p53 accumulation is due to the inhibition of E6-mediated p53 degradation. Because E6AP is the crucial factor recruited by HPV-E6 to ubiquitinate p53 (22), we tested the effects of RITA on the interaction between p53 and E6AP. As shown in Fig. 3E, RITA treatment decreased the amount of p53 bound to GST-E6AP in both HeLa and HCT116 colon carcinoma cells (Fig. 3C). Furthermore, RITA decreased the complex formation between endogenous E6AP and p53 in HeLa cells (Fig. 3D). The presence of MDM2 in p53 immunoprecipitates was barely detectable, which supports previously published data showing that p53 inactivation in these cells is largely due to E6/E6AP (6). Taken together, our results suggest that upon RITA treatment, p53 is protected from proteasomal degradation due to decreased complex formation with E6AP.

RITA restored the transcriptional function of p53.
Having discovered that RITA can rescue p53 from the

destruction by E6/E6AP, we asked whether it restores the transcriptional function of the stabilized p53. In line with apoptosis induction as described above, we used detected activated expression RITA and of p53 proapoptotic targets *PMAIP1* (Noxa), BAX, and *BBC3* (PUMA) by quantitative real-time PCR and immunoblotting (Fig. 4A and B). In addition, genes involved in G_2 arrest (23), *CDKN1A* (p21) and *GADD45* were also induced. Further, we found that RITA treatment resulted in the downregulation of mRNA levels of p53 targets involved in the G_2-M transition, namely *CDC2* (encoding CDK1), *CDC25C*, and *CCNB1* (encoding Cyclin B1; Fig. 4C; ref. 23), which agrees with the observed accumulation of cells in G_2 by FACS upon RITA treatment (Fig. 2A and B). Thus, our results clearly show that treatment with RITA induced the expression of p53 target genes involved in growth arrest and apoptosis, as well as transcriptional repression of genes required for cell proliferation.

