Silencing of HPV16 E6 and E7 oncogenic activities by small interference RNA induces autophagy and apoptosis in human cervical cancer cells.

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Abstract

Cervical cancer is the second most common form of death by cancer in women worldwide and has special attention for the development of new treatment strategies. Human Papilloma Virus (HPV) persistent infection is the main etiological agent of this neoplasia, and the main cellular transformation mechanism is by disruption of p53 and pRb function by interaction with HPV *E6* and *E7* oncoproteins. This generates alterations in cellular differentiation and cellular death inhibition. Thus, HPV E6 and E7 oncogenes represent suitable targets for the development of gene therapy strategies against cervical cancer. An attractive technology platform is developing for post-transcriptional selective silencing of gene expression, using small interference RNA (siRNA). Therefore, in the present study, we used SiHa cells (HPV16+) transiently transfected with specific siRNA expression plasmids for HPV16 *E6* and *E7* oncogenes. In this model we detected repression of *E6* and *E7* oncogene and oncoprotein expression, an increase in p53 and hypophosphorylated pRb isoform protein expression, and autophagy and apoptosis morphology features. These findings suggest that selective silencing of HPV16 *E6* and *E7* oncogenes by siRNAs, has significant biological effects on the survival of human cancer cells and is a potential gene therapy strategy against cervical cancer.

Introduction

Clinical, epidemiological and molecular data associate the high-risk HPV persistent infection with cervical cancer development and show that it is the etiologic agent for this neoplasia. Furthermore, HPV16 and HPV18 have been related with pre-cancerous lesions and ano-genital carcinoma development.[1,2] A relevant event in HPV-associated carcinogenesis is a global disruption of differential cellular gene expression by HPV E6 and E7 oncoproteins, and molecular analysis has revealed cellular protein targets for these oncoproteins.[1] *E6* oncoprotein induces the rapid degradation of p53 protein by the proteasome pathway. The p53 protein integrates responses to genotoxic stress and DNA damage with cell cycle control, autophagy and apoptosis. Therefore, the loss of p53 leads to increased genetic instability.[3,4] *E7* oncoprotein binds to hypophosphorylated pRb isoform, causing release of E2F transcription factors, which in turn stimulates expression of multiple genes involved in cell cycle progression.[5] In addition, during the cervical cancer process occurs the induction of TGF-beta1 gene expression by HPV16 *E6* and *E7* oncoproteins, which is a cytokine that regulate the anti-tumor immune response.[6] Thus, E6 and E7 together exert their effects on perturbation of immune response against cancer, on cell cycle control, on disruption of gene expression; and in combination they efficiently immortalize and transform the human keratinocytes to promote the carcinogenesis process. Due to these properties of HPV *E6* and *E7* oncogenes, they have relevant attention because allow the understanding of the molecular mechanism of human viral oncogenesis and are considered suitable targets for gene therapy strategy for cervical cancer treatment.

There have been significant efforts in the design of novel drugs and gene therapy approaches against cervical cancer.[7,8] However, specific clinical treatment protocols are still not available. Several studies have analyzed the HPV *E6* and *E7* mRNA expression inhibition using ribozymes and antisense oligonucleotides as treatment strategy.[9-11] Nevertheless, these approaches have low efficiency, short time of stability and high cost for design and administration. Alternatively, an effective strategy that has been used to knock-down gene expression in a sequence specific way and that represses in considerable magnitude the viral oncogenes at post-transcriptional level, is by the RNA interference (RNAi).[12,13] The RNAi mechanism is a natural process by which gene expression in eukaryote cells is silenced by microRNAs to cleave target mRNA.[14] Currently, we know that the biogenesis process of microRNAs begins in the cellular nuclei, when the genes that encode the microRNAs are transcribed as primary microRNAs (pri-microRNAs). Pri-microRNAs are processed by a microprocessor which contains a RNase type III endonuclease called Drosha to generate the precursor microRNAs (pre-microRNAs). Pre-microRNAs are then exported to the cytoplasm by an anti-port transport called Exportin-5 and are then processed by another RNase type III endonuclease known as Dicer to generate microRNAs. MicroRNAs that are 21-25 nucleotides of length are processed to produce mature microRNAs

