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

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Abstract

p53 is degraded in cervical cancer cells by the human papillomavirus E6 and can be stabilized with short interfering RNA (siRNA) molecules targeting E6 mRNA. In this *in vitro* study, we show that E6 siRNA–induced p53 activation is transient in HeLa cervical cancer cells despite continuous suppression of E6 mRNA; activation can be sustained if the endogenous p53 antagonists COP1, MDM2, Pirh2, and c-*Jun*-NH_2-kinase are also targeted by siRNAs or by inhibiting the nuclear export of p53 with leptomycin B. The direct targeting of any one of these four cellular p53 antagonists had no effect on p53 activity when E6 was intact, but inhibited the fading off of E6 siRNA–induced p53 activation in nonstress conditions. The effect was additive when multiple cellular antagonists were concomitantly inhibited, indicating that all these proteins degrade p53 when E6 is inactivated. The antiproliferative effect induced by E6 silencing was enhanced when the endogenous p53 antagonists were additionally targeted. In conclusion, if human papillomavirus E6 is inhibited under nonstress conditions, the subsequent p53 activation is quickly reversed by the endogenous p53 degenerative machinery. The present results indicate that several cellular p53 antagonists must be inhibited for sustained p53 activity if E6 siRNA therapy is attempted and if no combined genotoxic therapy is applied. (Cancer Res 2006; 66(24): 11817-24)

Introduction

High-risk human papillomaviruses (HPV) play a pivotal role in the pathogenesis of cervical cancer. After infecting the genital mucosa and becoming integrated into the host genome, they start to overexpress E6 and E7 oncoproteins, which then immortalize the host cells by disrupting p53 and pRb function, respectively (1). E6 binds to p53 and targets it for ubiquitin-mediated degradation; E7 inactivates pRb in a similar manner. p53 coordinates cellular responses to different forms of stress, e.g., DNA damage. Activation of p53 may trigger apoptosis, cell cycle arrest, or attempts to repair the damaged DNA, depending on how extensive the damage is (2).

As current treatment modalities are rather ineffective against advanced cervical cancer, new therapeutic approaches are needed. Because the p53 pathway is not irreversibly damaged in cervical cancer, means of reactivating p53 in this malignancy have been actively studied. Previously, gamma-irradiation, certain cytotoxic drugs, small molecule compounds, and some direct anti-E6 approaches, such as ones based on antisense and ribozyme techniques, have been studied and reported to activate p53 in cervical cancer cells (3–7).

The discovery of RNA interference has enabled selective, highly efficient, and sustained degradation of the desired target mRNA. In RNA interference, small double-stranded RNA molecules, called short interfering RNA (siRNA), induce the degradation of homologous mRNA as part of the RNA-induced silencing complex (8, 9). The viral E6 expression in cervical cancer cells can be successfully silenced using siRNA, leading to the reactivation of p53 (10–14). However, these studies present controversial data on the biological consequences of E6 silencing: in one study, massive apoptosis was seen (14), whereas the main outcome of E6 siRNA treatment in other studies was either inhibition of cell growth or induction of cell senescence (10–13).

In our previous study, siRNA-mediated E6 silencing produced only transient p53 activation in HeLa cervical cancer cells despite continuous suppression of E6 mRNA, together with a modest antiproliferative effect (13). These findings suggested that cellular p53-antagonizing mechanisms were activated in response to E6 depletion. There have been efforts to develop means of targeting HPV E6, with the hope that anti-E6 therapies would be clinically effective against cervical cancer. However, the quickly decreasing p53 activation may significantly limit the clinical usefulness of E6 siRNA monotherapy.

Cellular negative regulation of p53 is principally mediated by certain ubiquitin ligases, such as MDM2. MDM2 forms a tight negative feedback loop with p53: active p53 stimulates MDM2 gene expression, and the resulting MDM2 protein binds to p53, exports it out of the nucleus and targets it for ubiquitin-mediated degradation (15, 16). Expression of E6 renders the MDM2 pathway silent in cervical cancer cells, but MDM2 becomes active in these cells in response to p53 activation induced by the small molecule compounds, actinomycin D and leptomycin B (LMB; refs. 5, 17). Also, the RING finger proteins, COP1 and Pirh2, can act as negative p53 regulators by working in a similar manner as MDM2, but independently of it (18, 19). c-*Jun*-NH_2-kinase (JNK), a p53 activator in stress conditions, targets p53 for ubiquitination and degradation in nonstressed cells (20). Before being degraded in the proteasome, p53 is transported from the nucleus to the cytoplasm. CRM1 is a central mediator of this nuclear export and works by binding to the nuclear export signal of the proteins to be exported, and guiding them to nuclear pores containing the actual export machinery (21).

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doi:10.1158/0008-5472.CAN-06-2185

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LMB, a specific inhibitor of CRM1, can disrupt the nucleocytoplasmic shuttling of p53 (22). Here, we have studied the functional role of the p53 antagonists COP1, JNK, MDM2, and Pirh2 in the suppression of E6 siRNA–induced p53 activation in cervical cancer cells *in vitro*.

Materials and Methods

Cell lines and plasmids. The HeLa and SiHa human cervical cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). The cells were cultured in DMEM (Euroclone, Wetherby, United Kingdom) containing 10% fetal bovine serum, 2 mmol/L L-glutamine, 1× nonessential amino acids (Euroclone), and 50 μg/mL of gentamicin (Calbiochem, San Diego, CA). The HeLa p53 reporter cell line, carrying the p53 reporter plasmid ptkGC$_3$p53luc-bsd (23), has been described previously (6). The HeLa DDp53 cell line is stably transfected with a plasmid expressing a truncated dominant-negative p53 under the control of a CMV promoter. The HeLa CMV was used as a control cell line, carrying the empty vector pCMV-neo (6).

siRNAs, transfections, and drugs. The 18E6 siRNA is targeted against HPV18 E6 mRNA, and in a previous study, we designated it 18E6-385 siRNA (13). The 16E6 siRNA (10) and the COP1, MDM2, and Pirh2 siRNAs (19) have also been previously described. Functionally validated commercial JNK1 and JNK2 siRNAs were purchased from Qiagen (Hilden, Germany), all the other siRNAs were purchased from Dharmacon (Lafayette, CO). The siRNA delivery was carried out with the OligofectAMINE (Invitrogen, Carlsbad, CA) transfection reagent according to the manufacturer's instructions. The siRNA transfection efficiency with this method is ~80% as determined under light microscopy of cells transfected with Eg5 siRNA molecules.[7] siRNA-mediated inhibition of Eg5, a kinesin-related motor protein, causes mitotic arrest and a characteristic round cellular phenotype that can be followed for monitoring siRNA transfection efficiency (24). LMB was ordered from Sigma-Aldrich (St. Louis, MO) and SP600125 was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA).

