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RNA Interference against Human Papillomavirus Oncogenes in Cervical Cancer Cells Results in Increased Sensitivity to Cisplatin

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ABSTRACT

Targeted inhibition of oncogenes in tumor cells is a rational approach toward the development of cancer therapies based on RNA interference (RNAi). Tumors caused by human papillomavirus (HPV) infection are an ideal model system for RNAi-based cancer therapies because the oncogenes that cause cervical cancer, E6 and E7, are expressed only in cancerous cells. We investigated whether targeting HPV E6 and E7 oncogenes yields cancer cells more sensitive to chemotherapy by cisplatin, the chemotherapeutic agent currently used for the treatment of advanced cervical cancer. We have designed siRNAs directed against the HPV E6 oncogene that simultaneously targets both E6 and E7, which results in an 80% reduction in E7 protein and reactivation of the p53 pathway. The loss of E6 and E7 resulted in a reduction in cellular viability concurrent with the induction of cellular senescence. Interference was specific in that no effect on HPV-negative cells was observed. We demonstrate that RNAi against E6 and E7 oncogenes enhances the chemotherapeutic effect of cisplatin in HeLa cells. The IC_{50} for HeLa cells treated with cisplatin was 9.4 μM, but after the addition of a lentivirus-delivered shRNA against E6, the IC_{50} was reduced almost 4-fold to 2.4 μM. We also observed a decrease in E7 expression with a concurrent increase in p53 protein levels upon cotreatment with shRNA and cisplatin over that seen with individual treatment alone. Our results provide strong evidence that loss of E6 and E7 results in increased sensitivity to cisplatin, probably because of increased p53 levels.

Papillomaviruses are nonenveloped, double-stranded, DNA tumor viruses that cause a range of proliferative lesions upon infection of epithelial cells (Howley, 1996). These viruses are the critical factor in the formation of anogenital cancer (Zur Hausen, 1994). Infection with high-risk HPV types (i.e., 16, 18, 31, 45, 33, 35, etc.) is the predominant etiological factor in the development of cervical cancer, the second largest cause of cancer mortality in women worldwide with more than 270,000 deaths per year (Ferlay et al., 2004). HPV's transforming capabilities arise from the overexpression of the early genes E6 and E7, a result of the integration of the viral genome into the host genome, which disrupts the negative regulator of E6/7 transcription, E2 (Choo et al., 1987; Romanczuk and Howley, 1992).

The most well characterized target of HPV E6 is the p53 tumor suppressor protein. Under normal circumstances, p53 promotes cell cycle arrest or initiates apoptosis in response to cellular stress. However, the antiproliferative role of p53 is abrogated by E6, which promotes its accelerated degradation via ubiquitination and subsequent proteosomal degradation (Scheffner et al., 1990). In the absence of p53, cell cycle arrest in response to genetic insult is abolished, allowing the accumulation of harmful mutations that may contribute to malignant progression. The principal role of E7 is to overcome regulatory mechanisms to promote cell cycle advancement and entry into S-phase. E7's most well characterized target in this regard is the retinoblastoma tumor suppressor protein (pRB), which it binds and destabilizes via ubiquitin-mediated proteosomal degradation (Boyer et al., 1996).

Current therapies for the treatment of advanced cervical

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ABBREVIATIONS: RNAi, RNA interference; DMEM, Dulbecco's modified Eagle's medium; FACS, fluorescence-activated cell sorting; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; HPV, human papillomavirus; PARP, poly(ADP-ribose) polymerase; LV, lentivirus; shRNA, short hairpin RNA; siRNA, short interfering RNA; nt, nucleotide(s).

cancer involve the use of *cis*-diamminedichloroplatinum(II) (cisplatin), often in combination with radiotherapy. Cisplatin is believed to act via the formation of inter- and intrastrand cross-links in DNA, culminating in the initiation of cell death via caspases (Siddik, 2003). Unfortunately, the current cisplatin-based treatment for cervical cancer does not lead to a high disease-free survival rate in patients with bulky or locally-advanced disease. Although cisplatin is beneficial for the treatment of many cancers, side effects, including neurotoxicity, are limiting factors (Decatris et al., 2004). Development of resistance to cisplatin is also an issue (Siddik, 2002). Response rates to cisplatin therapy of around 50% can be achieved for cervical cancer, but the outlook for patients with persistent or recurrent squamous cell carcinoma is bleak, with only 20% survival at 12 months.

The overexpression of the HPV E6 and E7 oncogenes in cervical cells results in the development of cancer; therefore, these tumors seem to be ideal candidates for therapy using RNA interference (RNAi). Cervical cancer cells rely on the continual expression of E6 and E7 for growth, and loss of oncogene expression results in reduced DNA replication and senescence because of the re-establishment of these tumor suppressor pathways (Goodwin et al., 2000; DeFilippis et al., 2003). Moreover, because E6 and E7 genes are not present in normal cells, RNAi-based therapies would not affect them. Indeed, a number of groups have shown that HPV E6 and E7 can be targeted by RNAi, resulting in slowed growth (Yoshinouchi et al., 2003), apoptosis (Jiang and Milner, 2002; Butz et al., 2003) or senescence (Hall and Alexander, 2003) of the target cell. We hypothesized that the return of p53 after treatment with siRNA against E6 and E7 would yield cells more sensitive to chemotherapeutic drugs currently used to treat cervical cancer.

In this study, we tested the ability of siRNA to target the E6 and E7 oncoproteins simultaneously, to observe changes in cisplatin sensitivity resulting from oncogene silencing. Our results provide strong evidence that loss of E6 and E7 results in a 4-fold increase in sensitivity to cisplatin and suggest that the use of RNA interference in combination with cisplatin may be a useful adjuvant to current treatment options.