Interestingly, attenuation of either E6 or E6AP by corresponding siRNAs in HeLa cells, followed by a prominent accumulation of p53, triggered only the slight increase of
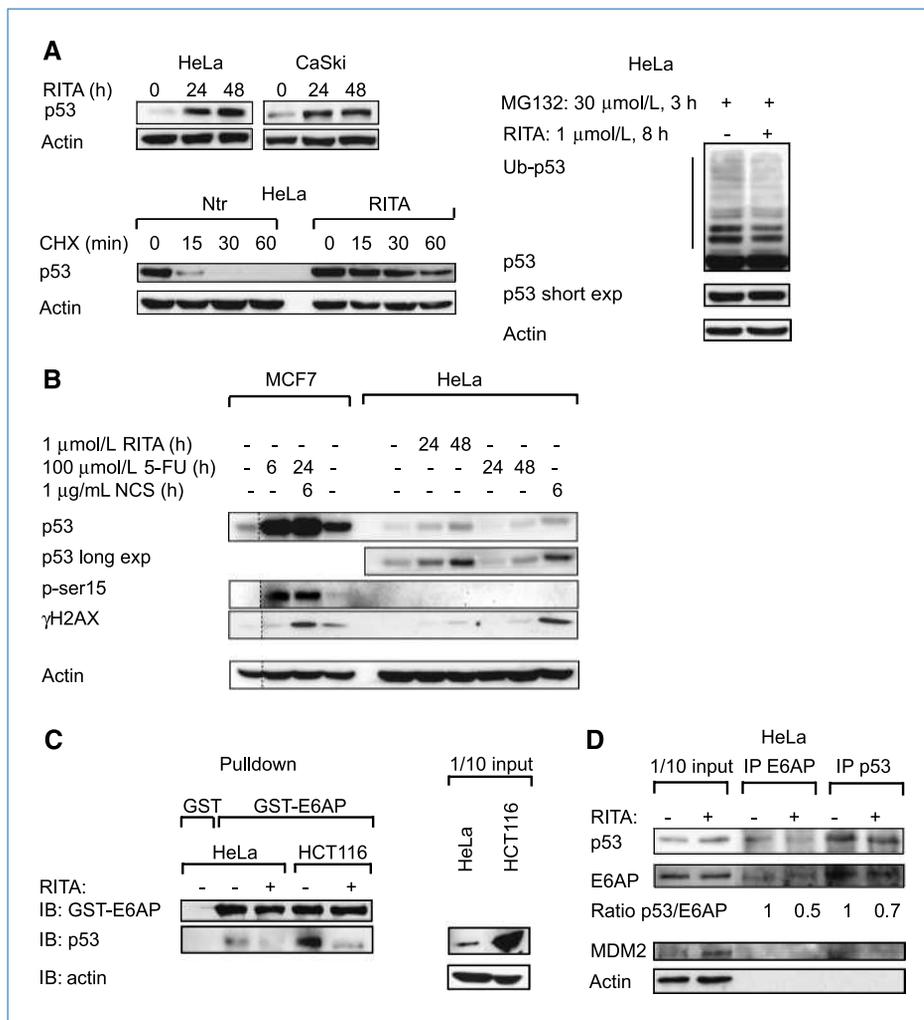
Figure 3. RITA stabilizes p53 in cervical carcinoma cells by inhibiting E6AP/p53 interaction. A, induction of p53 protein levels by RITA in cervical cancer cells was detected by Western blot. Half-life of p53 upon 24 h of RITA treatment was assessed using cycloheximide (CHX, 20 μg/mL). Ubiquitination of p53 in HeLa cells upon 8 h of RITA treatment was detected by immunoblotting (IB). Cells were treated with proteasome inhibitor MG132 for 3 h before harvesting. The ratio between p53 in nontreated (Ntr) and RITA-treated cells was 1.05:1. B, induction of p53, γH2AX, and Ser15-phosphorylated p53 in HeLa and MCF7 cells upon treatment with RITA and with drugs inducing DNA damage, neocarzinostatin (NCS), and 5-fluorouracil (5-FU), detected by Western blot. C, GST-pull down assay assessed the binding of E6AP to p53 in cell lysates of HeLa and colon carcinoma HCT116 cells treated with RITA. Bound proteins and input were detected by immunoblotting. D, the binding between endogenous p53/E6AP and p53/MDM2 upon RITA treatment was examined using coimmunoprecipitation (IP) followed by Western blot.
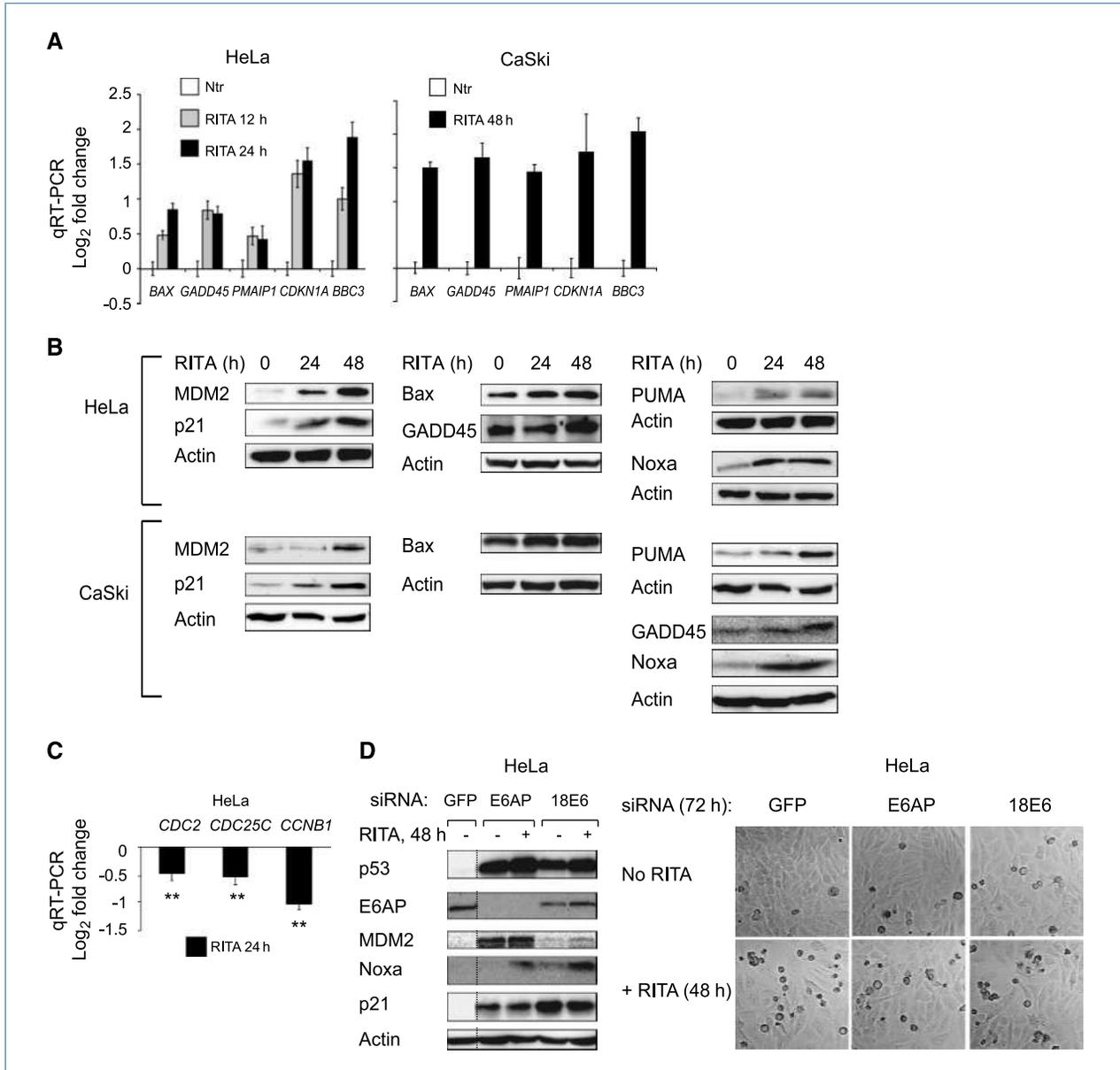
Figure 4. p53 activated by RITA is transcriptionally active. A, RITA induced p53 target genes *BAX, GADD45, PMAIP1* (NOXA), *CDKN1A* (p21), and *BBC3* (PUMA), detected by quantitative RT-PCR in HeLa and CaSki. Ntr, nontreated. B, RITA induced p53 targets MDM2, p21, BAX, Noxa, PUMA, and GADD45 in HeLa and CaSki cells, as detected by Western blot. C, upon RITA treatment, p53 repressed its target genes *CDC2* (CDK1), *CDC25C*, and *CCNB1* (Cyclin B1), responsible for cell cycle progression, as assessed by quantitative RT-PCR. Columns, mean ($n = 4$); bars, SEM; **, $P < 0.01$ by two-tailed *t* test. D, induction of p53 and its targets MDM2, p21, and Noxa upon depletion of E6AP and 18E6 with siRNA with or without RITA treatment was assessed by immunoblotting. Microscopy analysis of HeLa cells transfected with siRNA against E6AP and 18E6 siRNA for 72 h, with or without 48 h of RITA treatment.