Western blotting. The cells were washed twice with ice-cold PBS, overlayed with 200 μL of standard 2× SDS sample buffer, and harvested with a cell scraper. The resulting whole cell extracts were boiled for 5 minutes, separated by 10% SDS-PAGE and transferred to Immobilon-P polyvinylidene fluoride membranes (Millipore, Billerica, MA). The membranes were probed with the following primary antibodies: the monoclonal p53 antibody DO-1 (Santa Cruz Biotechnology, Santa Cruz, CA), a polyclonal COP1 antibody (BIOMOL), the monoclonal MDM2 antibody 2A10 (Oncogene Research Products, San Diego, CA), the polyclonal Pirh2 antibody BL588 (Bethyl Laboratories, Montgomery, TX), the polyclonal poly(ADP-ribose) polymerase (PARP) antibody H-250 (Santa Cruz Biotechnology), and the polyclonal horseradish peroxidase–conjugated actin antibody C-11 (Santa Cruz Biotechnology). Secondary horseradish peroxidase–conjugated sheep anti-mouse and swine anti-rabbit antibodies were purchased from Amersham Biosciences (Piscataway, NJ) and DakoCytomation (Glostrup, Denmark), respectively. The protein bands were detected with enhanced chemiluminescence reagents (Amersham). The band intensities were quantified through MCID image analysis software from Imaging Research, St. Catharines, Ontario, Canada. The relative protein levels were obtained by normalizing each protein band of interest to the density of the corresponding actin band.

p53 reporter assay. The ptkGC$_3$p53luc-bsd HeLa p53 reporter cells (6, 23) were plated in triplicate into 96-well plates (~3.5 × 10^3 cells/well). The number of living cells in each well after treatment was estimated colorimetrically with the WST-1 assay (Roche, Mannheim, Germany), and the luciferase activity was determined with the luciferase assay reagent Bright-Glo (Promega, Madison, WI). In both assays, the plates were read in the Wallac Victor^2 microplate reader (Perkin-Elmer Wallac, Turku, Finland). The p53 readings were normalized against the WST-1 values to obtain relative p53 reporter activities.

Cell proliferation assay. HeLa CMV and HeLa DDp53 cells were plated in triplicate into 96-well plates (500 cells/well) and were allowed to attach for 24 hours. The cells were then transfected with one or more siRNAs (20 nmol/L each), targeting HPV16 E6, HPV18 E6, COP1, MDM2 or Pirh2 mRNA, and/or were treated with 2 μmol/L of SP600125. Control cells were treated with medium. After 5 days, the amount of surviving cells in each well was quantified colorimetrically with the WST-1 assay. The absorbance readings of treated wells were compared with the readings of control wells to yield relative values. Both the siRNAs and the SP600125 were left in the cell culture medium for the entire experiment.

Results

E6 siRNA–induced p53 activation is transient in HeLa cervical cancer cells. Despite sustained suppression of E6 mRNA levels, E6 siRNA treatment induced only transient p53 protein accumulation and p53 reporter activation in HPV18-positive HeLa cervical cancer cells (ref. 13; Fig. 1). Once the HeLa cells had been transfected with 18E6 siRNA, the p53 protein levels increased 15-, 11-, 6-, and 5-fold at 24, 48, 72, and 96 hours posttransfection, respectively (Figs. 1 and 2). The nonhomologous 16E6 siRNA induced a modest and transient increase in p53 protein levels which is probably explained by nonspecific stress caused by the transfection process. Neither 16E6 nor 18E6 siRNA induced PARP protein cleavage, indicating that there is no apoptosis-related caspase activity (Fig. 2). There was a 48-hour lag between stabilization and transcriptional activation of p53. This is in line with previous data showing that the transcriptional activation of p53 is critically dependent, for example, on direct interaction between p53 and the prolyl isomerase Pin1. This is something that does not readily occur in the absence of DNA damage-induced phosphorylation of p53 (25–27). Thus, the lack of genotoxic damage after E6 siRNA treatment could at least partially explain the slow transcriptional activation of p53.

Endogenous degradation of p53 is activated after E6 downmodulation. In various cell types, COP1, MDM2, and Pirh2 protein levels increase in response to p53 activation, leading to accelerated proteasomal p53 degradation (15, 16, 18, 19), whereas JNK is not induced by p53 and degrades p53 specifically in nonstressed cells (20). In the present study, COP1, MDM2, and Pirh2 protein levels were increased in HeLa cells in response to 18E6 siRNA–induced p53 activation (Fig. 2). The induction of COP1, MDM2, and Pirh2 was maximal at 24 hours, and it decreased along with the decreasing p53 protein levels over time. At 96 hours, COP1, MDM2, and Pirh2 proteins reached their initial levels, and the amount of p53 protein did not decrease further. These findings indicate a reciprocal relationship between these proteins. Also, the nonhomologous 16E6 siRNA raised COP1, MDM2, and Pirh2 protein levels slightly. COP1 and MDM2 were almost undetectable in untreated HeLa cells, but Pirh2 was expressed in readily detectable quantities in the presence of E6 (Fig. 2). The JNK isoforms JNK1 and JNK2 were constitutively expressed in untreated HeLa cells (Fig. 2). In contrast to COP1, MDM2, and Pirh2, both JNK1 and JNK2 protein levels decreased after transfection with 18E6 siRNA. Essentially identical changes were also recorded after transfection with 16E6 siRNA. Because JNK activity correlates negatively with cell density (28), the reduced JNK protein levels may be a response to increasing cell confluency during the course of the experiment.

[7] Unpublished data.