Materials and Methods

Cells, Antibodies, and Drugs

CaSki, SiHa, HeLa, C-33 A, and 293T cell lines were obtained from the American Type Culture Collection (Manassas, VA). HeLa 16E6E7 cells have previously been described in Horner et al. (2004). Cells were maintained in Dulbecco's modified Eagle's medium, (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin sulfate, and 0.29 mg/ml L-glutamine (Invitrogen). p53, tubulin, and actin monoclonal antibodies were purchased from Sigma (Sydney, Australia). HPV 16E7, HPV 18E7, and p21 monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cisplatin was purchased from Sigma (Sydney, Australia) and dissolved in dH_2O to 5 mM.

siRNA and siRNA Transfection

siRNAs were synthesized as duplexes by Proligo (Lismore, Australia). The sequences of the siRNAs are shown in Table 1.

On the day before transfection, cells were plated out at a density of 150,000 cells/well in a six-well plate. siRNA was diluted to concentrations of 1 to 40 nM in dH_2O to a final volume of 5 μl, to which 80 μl of Opti-MEM I reduced-serum media (Invitrogen) was added. Oligofectamine reagent (Invitrogen) (2 μl) was added to 13 μl of Opti-MEM I, and the mixture was incubated at room temperature for 5 to 10 min. The Oligofectamine/Opti-MEM I solution was added to the siRNA solution for a final volume of 100 μl. Transfection complexes were added to wells containing 400 μl of Opti-MEM I and incubated for 4 h at 37°C. After incubation, transfection complexes were removed and replaced with complete DMEM.

Infection of Cells with RNAi Lentivirus

The production of third-generation, replication-defective RNAi lentiviruses was performed as described in Rubinson et al. (2003). In brief, we cotransfected 6.6 μg of transferring plasmid DNA (pLL3.7 or pLL3.7 with insert) and 3.3 μg of each packaging plasmid (pVSVG, pRSV-REV, and pRRE) in 133 μl of 1.25M $CaCl_2$, 0.5 ml H_2O, and 0.66 ml of 2× HEPES-buffered saline in a T75 flask. Four hours later, the medium was removed, and the cells were washed with warm PBS. Complete DMEM (7 ml) was added to the cells, and the cell culture supernatant was harvested 36 to 48 h later by centrifugation at 850*g* for 7 min at 4°C, followed by filtration of the supernatant through a 0.45-μm filter. Titers were determined by infecting 293T cells with serial dilutions of concentrated lentivirus. We determined green fluorescent protein expression of infected cells by flow cytometry 48 h after infection; for a typical preparation, the titer was approximately 4 to 10×10^8 infectious units per ml. Cells (25-cm^2 flask) were infected with 10 infectious units per cell in 1 ml of complete DMEM/polybrene (8 μg/ml) for 60 min at 37°C before the addition of 4 ml of complete DMEM/polybrene. Incubation was continued for 24 h before cells were washed three times in complete DMEM.

FACS Analysis and Cell Sorting

Cells transfected with fluorescent-tagged siRNA were trypsinized, collected in a 15-ml tube, and washed three times with PBS before being resuspended in a final volume of 500 μl of PBS. Cells were sorted using a MoFlo FACS. Sorted cells were collected in a 15-ml tube containing 10 to 12 ml of complete DMEM (10% fetal calf serum, 100 units/ml penicillin G, 100 μg/ml streptomycin sulfate, and 0.29 mg/ml L-glutamine) and pelleted by centrifugation at 1500*g* for 10 min. Cells were then resuspended in tissue culture media and counted using a hemocytometer before seeding into a flask or tissue culture plate.

Western Blot Analysis

Cell extracts were prepared in radioimmunoprecipitation assay buffer (150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, and 50 mM Tris, pH 8.0), with the addition of 2 mM phenylmethylsulfonyl fluoride and 1 μl/ml protease inhibitory cocktail (Sigma). Lysis buffer was freshly prepared and added to trans-

TABLE 1
siRNA sequences

siRNAs	Sequences
Si16E6-Seq 10	
Sense	5′-GCAACAGUUACUGCGACGUUU-3′
Antisense	3′-UUCGUUGUCAAUGACGCUGCA-5′
Si16E6-Seq 37	
Sense	5′ CACGUAGAGAAACCCAGCUUU-3′
Antisense	3′-UUGUGCAUCUCUUUGGGUCGA-5′
SiScrambled	
Sense	5′-GACCUGUUAAUGACGGCACUU-3′
Antisense	3′-UUCUGGACAAUUACUGCCGUG-5′
SiGFP	
Sense	5′-GCACGACUUCUUCAAGUCCUU-3′
Antisense	3′-UUCGUGCUGAAGAAGUUCAGG-5′
HPV18 E6	
shRNA Target (nt 128–145)	5′-AGGTATTTGAATTTGCAT-3′

fected cells in wells of six-well plates (100 μl/well) on ice, which were then incubated for 10 min. Protein concentrations were determined by protein assay kit (Bio-Rad, Hercules, CA). Cell extracts were boiled for 10 min in loading buffer before being separated on 8 to 15% SDS-PAGE gels. Separated proteins were transferred to polyvinylidene difluoride membranes at 100 V for 1 h before membrane blocking in 5% skim milk powder in PBS with 0.1% Tween 20. Primary and secondary antibodies were diluted 1/1000 in PBS with 0.1% Tween 20 and incubated for 60 min at room temperature (or 2 days at 4°C for HPV16E7) with three washes between each step. Protein bands were visualized by enhanced chemiluminescence.

Cell Viability Assays

MTT Assay. After transfection of cells, 250 μl of 2 μg/ml MTT reagent and 750 μl of 1× PBS was overlaid onto cells that were then incubated for 3 h at 37°C. Dimethyl sulfoxide (750 μl) was added to each well and incubated for 10 min before the supernatant was transferred to a 96-well plates and absorbance measured at 595 nm.