Noxa levels. In contrast, two other p53 targets, MDM2 and p21, were significantly upregulated (Fig. 4D). RITA enhanced Noxa induction upon E6 or E6AP inhibition, correlating with a more efficient induction of apoptosis (Fig. 4D). These data suggest that p53 activated by RITA is more active as a transcriptional regulator of proapoptotic genes.

MDM2 does not seem to play a role in p53 regulation in cervical carcinoma (6). Indeed, the level of p53 or its target genes was not affected by siRNA-mediated MDM2 inhibition, nor did it enhance the biological effect of E6/E6AP attenuation (Supplementary Fig. S2). Similarly, MDM2 depletion did not affect the apoptosis induced by RITA, suggesting that MDM2 inhibition by RITA does not significantly contribute to p53 activation in cervical cancer cells.

Effects of RITA in cervical carcinoma cells were p53 dependent. Next, we addressed the question of whether

RITA-induced changes in gene expression and biological effects were mediated by p53. We established a p53-knockdown cell line by a stable transfection of pSUPERshp53 (Fig. 5A). Attenuation of p53 by shRNA conferred partial protection against RITA-induced growth suppression. This is shown by an assessment in a short-term cell proliferation assay using WST-1 as well as a long-term viability assay (Fig. 5B).

p53 depletion by shRNA resulted in the lower level of induction of p53 target Noxa upon RITA treatment (Fig. 5C) and also rescued RITA-induced apoptosis, manifested by the decreased amount of sub-G_1 fraction by FACS (Fig. 5C). However, FACS analysis also showed that prevention of G_2 arrest was much less efficient (Fig. 5C). Although p53 depletion was easily detectable, it was not complete and thus could not prevent a weak induction of p53 upon RITA treatment (Fig. 5A). Because p53-induced changes in the expression of *CCNB1, CDC2,* and *CDC25C* were only partially prevented by p53 depletion (Fig. 5D), it is likely that the observed G_2 arrest in HeLa cells upon p53 knockdown is due to the repression of these genes by residual p53.

Thus, we concluded that the effects of RITA on gene expression and induction of apoptosis are largely p53 dependent.