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siRNA-mediated degradation of COP1, JNK, MDM2, and Pirh2 mRNA prevents the fading off of E6 siRNA–induced p53 activity. We next studied how direct targeting of COP1, JNK, MDM2, and Pirh2 modulates 18E6 siRNA–induced p53 activation in HeLa cells. The p53 activities were measured at 96 hours after treatment, when the p53 activation induced by 18E6 siRNA alone had already returned to near control levels.

COP1, MDM2, and Pirh2 siRNAs alone induced only marginal p53 reporter activations at 96 hours (Fig. 3*A*). However, each of these three siRNAs inhibited the decrease of 18E6 siRNA–induced p53 activation, and the effect was more pronounced when two or three cellular p53 antagonists were targeted, rather than one (Fig. 3*A*). In contrast to 18E6 siRNA, the nonhomologous 16E6 siRNA did not affect the p53-activating properties of COP1, MDM2, and Pirh2 siRNAs (Fig. 3*A*), nor was 18E6 siRNA–induced p53 reporter activation significantly affected by cotransfection with 16E6 siRNA (data not shown). This indicates that the effect of COP1, MDM2, and Pirh2 siRNAs is not explained by nonspecific p53-activating stress, but rather that, under normal conditions, viral E6 is the predominant p53 antagonist in HeLa cervical cancer cells, whereas the endogenous p53 antagonists COP1, MDM2, and Pirh2 are more or less inactive. However, when E6 expression is abrogated with siRNA, these three endogenous p53 antagonists begin to reverse the siRNA-induced p53 activation.

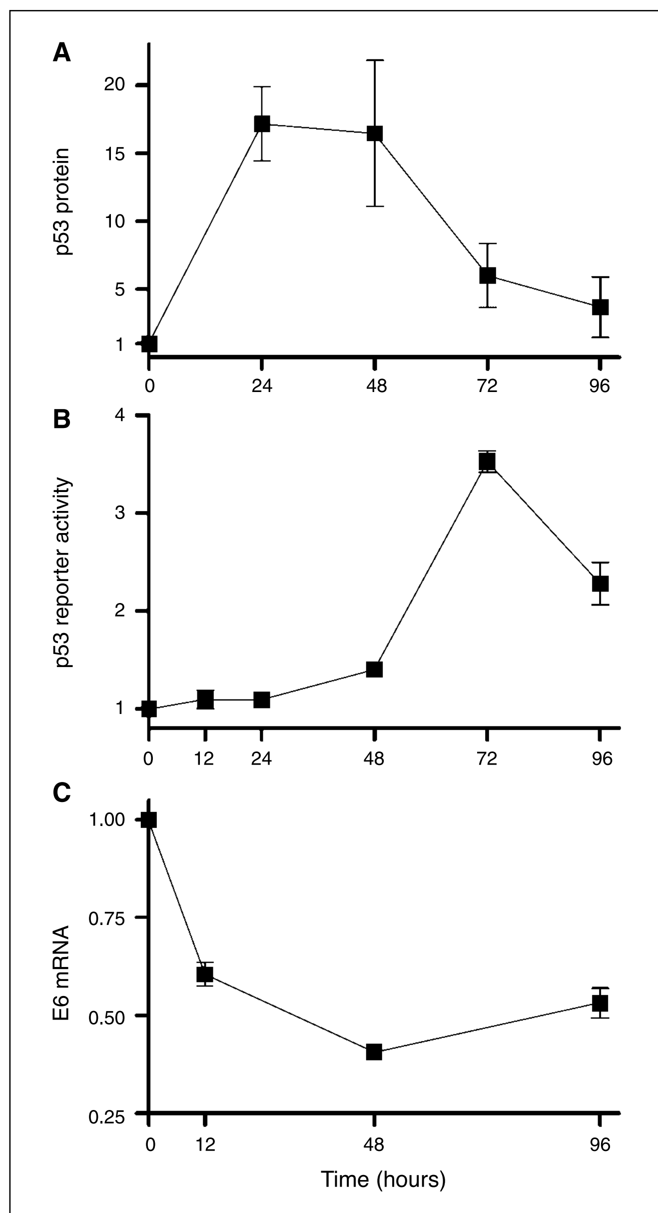

Figure 1. Kinetic changes in p53 protein levels (*A*), p53 reporter activity (*B*), and amount of HPV18 E6 mRNA (*C*) in HPV18-positive HeLa cervical cancer cells after transfection with 18E6 siRNA. The p53 reporter activities and the E6 mRNA data, obtained by quantitative reverse transcription-PCR, were derived from our previous study (13). The relative p53 protein values are plotted from time course Western blot experiments done in the present study; for a representative blot, see Fig. 2. *Points,* means from triplicate experiments; *bars,* ±SD.