CellTiter-Blue Assay. Cell viability was measured according to the manufacturer's instructions (Promega, Madison, WI). Fluorescence was read using a Prism 7700 sequence detector (Applied Biosystems, Foster City, CA).

Senescence-Associated β-Galactosidase Assay. The senescence-associated β-galactosidase assay was performed as described by Dimri et al. (1995). Cells were seeded into wells containing coverslips before transfection with siRNA. Cells were fixed using 3% paraformaldehyde/PBS for 5 min at room temperature, then washed three times with PBS. Coverslips were placed cell-side down onto one drop of 5-bromo-4-chloro-3-indolyl-β-D-galactosidase solution on Parafilm in a humidified chamber and incubated at 37°C overnight (or until blue precipitate was visible inside cells). Coverslips were washed three times in PBS followed by a rinse in dH_2O, dried, and mounted on slides.

Results

To undertake our study, we first designed and tested siRNAs directed against the HPV 16 E6/E7 mRNA. The E6 and E7 genes can be expressed as a bicistronic message, producing both full-length E6 and E7, or as a poly-cistronic message, producing a truncated E6 (E6*) and full-length E7 (Fig. 1A) (Smotkin et al., 1989; Doorbar et al., 1990; Sherman and Alloul, 1992). Both types of transcripts express the E7 message with equal efficiency (Stacey et al., 1995). It has been demonstrated that the polycistronic message expressing E6* and E7 is the predominant transcript found in both cervical tumors and tumor-derived cell lines (Taniguchi and Yasumoto, 1990). We therefore selected siRNA sequences that were not within the portion of E6 that is alternately spliced so as to target both full-length E6, E6*, and E7 mRNA expressed from both transcripts (Fig. 1B).

To screen potential candidates, E6 siRNAs were transfected into the HPV16-positive cervical cancer cell line CaSki. Because E6 is expressed in CaSki cells at a level too low to be detected by Western blot with available commercial antibodies, E6 siRNAs were screened based on their ability to reduce E7 protein levels, given that E6 and E7 are expressed on the same mRNA. Two E6 siRNAs (sequences 10 and 37) were found to reduce E7 protein levels in a dose-dependent manner, with IC_{50} values of 6.1 and 4.9 nM, respectively (Fig. 2A). At 40 nM, E7 protein levels were reduced by 81% (seq 10) and 79% (seq 37), whereas a scrambled siRNA sequence did not affect E7 levels at all. To measure the duration of the siRNA effect, E7 protein levels were measured by Western blot at 24, 48, and 72 h after a single 40 nM transfection of siRNA. The greatest silencing effect was observed at 24 h, and this was sustained through 48 h (Fig. 2B). However, silencing was much reduced by 72 h. We concluded that our siRNAs were equally potent and were able to efficiently knock down E7 expression.

Interaction between E6 and p53 results in the specific degradation of p53 via the ubiquitin-proteasome pathway; therefore, any loss in E6 will result in an accumulation of p53 protein. At 24 h after a single treatment with either of our 16E6 siRNAs, we observed a concentration-dependent increase in p53 protein levels by Western blot, whereas a scrambled control siRNA had only a small effect (Fig. 3A). This increase was accompanied by an increase in the cyclin-dependent kinase inhibitor, $p21^{CIP1/WAF1}$, a transcription target and downstream effector protein of p53 (Fig. 3B). These results suggest that the level of E6 protein is being reduced by the siRNA treatment and that p53 not only accumulates but also is functionally active.

It is not clear from previous studies what the effect of reducing E6 or E7 by siRNA would be, because results have been inconsistent, with either reduced growth, apoptosis, or senescence observed (Jiang and Milner, 2002; Butz et al., 2003; Hall and Alexander, 2003; Yoshinouchi et al., 2003). We therefore performed a number of assays to identify which

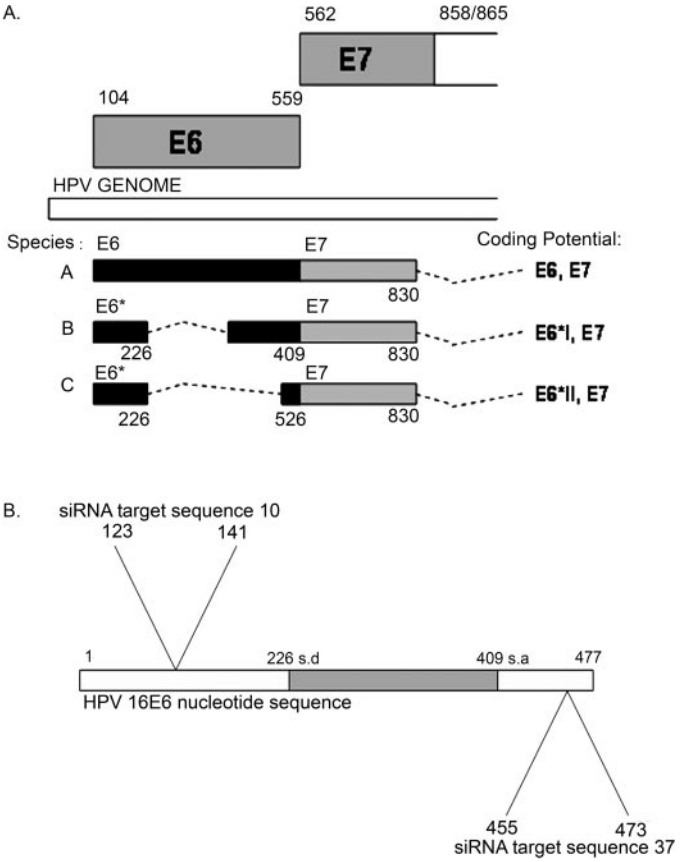

Fig. 1. Targeting of E6 and E7 using siRNA. A, diagrammatic representation of the various spliced mRNAs that encode E6 and E7. Coding regions are indicated by filled boxes and introns by dotted lines. Transcript (mRNA A), the only message from which full-length E6 is expressed, is present at very low levels (Smotkin et al., 1989). The predominant transcript expressed in cervical tumors and cell lines (mRNA B) expresses truncated E6* and E7. B, HPV 16 E6 siRNA targets for seq 10 (nt 123–141) and seq 37 (nt 455–473). Note the spliced region in E6 (Transcript B) is marked as a gray-filled box (nt 226–409).