RITA efficiently suppressed cervical carcinoma growth in vivo. *In vivo* systemic administration is the most rigorous test to evaluate the effect of a drug against tumor growth. To test whether RITA can suppress the growth of cervical carcinoma *in vivo*, we treated SCID mice carrying HeLa xenografts with i.p. injections of 10 mg/kg RITA twice daily. As shown in Fig. 6, treatment with RITA resulted in a dramatic decrease in the growth rate of HeLa xenografts. Thus, we conclude that the reactivation of p53 in cervical cancer cells by small-molecule RITA can suppress the growth of tumors *in vivo*.

Discussion

Cervical cancer development involves oncogenic HPV-E6, which plays a central role by disabling the tumor suppressor function of p53. Hence, inhibiting the E6-mediated degradation
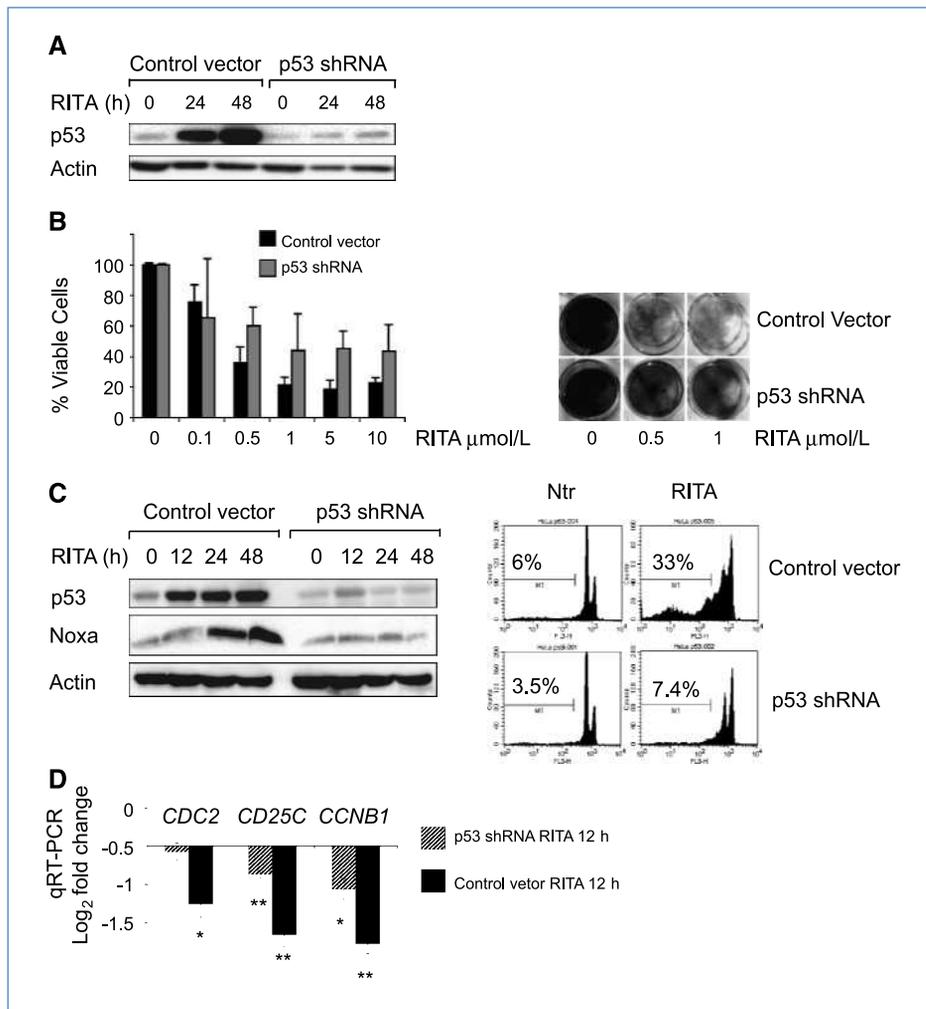
Figure 5. Growth suppression and RITA-induced transcriptional program is p53 dependent. A, decreased level of p53 in HeLa cells stably transfected with pSUPER p53shRNA as detected by Western blot. B, cell proliferation assay (WST-1) was applied to compare RITA-induced growth inhibition in p53shRNA cells and control-transfected cells. Long-term viability assay over 10 d was used to compare the effects of RITA in p53shRNA cells and cells transfected with control vector. C, immunoblotting detected induction of NOXA upon RITA treatment of p53shRNA cells and cells transfected with control vector. FACS analysis of propidium iodide–stained cells was applied to compare the cell cycle profiles and sub-G_1 apoptotic fraction of p53shRNA cells and cells transfected with control vector upon RITA treatment. Ntr, nontreated. D, transcriptional repression of *CDC2, CDC25C,* and *CCNB1* upon RITA treatment of p53shRNA cells and cells transfected with control vector were examined using quantitative RT-PCR. Columns, mean ($n = 3$); bars, SEM; **, $P < 0.01$; *, $P < 0.05$, two-tailed t test.