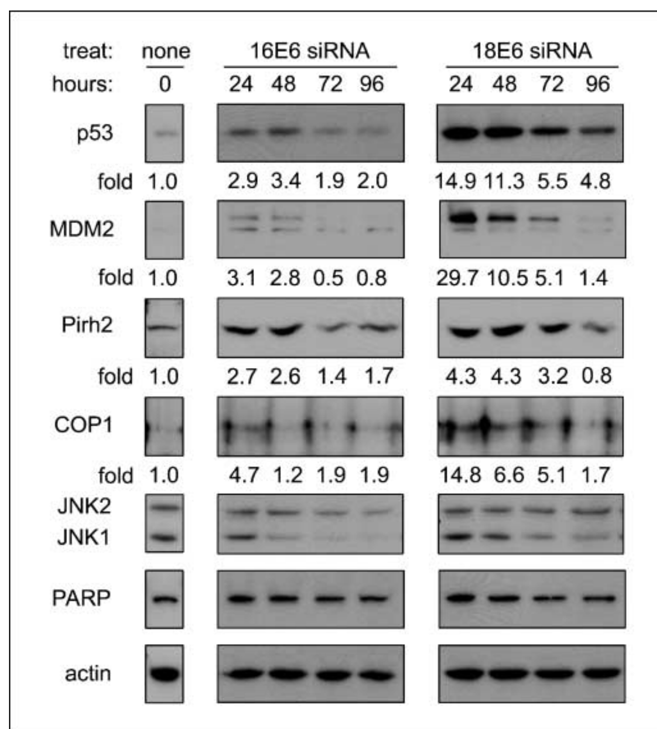

Figure 2. p53, MDM2, Pirh2, COP1, and JNK protein levels and PARP status in HeLa cells after siRNA-mediated abrogation of HPV E6 function. HPV18-positive HeLa cells were transfected with 30 nmol/L of siRNA targeting either HPV16 or HPV18 E6 mRNA, whereas reference cells (*left*) were left untreated. The cells were harvested at the indicated time points to obtain whole cell extracts, which were analyzed by Western blotting. Equal sample loading was confirmed by determining the actin expression levels. The densities of p53, MDM2, Pirh2, and COP1 bands were quantified digitally and normalized against the density of the corresponding actin band. PARP is cleaved in the early stages of apoptosis by certain caspases, in which case, the resulting 89 kDa fragment could be visualized with a PARP antibody along with the intact 113 kDa protein. Here, the PARP protein remains intact in all lanes, indicating the absence of apoptosis. Representative blots from at least two independent experiments: the kinetic changes in the levels of each studied protein were similar based on the SD values (for an example, see p53 in Fig. 1).

JNK1 and JNK2 are the most abundant of the 10 currently known JNK isoforms. To study the role of JNK as a p53 antagonist in 18E6-depleted HeLa cells, we did experiments with JNK1 and JNK2 siRNAs, and a plasmid expressing MKK4, an upstream positive regulator of JNK. Because there was a moderate antiproliferative effect at 96 hours after transfection with 18E6 and JNK siRNAs, probably in part because of reduced JNK-mediated p53-independent pro-proliferative signaling (29–31), the 48-hour time point was chosen as the time point to analyze, instead of 96 hours. Importantly, JNK1 and JNK2 siRNAs enhanced 18E6 siRNA–induced p53

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protein stabilization compared with the nonhomologous 16E6 siRNA (Fig. 3*B*). Consistently, overexpression of MKK4, but not transfection with a plasmid expressing green fluorescent protein, suppressed 18E6 siRNA–induced p53 reporter activity at 96 hours (Fig. 3*C*). These data suggest that JNK degrades p53 after siRNA-mediated E6 silencing. Interestingly, 18E6 siRNA–induced p53 reporter activation was suppressed when either JNK1 or JNK2 was degraded by siRNA, but not when both JNK isoforms were targeted (data not shown). Therefore, although E6 siRNA–induced p53 protein stabilization can be increased by degrading either JNK1 or JNK2, the newly stabilized p53 protein does not seem to become transcriptionally active, unless both main JNK isoforms are concomitantly targeted.

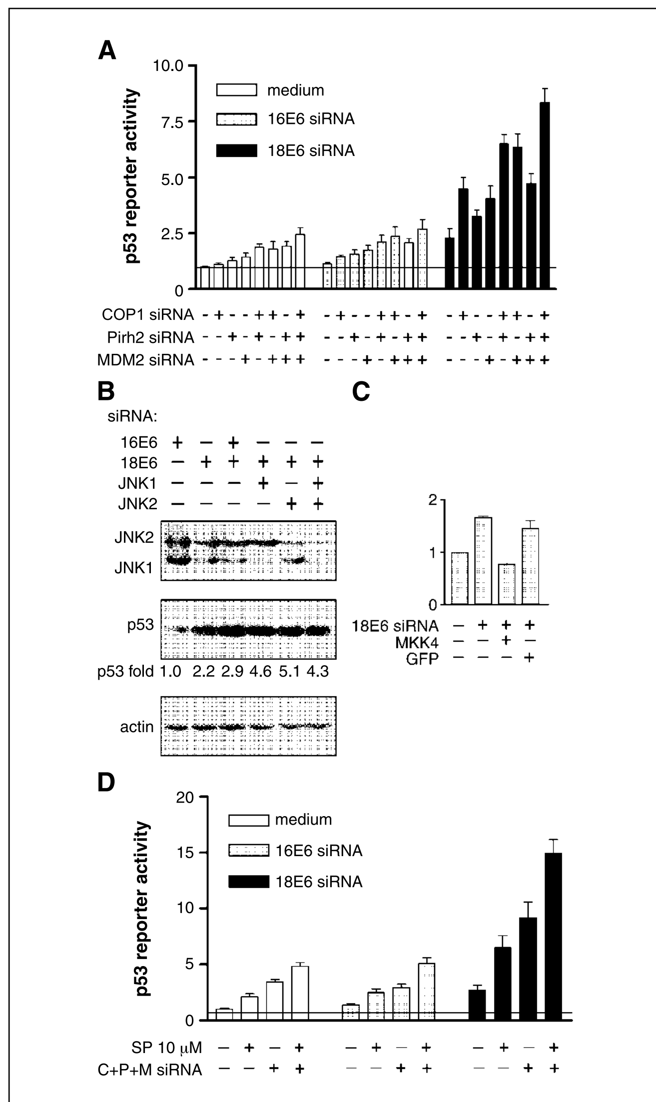

Figure 3. Targeting of the endogenous p53-antagonizing machinery enhances 18E6 siRNA–induced p53 activation in HeLa cells. *A,* p53 reporter activity in HeLa cells after degrading some or all of the following mRNAs with siRNA molecules: HPV18 E6, COP1, Pirh2, and MDM2 mRNA. HeLa p53 reporter cells were transfected with the indicated siRNAs (30 nmol/L each), and after 96 hours, the luciferase activities were measured and normalized against the relative cell number obtained with the colorimetric WST-1 assay. Note that 16E6 siRNA has no target in HPV18-positive HeLa cells. *B,* effect of JNK1 and JNK2 siRNAs on 18E6 siRNA–induced p53 protein stabilization in HeLa cells. The cells were harvested 48 hours after transfection with 30 nmol/L of siRNAs. The p53 protein bands were quantified and normalized against the corresponding actin bands to obtain relative p53 expression levels. *C,* effect of MKK4-mediated JNK stimulation on 18E6 siRNA–induced p53 reporter activation in HeLa cells. HeLa cells were transfected with 30 nmol/L 18E6 siRNA and incubated for 48 hours, after which, the cells were transfected with a plasmid expressing MKK4 or GFP. The p53 reporter activities were measured at 96 hours. *D,* HeLa p53 reporter activity after targeting HPV18 E6 and endogenous p53 regulators in different combinations. The cells were transfected with the indicated siRNAs (30 nmol/L each), and were incubated for 72 hours before adding the JNK1 and JNK2 inhibitor SP600125 to the cell culture medium. The p53 reporter activities were measured at 96 hours. *A* and *D,* the basal p53 reporter activity was adjusted to 1 (*horizontal line*). *Columns,* means from three independent experiments each done in triplicate wells; *bars,* +1 SD.