of these phenotypes were present after treatment with our siRNAs. CaSki or HPV negative C-33 A cervical cancer cells were transfected with 40 nM fluorescently tagged 16E6 siRNA. After transfection, cells were sorted using a MoFlo FACS into siRNA-positive and -negative populations. Cellular viability was measured at day 5 after transfection by MTT and CellTiter-Blue assays. We observed a significant growth reduction in CaSki cells, but not in C-33 A cells, after treatment with 16E6 siRNA, demonstrating that E6 siRNA was exerting a specific effect on the HPV16-positive CaSki cells (Fig. 4A).

We hypothesized that this occurred via the induction of senescence based on the outcome of previous studies in which E6 and E7 were repressed concurrently (Hall and Alexander, 2003; Horner et al., 2004; Psyrri et al., 2004). To investigate whether senescence was induced, we assayed for the presence of senescence-associated β-galactosidase activity, a well characterized marker of senescent cells (Dimri et al., 1995). Staining was performed 7 days after transfection, and numerous strongly stained cells were observed (Fig. 4C). Counting of these cells in triplicate experiments indicated a 5-fold increase in senescent cells compared with cells treated with a scrambled siRNA (Fig. 4B), but not all cells were stained.

To determine whether apoptosis was occurring concurrently with the development of senescence in these cells, a Western blot for the cleaved 85-kDa form of poly(ADP-ribose) polymerase (PARP) was performed at both 24 and 48 h, the times at which silencing of E7 was most efficient. Although exposure to UV-B induced the cleavage of PARP, no cleavage was observed in any siRNA-treated cells, indicating that senescence was occurring in the absence of apoptosis (Fig. 4D).

At present, treatment of advanced cervical cancer involves the use of cisplatin in combination with radiotherapy (Loizzi et al., 2003). To examine the hypothesis that treatment of HPV-positive cancer cells with siRNA would enhance the effect of cisplatin, we transfected CaSki cells with 40 nM E6 siRNA or scrambled control siRNA. The following day, transfected cells were treated with various doses of cisplatin and incubated for 24 h. After this time, the drug was removed and cells were maintained in complete medium for 48 h before a cellular viability assay with CellTiter-Blue was performed. Our results demonstrated a 37% decrease in the IC_{50} of cisplatin, from 3.6 to 2.3 μM (data not shown). This result was not statistically significant, and we speculated that this was because the siRNA treatment was beginning to lose efficacy after 24 h and a transfection efficiency of only 80%. Therefore, we next tested lentivirus (LV)-based delivery of a DNA plasmid that expressed a short hairpin RNA (shRNA) molecule against the HPV18 E6 gene (LV 18E6–1) in HeLa cells. In this system, shRNA is driven from the U6 promoter using a vector and packaging system described previously (Rubinson et al., 2003). Using this strategy, our transfection rate was now 100% (compared with 80% previously), and RNA interference was constant. We observed that E7 levels were reduced by more than 90% using this treatment, whereas control lentivirus has no effect (Fig. 5A). Using this system in HeLa cells results in the same biological effects we observed using siRNA in CaSki cells (W. Gu, L. Putral, K. Hengst, K. Minto, N. Saunders, G. Leggatt, N. McMillan, submitted for publication). HeLa cells were infected with 20

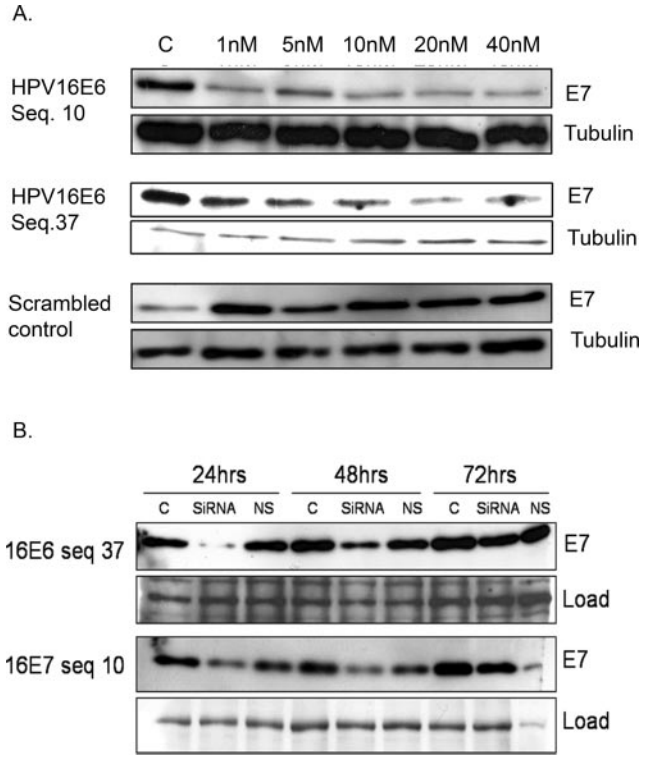

Fig. 2. siRNAs against E6 reduce E7 protein levels in cervical cancer cells. A, CaSki cells were transfected with 1 to 40 nM concentrations of each E6 siRNA (seq 10 and 37) or a scrambled control siRNA, and cell extracts were collected after 24 h. E7 and tubulin protein levels were determined by Western blot. B, determination of the duration of the siRNA effect for E6 seq 10 and 37 siRNA. Cells were transfected with 40 nM concentrations of each E6 siRNA. After 24, 48, or 72 h, cell extracts were collected, and E7 levels were determined by Western blot. Loading controls are β-tubulin.