Figure 6. RITA suppresses the growth of cervical tumors *in vivo*. Antitumor effect of RITA was assessed by i.p. treatment of SCID mice carrying HeLa tumor xenografts with 10 mg/kg of RITA or PBS as control. Mice were treated twice daily over a period of 12 d followed by a 2-d pause, continued treatment for 5 d followed by another 2-d pause and another 2 d of treatment. Left, tumor volumes in the control mice; right, RITA-treated mice.
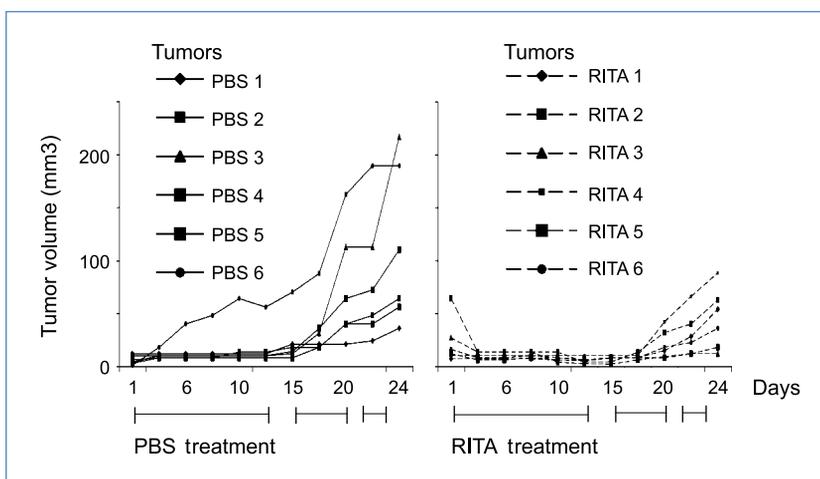
of p53 could be a good strategy to combat cervical cancer. We present a new approach to protect p53 from E6-mediated degradation by using a small molecule that targets p53.

Previously published studies put forward the idea that preventing p53 degradation in cervical carcinoma by silencing E6 or E6AP can resurrect the tumor suppressor function of p53 and kill HPV-infected cancer cells. Comparing the difficulties faced by the approaches of gene therapy, small molecules have great advantages as potential drugs as they are easily taken up by cells and do not induce immune response. To date, only a few substances have been reported that are able to reactivate p53 in HPV-infected cells. An example is the combination of leptomycin B and actinomycin D that greatly reduces the amount of HPV-E6 mRNA, stabilizes p53, and activates the expression of p21 and MDM2, resulting in apoptosis induction (14). Similar results were observed with a lignan from the creosote bush (24). However, these compounds are not specific for HPV-infected cells; along with inhibiting E6 expression, they display cytotoxicity in a broad range of cells. Recently, a chemical library screen identified two small molecules that selectively suppress the growth of cervical carcinoma cells by inhibiting the *E6* gene (15). In addition, a synthetic peptide that binds E6 and blocks its activity has been identified and may be used as a starting point to develop a small-molecule peptide mimetic (10).

Recent success in identifying several classes of small molecules that prevent the interaction of p53 with its inhibitor protein MDM2 strongly suggest that it is a promising strategy in the development of inhibitors of protein-protein interactions (25). Our study further promotes this notion by showing that RITA, a small molecule targeting p53 itself, can simultaneously counteract more than one inhibitor that inactivates wild-type p53 in cancer. Application of RITA and/or other small molecules with similar properties may be a very attractive strategy to combat a broad range of cancers. Further studies are needed to define the structural determinants of the complexes targeted by RITA to facilitate the development of more potent and efficient compounds.

We found that RITA can protect p53 from E6/E6AP-mediated ubiquitination, increases p53 half-life, and induces

its accumulation and transcriptional activity. Thus, our study provides a proof of principle that it is possible to reactivate the dormant p53 function in HPV16- and HPV18-infected cervical carcinoma cells by targeting p53 itself. Interestingly, RITA prevents the interaction between E6AP and p53 also in non–HPV-infected cells. Although the p53/E6AP binding and E6AP-mediated p53 degradation in non–HPV-infected cells has been previously reported (26), the contribution of this phenomenon to cell survival is not known at the moment. It would be interesting to investigate in the future the biological effect of the prevention of E6AP/p53 interaction in non–HPV-infected cells.