In line with the JNK siRNA data, the broad-range JNK inhibitor SP600125, which inhibits both JNK1 and JNK2, produced only modest p53 reporter activation when E6 expression was intact, but induced synergistic p53 activation with 18E6 siRNA (Fig. 3*D*). In contrast to 18E6 siRNA, 16E6 siRNA did not enhance the effect of SP600125. To study whether protein kinases in general could regulate p53 activity in cervical cancer cells, we tested three specific kinase inhibitors: the HER2 inhibitor, trastuzumab, the BCR-ABL inhibitor, imatinib, and the vascular endothelial growth factor receptor 1 and 2 inhibitor, bevacizumab. None of these three kinase inhibitors stimulated the p53 reporter when E6 expression was left intact, and importantly, each inhibitor did not prevent 18E6 siRNA–induced p53 activation from decreasing towards control levels (data not shown). SP600125 was used in the subsequent JNK targeting experiments to eliminate the need for multiple JNK siRNAs to obtain full JNK silencing. SP600125 further increased the p53 reporter activation induced by down-modulation of COP1, MDM2, and Pirh2 ± 18E6 (Fig. 3*D*). Therefore, inhibition of JNK seems to activate p53 in HeLa cells via a different pathway than inhibition of COP1, MDM2, and Pirh2.

Abrogation of nuclear export inhibits the fading off of E6 siRNA–induced p53 activation. COP1, MDM2, Pirh2, and JNK must export p53 out of the nucleus before p53 can be degraded in the proteasome. If these p53 regulators oppose E6 siRNA–induced p53 activation in HeLa cells, one should be able to prevent the fading off of the p53 activation by abrogating the nuclear export of p53. Indeed, when HeLa cells were treated with 18E6 siRNA and the nuclear export inhibitor LMB, both p53 protein levels and p53 reporter activity were synergistically enhanced (Fig. 4*A* and *B*). In contrast to 18E6 siRNA, the nonhomologous 16E6 siRNA did not enhance the p53-activating effect of LMB (Fig. 4*A* and *B*). Although the transfection efficiency of 18E6 siRNA is incomplete (~80%, see Materials and Methods), the effect of LMB is far too dramatic to be explained by the inhibition of the remaining E6 function alone. Rather, the above data strongly suggest that a cellular nuclear export–dependent process antagonizes E6 siRNA–induced p53 activation in HeLa cells. Also, because LMB could not further enhance the combined p53-activating effect of 18E6, COP1, MDM2, and Pirh2 siRNAs and SP600125 (Fig. 4*B*), there seems to be no additional endogenous nuclear export–dependent p53 antagonists to oppose the E6 siRNA–induced p53 activation in HeLa cells.

Cellular p53 antagonists inhibit both HPV16 and HPV18 E6 depletion-induced p53 activation. In both HPV18-positive HeLa and HPV16-positive SiHa cells, targeting of either the viral E6 or the endogenous p53 antagonists produced only slight increases in p53 protein levels at 96 hours (Fig. 5). In contrast, the simultaneous targeting of both E6 and the endogenous p53 antagonists induced strong elevations of the p53 protein levels in both cell lines (Fig. 5); thus, HeLa is not the only cervical cancer cell line in which endogenous p53 regulators oppose E6 siRNA–induced p53 activation. Interestingly, PARP remained uncleaved in every lane, indicating that—despite the enhanced p53 activation—not even

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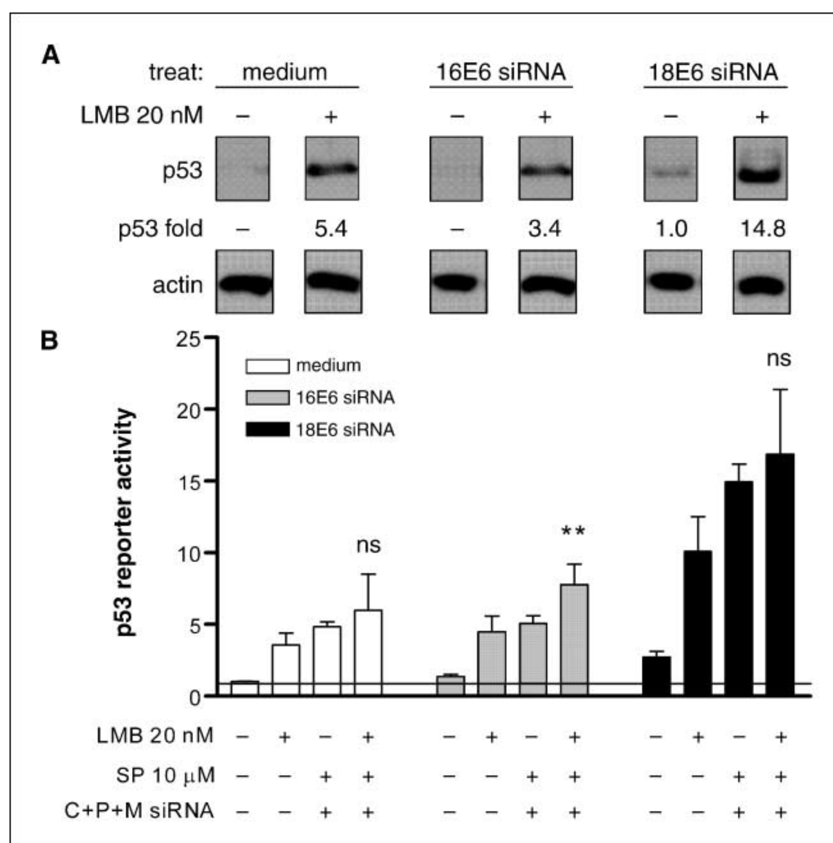