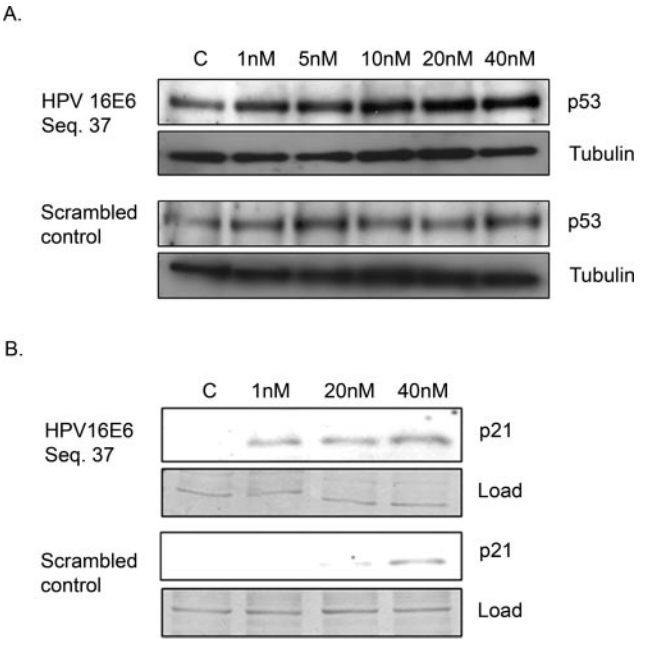

Fig. 3. Treatment with E6 siRNA induces p53 and p21. A, CaSki cells were treated with 1 to 40 nM seq 37 E6 siRNA or a scrambled control siRNA. p53 protein levels were determined by Western blot after 24 h. Loading control is β-tubulin. B, CaSki cells were treated with 1 to 40 nM seq 37 E6 siRNA or a scrambled control siRNA, and p21 protein levels were determined by Western blot after 24 h. Loading control is Coomassie blue stains of the transferred blot.

infectious units of lentivirus per cell for 24 h before being treated with cisplatin for a further 24 h. After treatment, cisplatin was removed and cells were maintained in complete medium for a further 24 h before cell viability was assayed using CellTiter-Blue. Comparisons between treatments were analyzed statistically using an F test. We observed that the IC_{50} for cisplatin in HeLa cells was 9.4 μM but was decreased almost 4-fold to 2.4 μM in cells treated with the shRNA against E6 (Fig. 5B). We also observed a significant reduction in the viability of cells treated with empty vector, pLL (IC_{50}, 6.8 μM) compared with HeLa cells. This suggests that the act of infecting cells with virus induces cellular stress, rendering them more sensitive to cisplatin. However, statistical analysis showed the difference between pLL lentivirus and lenti-