Despite the fact that MDM2 plays a negligible role as a p53 inhibitor in HPV-infected cells, a recent report suggests that continuous silencing of E6 gives MDM2 a chance to take over and degrade p53; therefore, targeting E6 along with depletion of MDM2 prevents the fading of p53 (27). We detected MDM2 induction upon E6/E6AP depletion by siRNA, as well as upon RITA treatment. However, we did not observe an additive effect of a concomitant depletion of E6/E6AP and MDM2 on p53 levels and induction of p53 target p21, nor any facilitation of RITA-induced apoptosis by MDM2 attenuation. Therefore, we believe that the effect of RITA on p53/MDM2 interaction is unlikely to be a major mechanism for the induction of p53-dependent apoptosis in cervical cancer cells, although the minor contribution of RITA-mediated prevention of p53/MDM2 interaction cannot be excluded.

It is plausible that upon RITA treatment p53 becomes highly active as a transcription factor and induces a robust proapoptotic transcriptional program. We have previously reported that the ability of p53 to induce apoptosis is much more pronounced upon RITA treatment than after nutlin-3A treatment (19). The difference in the biological response seems to be due to the different transcriptional programs induced by p53 upon reactivation by nutlin-3A and RITA, including the induction of a broader set of proapoptotic genes and the repression of antiapoptotic genes (19, 28). The idea of higher transcriptional activity of p53 induced by RITA in cervical carcinoma cells is supported by our observation that in spite of a strong p53 accumulation upon E6

or E6AP depletion, a rather weak induction of Noxa was observed. However, RITA markedly enhanced the induction of Noxa in these cells.

We have already identified several factors that can contribute to the enhanced activation of proapoptotic genes by p53, including hnRNP K, HIPK2, and TrxR1 (19, 28, 29). Further research, assisted by systems biology methods, must be done to get a deeper understanding of this phenomenon. In the future, it would be interesting to identify those crucial differences in the transcriptional programs in HeLa cells upon p53 activation by E6/E6AP siRNA versus RITA by applying the genome-wide comparison of gene expression profiles.

We found that the induction of p53 level and the onset of apoptosis by RITA in cervical carcinoma cells was delayed in comparison with HPV-negative cancer cells. Moreover, p53 accumulation in HPV-infected cells, although induced, never did reach the same level as in non–HPV-infected lines (Fig. 3B). Most likely, this is due to the much more potent p53 degradation by E6/E6AP compared with MDM2, resulting in an extremely low initial level of p53 (6). Nevertheless, cervical carcinoma cells eventually die after treatment, suggesting that even partial rescue of p53 is sufficient for apoptosis induction.

In cervical carcinoma cells p53 target p21 was induced both on mRNA and on protein levels. In contrast, RITA treatment of noninfected cancer cells increases p21 mRNA levels, whereas the protein level is reduced due to the proteasome degradation by the MDM2 that is released from p53 (19). In cervical carcinoma, however, RITA did not cause p53 to release a substantial amount of MDM2. In line with this, we did not detect p21 downregulation; instead, p21 protein levels rose. It is thus plausible that the p53-induced p21, as well as GADD45, along with repressed $CCNB1$ (cyclin B1), $CDC2$, and $CDC25C$ induce growth arrest, which in turn delays the onset of apoptosis. In fact, we did observe that the accumulation of cells in G_2 precedes the induction of apoptosis upon RITA treatment.

We found that RITA induced apoptosis in a p53-dependent manner, as it was significantly decreased upon depletion of p53 by shRNA. Interestingly, RITA-induced growth arrest was less efficiently prevented by p53 depletion, as well as repression of $CDC2$, $CDC25C$, and $CCNB1$. These data are in line with the requirement of higher p53 levels of induction of its proapoptotic targets than for regulating cell cycle genes (30).

We observed a suppression of growth in HeLa xenografts *in vivo* following i.p. treatment of 10 mg/kg of RITA, suggesting that compounds based on RITA might be applicable in a future for the treatment of disseminated disease. Further studies are needed to optimize the treatment protocol, i.e., the dose and duration of treatment, drug delivery, etc.