Figure 4. Abrogation of nuclear export by LMB inhibits the fading of 18E6 siRNA–induced p53 activation in HeLa cells. *A,* synergistic increase in p53 protein levels after concurrent treatment with 18E6 siRNA and LMB. HeLa cells were first transfected with 30 nmol/L of siRNA targeting either HPV16 or HPV18 E6 mRNA, or were treated with plain medium. LMB was added to the cell culture medium at 72 hours posttransfection. The cells were harvested at 96 hours, and the resulting whole cell extracts were analyzed by Western blotting. Representative blots from two independent experiments. *B,* p53 reporter activity after LMB-mediated inhibition of nuclear export, with or without simultaneous down-modulation of 18E6, COP1, MDM2, Pirh2, and JNK. HeLa p53 reporter cells were transfected with 30 nmol/L of siRNA or were overlaid with plain medium; LMB and the JNK inhibitor SP600125 were added to the cell culture medium 72 hours later. The relative p53 reporter activities were determined at 96 hours and the basal p53 reporter activity was adjusted to 1 (*horizontal line*). *Columns,* means from three independent experiments each done in triplicate wells; *bars,* +1 SD. The statistical comparison of p53 reporter activities induced by LMB + SP + COP1, MDM2, and Pirh2 siRNAs versus the same treatment without LMB was carried out with paired two-tailed *t* tests. *ns*, not significant; **, $P < 0.01$.

combined targeting of E6 and cellular p53 antagonists was able to induce apoptosis in these cells (Fig. 5).

Antiproliferative effect of E6 siRNA treatment is enhanced when endogenous p53 antagonists COP1, JNK, MDM2, and Pirh2 are also targeted. We next sought to determine whether boosting of the E6 siRNA–induced p53 activation further increases the antiproliferative effect of E6 silencing because it does not seem to stimulate the apoptotic pathway. We did cell proliferation assays with HeLa DDp53 and HeLa CMV cells carrying a dominant-negative p53 (DDp53) and an empty vector, respectively.

18E6 siRNA decreased HeLa CMV cell survival by 38% when given alone, and by 50% to 59% when cotransfected with COP1, MDM2, or Pirh2 siRNA (Fig. 6*A*). The combined down-modulation of 18E6, COP1, MDM2, and Pirh2 reduced HeLa CMV cell survival by 67%. However, simultaneous treatment with 18E6 siRNA and the JNK inhibitor SP600125 produced the most dramatic effect: only 9% of HeLa CMV cells survived this treatment (Fig. 6*A*). In contrast to 18E6 siRNA, the effect of the nonhomologous 16E6 siRNA (21% reduction in HeLa CMV cell survival) was not amplified when COP1, MDM2, or Pirh2 was additionally down-modulated (Fig. 6*A*). SP600125 and SP600125 + 16E6 siRNA reduced HeLa CMV cell survival by only 33% and 58%, respectively, compared with the 91% reduction after treatment with 18E6 siRNA and SP600125 (Fig. 6*A*). The effect was identical when medium was used instead of 16E6 siRNA in each treatment combination.

Disruption of wild-type p53 function by DDp53 expression abrogated most of the antiproliferative effect induced by 18E6 siRNA alone, and blocked approximately half of the growth inhibition seen after targeting both 18E6 and endogenous p53 antagonists (Fig. 6*B*). Therefore, COP1, MDM2, and Pirh2 siRNAs potentiate the effect of 18E6 siRNA in a p53-dependent manner, as expected. The finding that the antiproliferative effect of SP600125 ± 18E6 siRNA is only partially p53-dependent is in line with three previous studies showing that inhibition of the basal JNK activity decreases cell proliferation independently of p53 (29–31), possibly through diminished c-*Jun*-mediated transactivation of genes, such as endothelial growth factor receptor, which promote cellular growth (32). These findings suggest that in cervical cancer cells, JNK not only counteracts E6 siRNA–induced p53 activation, but also stimulates cellular proliferation, regardless of p53 or E6 status.

Discussion

This study shows that the release of p53 from E6 degradation after E6 siRNA treatment is rapidly reversed by host endogenous p53 degradation in nonstressed cervical cancer cells. This finding provides a mechanistic explanation why siRNA-mediated E6 silencing has generally given only modest growth inhibitory responses. We have previously shown that E6 siRNAs can induce sustained p53 activation when coupled with genotoxic treatments (13). Several other studies have also shown that E6 siRNAs could activate p53 in cervical cancer cells, but in these studies, the investigators did not extend their p53 analyses beyond 48 or 72 hours posttransfection (10–12), with one exception (14). In that article, p53 immunostaining in HeLa and SiHa cells seemed to be more intense at 24 hours than at 96 hours posttransfection with E6 siRNA, but this observation was not discussed.

Targeting of COP1, MDM2, Pirh2, and JNK produced additive effects in the p53 reporter experiments. These cellular p53

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regulators seem to antagonize E6 siRNA–induced p53 activation independently of each other. This finding is consistent with previous observations: ectopically expressed Pirh2 shortens the half-life of the p53 protein in MDM2-null mouse embryonic fibroblasts (18), and COP1 negatively regulates p53 in the absence of MDM2 and Pirh2 in mouse embryonic fibroblasts and Saos-2 cells (19). Our finding that MDM2 protein levels are low and MDM2 siRNA induces only negligible p53 activation when E6 expression is intact is in line with previous studies that have shown that the MDM2 pathway is not operational in cervical cancer cells expressing HPV E6 (5, 17). Because COP1 and Pirh2 share considerable functional homology with MDM2, it was not surprising that neither COP1 nor Pirh2 siRNA induced any significant p53 activation in HeLa cells in the presence of E6. Similarly, the JNK inhibitor SP600125 alone causes only weak p53 activation in the presence of E6.

All experiments conducted with SP600125 should be interpreted with some caution because SP600125 is not an entirely specific inhibitor of JNK (33). However, in support of the SP600125 data, suppression of JNK activity by siRNA enhanced E6 siRNA–induced p53 stabilization, whereas MKK4-mediated stimulation of JNK activity suppressed E6 siRNA–induced p53 activation. In line with the data obtained by direct targeting of COP1, Pirh2, MDM2, and JNK, we observed that LMB-mediated inhibition of nuclear export of p53 reverses the decrease of E6 siRNA–induced p53 activation, but does not further increase p53 activity when E6 and all four cellular p53 antagonists are already down-modulated. Thus, these four cellular proteins seem to be the predominant effectors responsible for the fading off of E6 siRNA–induced p53 activation in the cells we have studied.