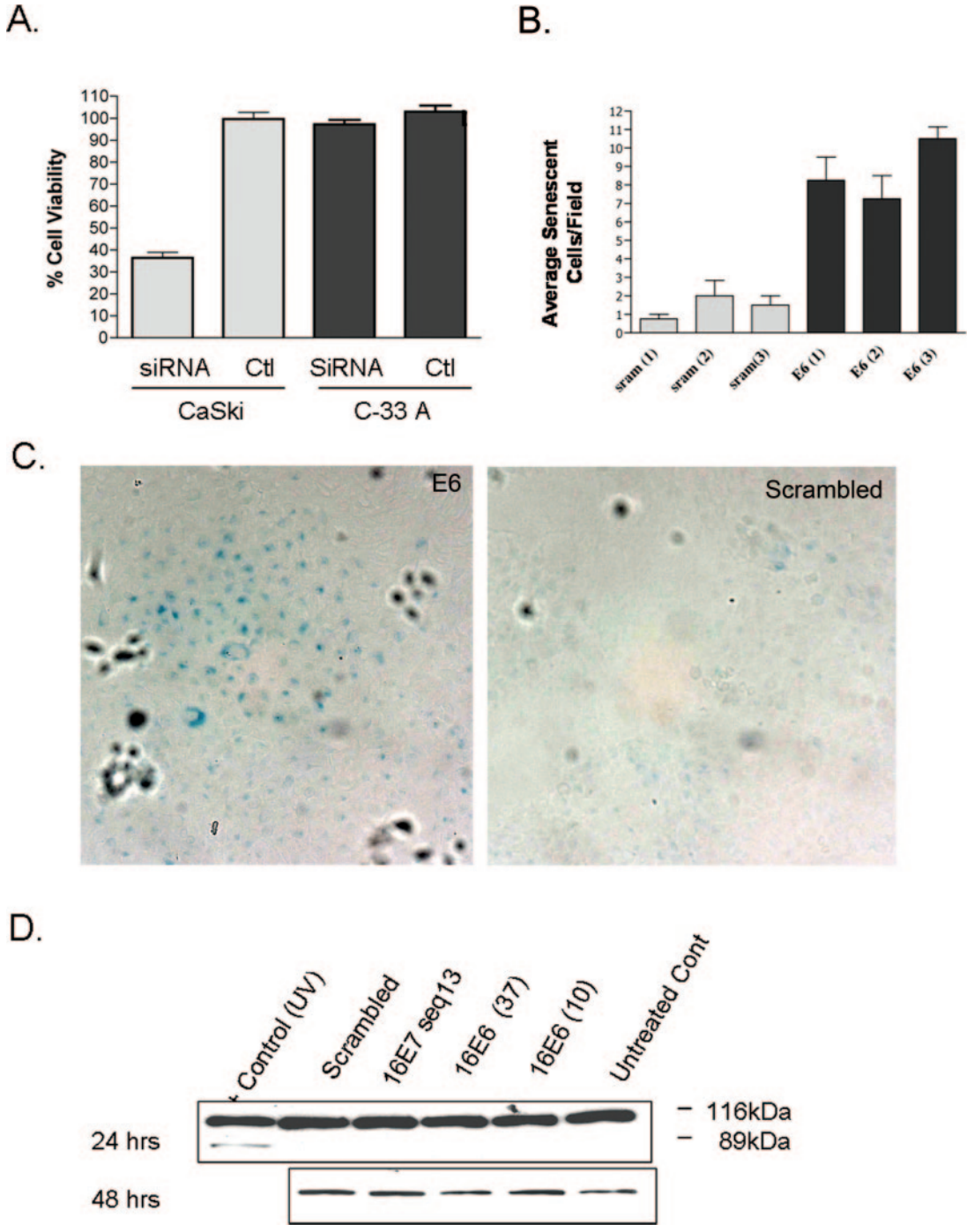

Fig. 4. Treatment with E6 siRNA results in reduced growth and induction of senescence. A, CaSki or C-33 A cells were transfected with fluorescently tagged E6 siRNA (seq 10) before being sorted by FACS into transfected (5-carboxyfluorescein–positive) and nontransfected (5-carboxyfluorescein–negative) populations. Five days after treatment, cellular viability was measured. B, effect of E6 siRNA on senescence-associated β-galactosidase activity. CaSki cells were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactosidase at pH 6.0, 7 days after a single transfection with 16E6 or scrambled siRNA. Blue cells were counted from five fields for three separate experiments. C, bright-field photograph of cells assayed for senescence-associated β-galactosidase activity (100× magnification). Senescent cells appear blue. D, treatment with E6 siRNAs does not cause apoptosis. CaSki cells were treated with 40 nM E6 siRNA or a scrambled control siRNA. PARP cleavage was detected by Western blot using cell extracts collected at 24 and 48 h after transfection. Cells were exposed to UV light as a positive control.

virus 18E6–1 treatment to be highly significant ($P = <0.0001$), demonstrating the specificity of the interference. There was no effect on the mock-infected cells (HeLa control), which use polybrene to aid viral infection (IC_{50}, 9.8 μM).

To further demonstrate the potency of our effect, we measured the response of cells treated with 5 μM cisplatin to increasing doses of our lentivirus. The low-cisplatin dose was chosen to allow for the observation of any shRNA effects. As expected, the cisplatin-only treatment gave a 25% reduction in cell viability, and we observed that there was indeed a dose-dependent response; the highest dose (30 infectious units) resulted in the greatest reduction in cellular viability (Fig. 5C). We have found that at a dose of 20 infectious units, four copies of the shRNA-expressing plasmid are present in each cell (W. Gu, L. Putral, K. Hengst, K. Minto, N. Saunders, G. Leggatt, N. McMillan, submitted for publication). One concern is the issue of specificity, and we addressed this by transfecting the cervical cancer cell lines C-33 A (HPV-negative) and SiHa (HPV16-positive) as well as a HeLa cell line expressing the HPV16 E6 and E7 genes (HeLa 16E6E7 cells) with 10 infectious units of the 18E6–1 lentivirus, and measuring the effect of a single 10 μM dose of cisplatin on cell viability (Fig. 5D). We choose the 10 μM dose because this had previously been shown to give a reduction of approximately 50% in cell viability in HeLa cells and would allow us to observe any further reductions caused by shRNA expression. As expected, the normal HeLa cells were sensitive to cisplatin, with a reduction in cellular viability to 49% observed (untreated = 100%) and cotreatment with cisplatin and 18E6–1 together, resulting in a further decrease in cell viability to 19%. As expected, SiHa and C-33 A cells had different sensitivities to cisplatin-only treatment, with C-33 A being slightly more sensitive than HeLa cells and SiHa observed to be more resistant. It is noteworthy that when C-33 A and SiHa cells were cotreated with cisplatin and 18E6–1, we observed no change in cell viability over the cisplatin-only treatment. HeLa 16 E6E7 cells were used to confirm that the increased cisplatin sensitivity observed in HeLa cells after cotreatment with E6 shRNA occurred as a result of the suppression of endogenous HPV18E6 and E7 and was not caused by nonspecific effects of shRNA. We predicted that the increase in cisplatin sensitivity seen in