In conclusion, our study identifies a promising novel strategy for p53 rescue in cervical carcinoma, which can lead to the development of efficient therapies against HPV-positive cervical cancer. Moreover, our findings show that targeting p53 by small molecules may confer the protection of p53 from several inhibitors that inactivate p53 in cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Grant Support

References

1. zur Hausen H. Papillomaviruses in the causation of human cancers—a brief historical account. Virology 2009;384:260–5.
2. Walboomers JM, Jacobs MV, Manos MM, et al. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol 1999;189:12–9.
3. Beaudenon S, Huibregtse JM. HPV E6, E6AP and cervical cancer. BMC Biochem 2008;9 Suppl 1:S4.
4. Butz K, Denk C, Ullmann A, Scheffner M, Hoppe-Seyler F. Induction of apoptosis in human papillomaviruspositive cancer cells by peptide aptamers targeting the viral E6 oncoprotein. Proc Natl Acad Sci U S A 2000;97:6693–7.
5. Levine AJ. p53, the cellular gatekeeper for growth and division. Cell 1997;88:323–31.
6. Hengstermann A, Linares LK, Ciechanover A, Whitaker NJ, Scheffner M. Complete switch from Mdm2 to human papillomavirus E6-mediated degradation of p53 in cervical cancer cells. Proc Natl Acad Sci U S A 2001;98:1218–23.
7. Kastan MB. Wild-type p53: tumors can't stand it. Cell 2007;128:837–40.
8. Vassilev LT, Vu BT, Graves B, et al. *In vivo* activation of the p53 pathway by small-molecule antagonists of MDM2. Science 2004;303:844–8.
9. Issaeva N, Bozko P, Enge M, et al. Small molecule RITA binds to p53, blocks p53-HDM-2 interaction and activates p53 function in tumors. Nat Med 2004;10:1321–8.
10. Dymalla S, Scheffner M, Weber E, et al. A novel peptide motif binding to and blocking the intracellular activity of the human papillomavirus E6 oncoprotein. J Mol Med 2009;87:321–31.
11. von Knebel Doeberitz M, Rittmuller C, zur Hausen H, Durst M. Inhibition of tumorigenicity of cervical cancer cells in nude mice by HPV E6-7 anti-sense RNA. Int J Cancer 1992;51:831–4.
12. Jiang M, Milner J. Selective silencing of viral gene expression in HPV-positive human cervical carcinoma cells treated with siRNA, a primer of RNA interference. Oncogene 2002;21:6041–8.
13. Goodwin EC, DiMaio D. Repression of human papillomavirus oncogenes in HeLa cervical carcinoma cells causes the orderly reactivation of dormant tumor suppressor pathways. Proc Natl Acad Sci U S A 2000;97:12513–8.
14. Hietanen S, Lain S, Krausz E, Blattner C, Lane DP. Activation of p53 in cervical carcinoma cells by small molecules. Proc Natl Acad Sci U S A 2000;97:8501–6.
15. Kochetkov DV, Il'inskaia GV, Komarov PG, et al. Transcriptional inhibition of human papilloma virus in cervical carcinoma cells

reactivates functions of the tumor suppressor p53. Mol Biol (Mosk) 2007;41:515–23.

16. Kelley ML, Keiger KE, Lee CJ, Huibregtse JM. The global transcriptional effects of the human papillomavirus E6 protein in cervical carcinoma cell lines are mediated by the E6AP ubiquitin ligase. J Virol 2005;79:3737–47.

17. Talis AL, Huibregtse JM, Howley PM. The role of E6AP in the regulation of p53 protein levels in human papillomavirus (HPV)-positive and HPV-negative cells. J Biol Chem 1998;273:6439–45.

18. Gu W, Putral L, Hengst K, et al. Inhibition of cervical cancer cell growth *in vitro* and *in vivo* with lentiviral-vector delivered short hairpin RNA targeting human papillomavirus E6 and E7 oncogenes. Cancer Gene Ther 2006;13:1023–32.

19. Enge M, Bao W, Hedstrom E, Jackson SP, Moumen A, Selivanova G. MDM2-dependent downregulation of p21 and hnRNP K provides a switch between apoptosis and growth arrest induced by pharmacologically activated p53. Cancer Cell 2009;15:171–83.

20. Kroemer G, Galluzzi L, Vandenabeele P, et al. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. Cell Death Differ 2009;16:3–11.

21. Vakifahmetoglu H, Olsson M, Tamm C, Heidari N, Orrenius S, Zhivotovsky B. DNA damage induces two distinct modes of cell death in ovarian carcinomas. Cell Death Differ 2008;15:555–66.

22. Huibregtse JM, Scheffner M, Howley PM. Cloning and expression of the cDNA for E6-AP, a protein that mediates the interaction of the human papillomavirus E6 oncoprotein with p53. Mol Cell Biol 1993; 13:775–84.