COP1, MDM2, and Pirh2 siRNAs increased the antiproliferative effect of 18E6 siRNA from 38% to 50% to 59%, whereas combining the JNK inhibitor SP600125 with 18E6 siRNA reduced the survival of HeLa cells by 91%. However, SP600125 reduced HeLa cell survival by ~50% even when E6 was not simultaneously degraded with siRNA, whereas COP1, MDM2, and Pirh2 siRNAs alone caused only ~15% reductions in the cell survival rates. In addition to its role in p53 regulation, JNK is a p53-independent stimulator of cellular proliferation (29–31), and this fact might partially explain the strong antiproliferative effect of the SP600125 ± 18E6 siRNA treatment. SP600125 is a cell-permeable molecule and can affect the entire target cell population, but the effect of COP1, MDM2, and Pirh2 siRNAs is probably diluted to some degree, because the siRNA transfection efficiency never reaches 100% (it was ~80% in this study). Interestingly, there was no marked apoptosis in HeLa or SiHa cells, not even after simultaneous targeting of E6, COP1, JNK, MDM2, and Pirh2.

There may still be unidentified, nuclear export–independent cellular p53 antagonists that oppose the E6 siRNA–induced p53 activation in cervical cancer cells. These presumptive p53 antagonists could prevent apoptosis. On the other hand, the present data, and our previous observation, in which concurrent treatment with E6 siRNA and chemotherapy results in strong synergistic p53 activation and enhanced cytotoxicity (13), suggest that it may be frankly impossible to trigger any marked apoptosis in cervical cancer cells simply by targeting the p53-antagonizing

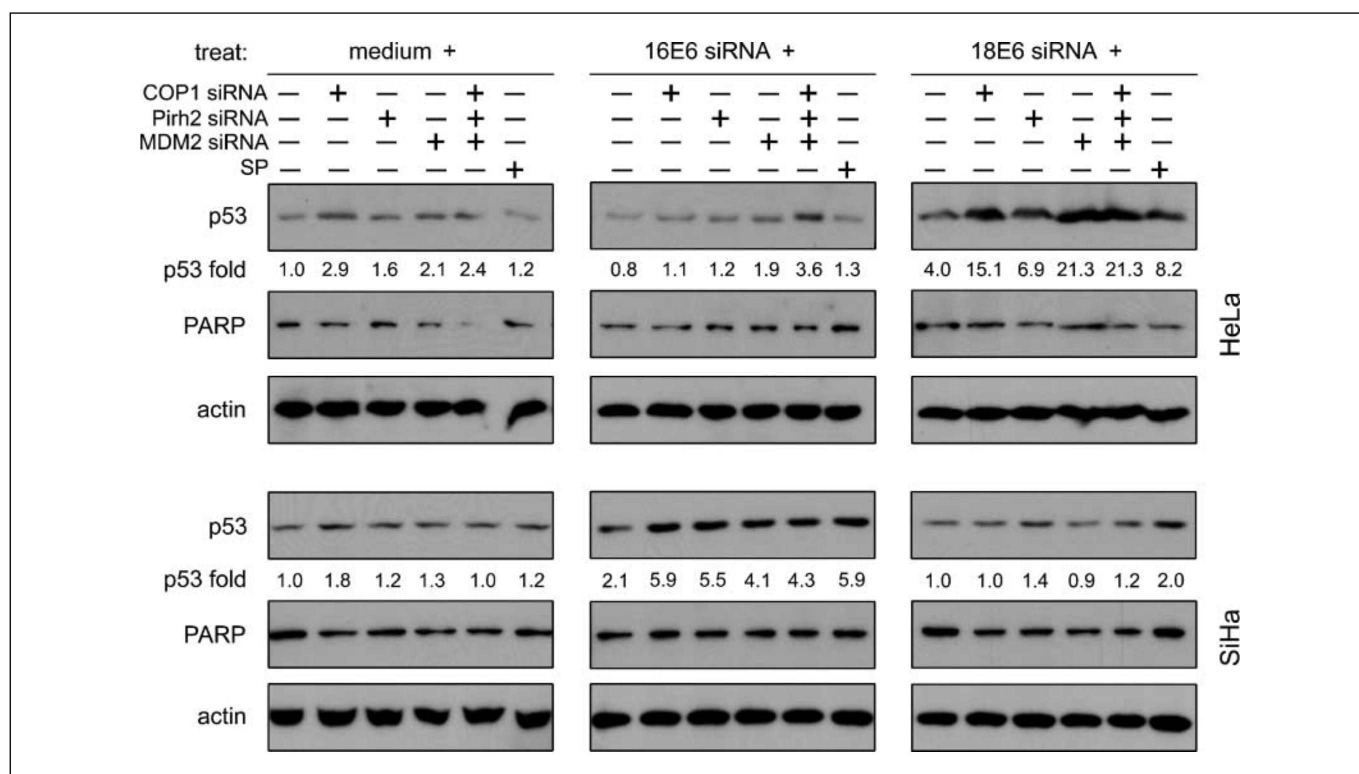

Figure 5. p53 protein levels and PARP status in HeLa and SiHa cervical cancer cell lines after targeting HPV E6 and endogenous p53 regulators in different combinations. HPV18-positive HeLa and HPV16-positive SiHa cells were transfected with the indicated siRNAs (30 nmol/L each), or were treated with cell culture medium alone. The JNK inhibitor, SP600125, was added 72 hours after the transfection. The cells were harvested at 96 hours, and the samples were analyzed by Western blotting. The density of each p53 band was quantified digitally and normalized against the density of the corresponding actin band. The resulting values are shown under the p53 bands. The experiment was done twice with essentially identical results.