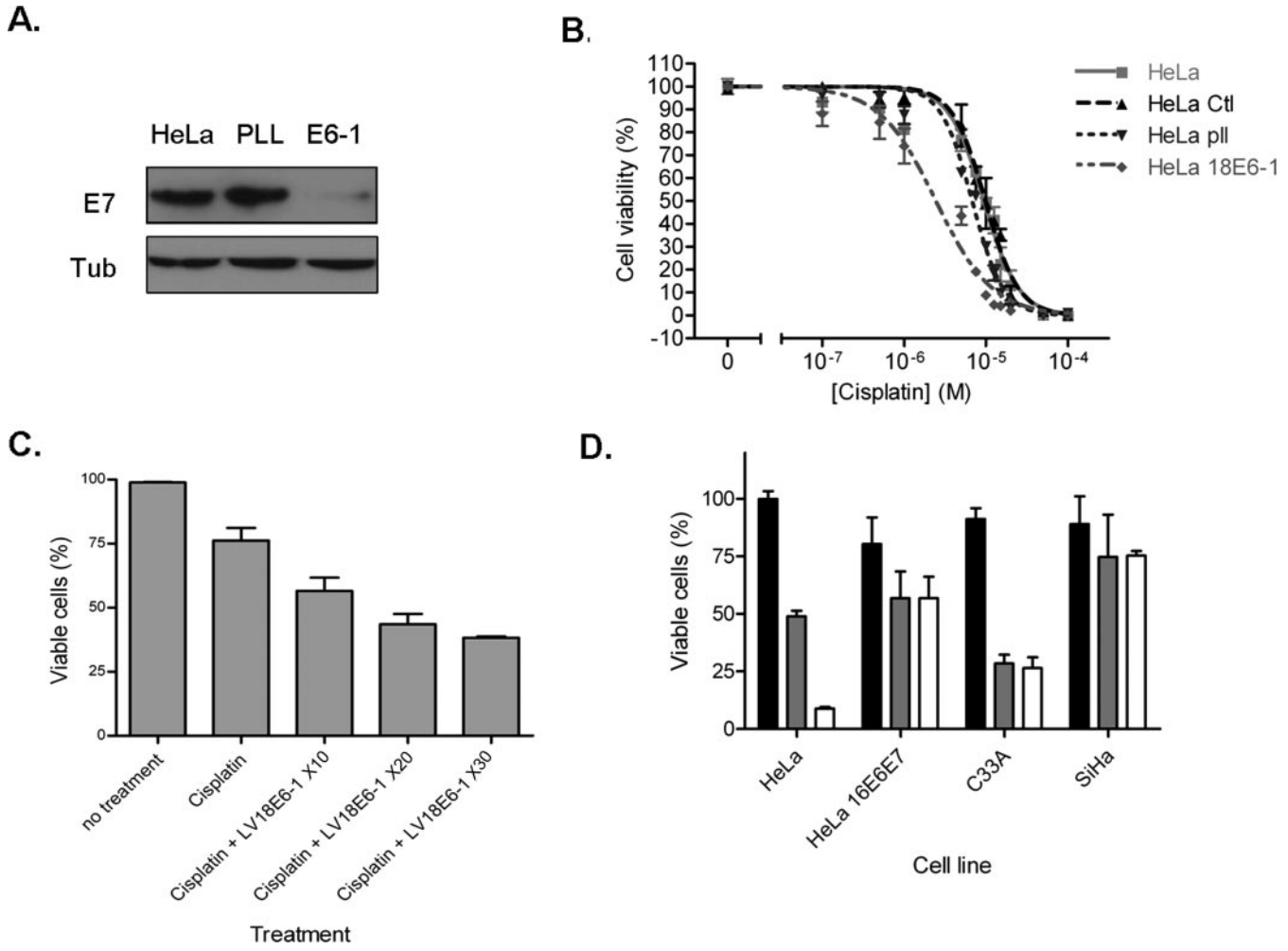

Fig. 5. Treatment of HeLa cells with shRNA against E6 induces sensitivity to cisplatin. A, HeLa cells were untreated or infected with 10 infectious units of control lentivirus (PLL) or LV-18E6–1 for 1 week before E7 protein levels were determined. B, HeLa cells were infected with lentiviruses at 20 infectious units for 24 h before being treated with various concentrations of cisplatin. Cisplatin was removed after 24 h of exposure, and cells were incubated for a further 24 h before cell viability was measured using the CellTiter-Blue assay. C, cells were infected with various multiplicities of infection of LV 18E6–1 before cisplatin treatment (5 μM) as described for A. D, cells were infected with 10 infectious units of LV 18E6–1 for 24 h before treatment with 10 μM cisplatin. Cell viability was measured 24 h later and normalized to untreated cells. Black bars, no cisplatin; gray bars, treated with 10 μM cisplatin; white bars, treated with cisplatin and LV18E6–1.

HeLa cells when cotreated with shRNA would be eliminated when HeLa 16E6E7 cells were used because of the presence of the type 16 E6 and E7 oncogenes. We observed that in the presence of 16E6 and E7, there was no significant difference in cisplatin sensitivity between those cells that received LV 18E6–1 and those that did not. Together, these data suggest that the shRNA against HPV18 E6 is highly specific in that it was only able to affect the HPV18-positive HeLa cells and that the effect was not a result of a nonspecific response to viral infection or shRNA.

Finally, to investigate the reason for this increased sensitivity of the combined shRNA and cisplatin treatment, we investigated the levels of p53. The p53 protein is known to be the target of E6, and its presence has been shown to correlate with sensitivity to cisplatin in a recent study using the National Cancer Institute set of 60 cancer cell lines (Vekris et al., 2004). We therefore performed a p53 Western blot to measure protein levels. Treatment with cisplatin or LV 18E6–1 shRNA alone resulted in a modest increase in p53 protein levels; however, upon cotreatment with LV 18E6–1 and cisplatin, p53 levels were markedly increased (Fig. 6). We also measured E7 protein levels and observed that cotreatment resulted in greater E7 silencing than was seen after treatment with cisplatin or shRNA alone, demonstrating a synergistic effect of shRNA and cisplatin (data not shown).

Discussion

RNAi is a promising new tool to suppress gene expression, and it is several orders of magnitude more efficient than ribozymes and antisense RNA. Tumor cells are typically resistant to growth suppressive signals and apoptosis, and in HPV-induced cancers, the viral oncogenes E6 and E7 provide this suppression. Therefore, a rational approach is to reinstate these pathways via RNAi, and a number of groups have recently shown that suppression of E6 and/or E7 results in the activation of p53 and retinoblastoma tumor suppressor protein pathways in cervical cancer cells (Jiang and Milner, 2002; Butz et al., 2003; Hall and Alexander, 2003; Yoshinouchi et al., 2003).

Growth suppression in CaSki cells was observed 5 days after treatment, and senescence was readily found at 7 days. These results suggest that despite the fact that our siRNAs had almost completely lost the ability to silence E7 at 72 h after transfection, even the temporary loss of E6 and E7 sets in motion the senescence pathway. These findings would be consistent with the situation in normal epithelial cells, which undergo irreversible senescence and differentiation as part of their normal life cycle. However, each system will differ depending on the siRNA dose given and the genetic background of the cells. For example, CaSki cells have 600 integrated copies of the HPV16 genome, whereas SiHa cells have only two.