23. Taylor WR, Stark GR. Regulation of the G2/M transition by p53. Oncogene 2001;20:1803–15.

24. Allen KL, Tschantz DR, Awad KS, Lynch WP, DeLucia AL. A plant lignan, 3′-O-methyl-nordihydroguaiaretic acid, suppresses papillomavirus E6 protein function, stabilizes p53 protein, and induces apoptosis in cervical tumor cells. Mol Carcinog 2007;46: 564–75.

25. Dey A, Verma CS, Lane DP. Updates on p53: modulation of p53 degradation as a therapeutic approach. Br J Cancer 2008;98:4–8.

26. Mishra A, Jana NR. Regulation of turnover of tumor suppressor p53 and cell growth by E6-AP, a ubiquitin protein ligase mutated in Angelman mental retardation syndrome. Cell Mol Life Sci 2008; 65:656–66.

27. Koivusalo R, Mialon A, Pitkanen H, Westermarck J, Hietanen S. Activation of p53 in cervical cancer cells by human papillomavirus E6 RNA interference is transient, but can be sustained by inhibiting endogenous nuclear export-dependent p53 antagonists. Cancer Res 2006;66:11817–24.

28. Grinkevich VV, Nikulenkov F, Shi Y, et al. Ablation of key oncogenic pathways by RITA-reactivated p53 is required for efficient apoptosis. Cancer Cell 2009;15:441–53.

29. Hedstrom E, Eriksson S, Zawacka-Pankau J, Arner ES, Selivanova G. p53-dependent inhibition of TrxR1 contributes to the tumor-specific induction of apoptosis by RITA. Cell Cycle 2009;8:3576–83.

30. Chen X, Ko LJ, Jayaraman L, Prives C. p53 levels, functional domains, and DNA damage determine the extent of the apoptotic response of tumor cells. Genes Dev 1996;10:2438–51.

Correction: Rescue of p53 Function by Small-Molecule RITA in Cervical Carcinoma by Blocking E6-Mediated Degradation

In this article (Cancer Res 2010;70:3372–81), which appeared in the April 15, 2010 issue of *Cancer Research* (1), there are errors in Fig. 2A and B. The actin blot, serving as a loading control for PARP in CaSki cells, was accidentally inserted in place of the panels showing loading control for p-histone H3 in Fig. 2B. In addition, the incorrect actin loading control for p-histone H3 in Fig. 2A was used. These mistakes were made during the assembly of the figure. The errors do not affect any of the findings reported in the article.

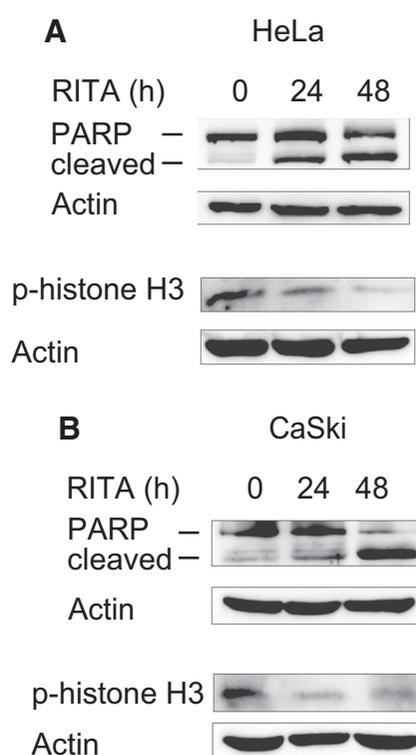The corrected panels appear below. The authors regret these errors.

Figure 2.

Reference

1. Zhao ZY, Szekely L, Bao W, Selivanova G. Rescue of p53 function by small-molecule RITA in cervical carcinoma by blocking E6-mediated degradation. Cancer Res 2010;70:3372–81.

Rescue of p53 Function by Small-Molecule RITA in Cervical Carcinoma by Blocking E6-Mediated Degradation

Carolyn Ying Zhao, Laszlo Szekely, Wenjie Bao, et al.

Updated version	Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/70/8/3372
Supplementary Material	Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2010/04/14/70.8.3372.DC1

Cited articles	This article cites 30 articles, 10 of which you can access for free at: http://cancerres.aacrjournals.org/content/70/8/3372.full#ref-list-1
Citing articles	This article has been cited by 5 HighWire-hosted articles. Access the articles at: http://cancerres.aacrjournals.org/content/70/8/3372.full#related-urls

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/70/8/3372. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.