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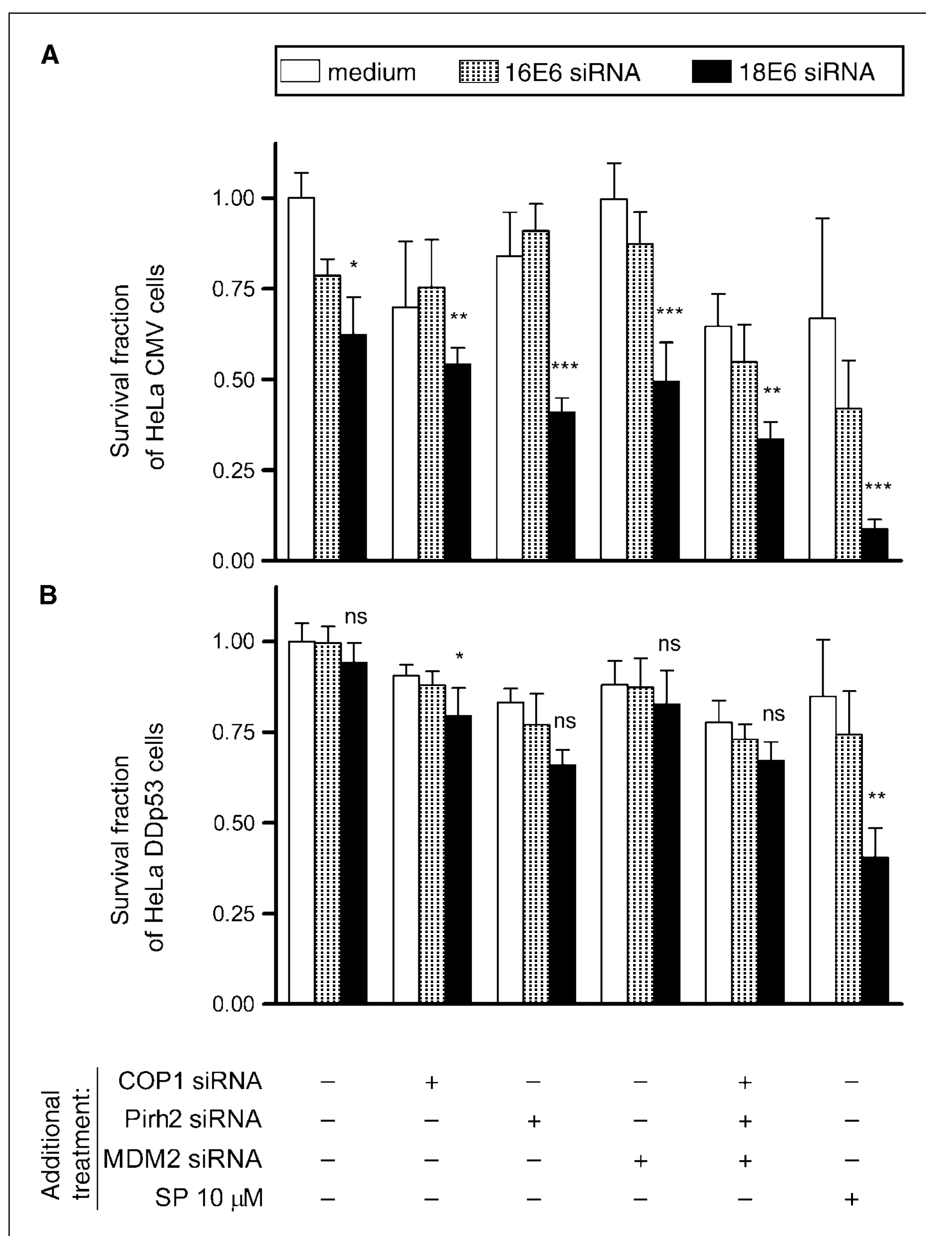

Figure 6. Survival of HeLa cells after targeting HPV E6 and endogenous p53 antagonists in different combinations. HeLa DDp53 cells (*B*), overexpressing a truncated dominant-negative p53 protein, and HeLa CMV cells (*A*), carrying the empty vector, were transfected with the indicated siRNAs (20 nmol/L each), and/or were treated with the JNK inhibitor SP600125 (2 μmol/L). Reference cells were treated with medium. The cells were allowed to grow for 5 days, after which, the amount of surviving cells was measured colorimetrically. *Columns,* means from two independent experiments both done in triplicate; *bars,* +1 SD. Statistical comparison of treatments including the reference siRNA 16E6 versus the corresponding treatments including the functional 18E6 siRNA was carried out with paired two-tailed *t* tests. *ns*, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. The differences in cell survival rates after using 16E6 siRNA versus DMEM in each treatment regimen were not statistically significant.

mechanisms without providing a proper stimulus for p53 at the same time, such as DNA damage by anticancer drugs or radiation.

Because HPV E6 plays a fundamental role in the pathogenesis of cervical cancer by disrupting the function of the p53 pathway, there have been hopes that anti-E6 approaches could be therapeutically effective in the treatment of cervical cancer. Here, we have presented evidence that the cellular p53 antagonists COP1, JNK, MDM2, and Pirh2 oppose the E6 siRNA–induced p53 activation in cervical cancer cells, and that both the p53 activation and the antiproliferative effect induced by the E6 siRNA treatment can be augmented by inhibiting the function of these endogenous p53 antagonists.

It is prudent to remember that although the present study was conducted with two common cervical cancer cell lines representing two of the most prevalent HPV types encountered in cervical cancer, the data derived from *in vitro* experiments cannot be transferred to an *in vivo* situation as such, let alone to the clinical setting. Nevertheless, the present results may have implications for the possible future use of E6 siRNAs for the treatment of cervical cancer: unless the endogenous p53 antagonizing machinery is targeted alongside HPV E6, and/or target cells are exposed to genotoxic stress, E6 siRNA–induced p53 activation fades quickly off. The findings in the present study may also have wider implications outside the context of cervical cancer, as endogenous p53 antagonists might also be activated in other cancer cell types in response to p53 reactivation therapies.

Acknowledgments

Received 6/15/2006; revised 8/30/2006; accepted 9/26/2006.

Grant support: Finnish Medical Society, State-Allocated Funding for Hospitals (EVO), AstraZeneca Finland, and Academy of Finland (Projects 878179 and 8212695).

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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

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Cancer Res 2006;66:11817-11824.

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