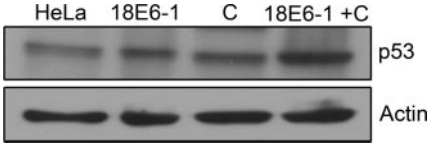

Fig. 6. Cotreatment with shRNA LV18E6–1 and cisplatin enhances p53 levels. Western blot analysis of p53 protein levels in HeLa cells after infection with 20 infectious units of LV 18E6–1 lentivirus and treatment with 5 mM cisplatin (C) respectively.

The question we wished to address is how RNAi against HPV E6 and E7 will work in combination with the current forms of treatment for cervical cancer. Current treatments for advanced cancers involve both radio- and chemotherapy (Loizzi et al., 2003). Chemotherapy typically uses cisplatin, and response rates are around 50%. Those with persistent, bulky, or recurrent squamous cell carcinoma of the cervix have a poor survival rate of 20% at 12 months. Problems associated with cisplatin therapy include dose-limiting side effects, the most important of which is neurotoxicity, resulting in peripheral neuropathy, tinnitus, and high-frequency hearing loss (Decatris et al., 2004). Development of resistance is also a problem (Siddik, 2002). Despite these issues, cisplatin currently remains the most effective therapy for the treatment of cervical cancer. Our data suggest a combination therapy using RNA interference may be beneficial in reducing the required dose of cisplatin for the same effect, thus reducing the extensive problems associated with toxicity. This strategy may also reduce the occurrence of drug resistance. The use of cisplatin in combination with siRNA is probably a more promising strategy than the use of siRNA alone, in that results of previous studies in which the E6 and E7 genes of HPV-positive cell lines have been silenced are variable; some report apoptosis but others report senescence. Based on our findings, siRNA and cisplatin is the combination most likely to result in the preferred outcome (i.e., apoptotic death of cancerous cells in particular).

Our data show that the loss of expression of E6 and E7 via RNAi seems to reactivate the dormant p53 tumor suppressor pathway and results in senescence of cervical cancer cells. Furthermore, our data show a clear, specific, and significant decrease in the IC_{50} for cisplatin when used in combination with RNAi. This represents an important first step in designing a rational, molecular cancer therapy using RNAi. Although others have shown that RNAi against various genes enhances cisplatin's effect, the major issue with many of these genes is that they are present in, and essential for, normal cell function and therefore not viable to be used therapeutically. For example, RNAi against EGF receptor has been shown to increase sensitivity of non–small-cell lung carcinoma cells to cisplatin treatment (Zhang et al., 2004), as has the targeting of Akt in uterine cancer cells (Gagnon et al., 2004) and bcl-2 in melanoma cells (Wacheck et al., 2003).

We hypothesized that cervical cancer cells might be rendered more sensitive to cisplatin as a result of the return of dormant p53 tumor suppressor pathways. In support of this, we observed a marked increase in the levels of p53 after cotreatment with E6 siRNA and cisplatin. Previous studies have produced largely conflicting results regarding the effect of p53 status upon cisplatin sensitivity (for review, see Weller, 1998; Brown and Wouters, 1999; Pirollo et al., 2000). It has been proposed that the presence of p53 may in fact decrease chemosensitivity because of interference with drug action. For example, activation of p53 may result in increased DNA repair, transcription of antiapoptotic genes, and cell cycle arrest, all of which may potentially decrease sensitivity to chemotherapeutic agents. In a study using the human ovarian cancer cell line A2780, the loss of functional p53 via the introduction of E6 resulted in increased cisplatin cytotoxicity with concurrent loss of G_1/S checkpoint control and

decreased repair of cisplatin-DNA adducts (Pestell et al., 2000). However, numerous studies have found that the presence of p53 correlates positively with cisplatin sensitivity (Makris et al., 1995; Mathieu et al., 1995; Cote et al., 1997; Kanamori et al., 1998; Vekris et al., 2004). In a panel of 60 National Cancer Institute cell lines, the presence of p53 showed a positive correlation with sensitivity to cisplatin (Vekris et al., 2004). It is likely that conflicting results regarding p53 and cisplatin sensitivity are cell type-dependent and based on cellular characteristics, including genomic instability. Indeed, it has previously been suggested that cisplatin cytotoxicity may be determined by the delicate balance between proteins that repair DNA adducts and those that interfere with DNA repair and initiate apoptosis (Gonzalez et al., 2001). This hypothesis supports the contrasting findings of previous studies involving the role of p53 on cisplatin sensitivity, because of the differing nature of the response to genotoxic stress in different cell lines. Our work demonstrates that the return of p53 may contribute to the chemosensitivity of cervical cancer cells but will require further study. An advantage of our experimental design over some previous studies examining the effect of p53 on cisplatin sensitivity is that it was performed in cervical cancer cell lines rather than cells in which E6, p53, or dominant negative p53 are expressed ectopically, which may not reflect the inherent regulation of p53 in cancer cells. It should be noted that while the manuscript of this article was under review, Koivusalo et al. (2005) described increased resistance of HeLa cells to cisplatin after treatment with siRNA, a finding that is in direct contrast to our results (Koivusalo et al., 2005). This discrepancy may result from the fact that in our study, shRNAs driven from a U6 promoter were used, providing a constant, high level of gene suppression, as opposed to siRNAs, which have a half-life of only a few days. We suggest that perhaps variation in p53 activation is involved in the "decision" of a cell to initiate apoptosis or, instead, cell cycle arrest and repair of DNA adducts.

The data presented here represent an important step in designing a rational, molecular cancer therapy using RNAi. First, we identify a single siRNA that targets both the major oncogenes of human papillomavirus. Second, our results show that the re-establishment of dormant tumor suppressor pathways via RNAi enhances the antitumor activity of cisplatin. Our data suggest that a combination therapy involving cisplatin and siRNA could be advantageous in promoting better prognostic outcomes for patients and reducing the severe, toxic side effects associated with cisplatin therapy. The challenge now is to turn these findings into meaningful clinical applications.

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