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which are associated with RNA-induced silencing complex (RISC), in order to recognize homologous mRNA sequences. The microRNAs mediate their effects at mRNA level by inhibiting translation or through cleavage of target mRNA by nucleotide complementarity between the microRNAs and the target mRNA. Perfect complementarity induces cleavage of mRNA, whereas several mismatches lead to translation arrest.[15-23] Thus, RNAi mechanism is a natural process of sequence specific post-transcriptional gene silencing in eukaryotic cells.

Biological effects of microRNAs may be mimetic, using siRNAs (small interference RNAs) which can be generated by cloning in molecular vectors.[24] In such vectors, oligonucleotide homologous sequences to the target gene of interest are expressed from RNA Pol-III promoters, resulting in biologically active siRNAs. In this system, different studies provide convincing evidences that a DNA insert can be transcribed like siRNAs of 21 nucleotides with identical characteristics to bioactive microRNAs, and can potently mediate specific gene exogenous silencing in mammalian cells during viral infections.[24] Thus, in the present study SiHa cells, which are human cervical cancer cells transformed with HPV16, were used like a cervical cancer model to investigate if siRNAs-mediated gene silencing specific to HPV16 *E6* and *E7* oncogenes expressed in DNA plasmids, can be used to silence the *E6* and *E7* oncogenes. In addition, we analyzed if these siRNAs could alter the expression of p53 and pRb target cellular proteins, and if siRNAs had biological effects on tumor cells. For this goal, we generated siRNAs expression plasmids for HPV16 *E6* and *E7* oncogenes, which have nucleotide complementarity in different regions of mRNA for each one of viral oncogenes. We found that HPV16 *E6* and *E7* oncogenes had a differential silencing by siRNAs. Furthermore, we identified that siRNAs were able to induce the reestablishment of p53 and pRb expression, as well as its biological effects, which depend of these tumor suppressor genes. Cellular death in SiHa cells was characterized using qualitative and quantitative assays, including proliferation assay, electron microscopy and cell cycle by flow cytometry. Our data indicate that SiHa cell death occurred by autophagy and apoptosis, by effect of silencing HPV16 *E6* and *E7* oncogene expression with siRNAs specific sequences. Taken together, these results demonstrate that siRNAs directed against HPV16 *E6* and *E7* oncogenes are excellent molecular tools to inhibit *E6* and *E7* oncogene expression and its activities in a specific manner, which has relevant biological effects on tumor cell progression.

Materials and Methods

Cell lines and culture conditions

Human cervical cancer cells transformed with HPV16 (SiHa cells) were obtained from the American Type Culture Collection (ATCC). The cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin (50 ug/mL), 2 mM L-glutamine, 250 ng/mL fungizone, and maintained at 37°C in 5% $C0_2$. The total RNA isolation was carried out with Trizol reagent (Invitrogen) for the RT-PCR assays. The cellular protein isolation was performed and protein concentration was determined by the BCA protein kit (Pierce, Rockford, IL, USA) for the Western Blot assays. In addition, the cells which were used in tranfection assays were fixed in ethanol for the flow cytometry assays and in paraformaldehyde for the electron microscopy assays.

siRNA expression plasmids for HPV16 E6 and E7 oncogenes

DNA inserts of hairpin siRNAs-encoding specific for HPV16 *E6* and *E7* oncogenes were designed using software from Applied Biosystems-Ambion,[25] and were cloned in *Apa* I and *Eco* RI restriction sites in the pSilencer1.0-U6 siRNA expression plasmid (Applied Biosystems, Foster, CA, USA), which contains the U6 RNA Pol- III promoter to generate small RNA transcripts. Four independent plasmids for *E6* oncogene (psiRNAE6A, psiRNAE6B, psiRNAE6C and psiRNAE6D) and four independent plasmids for *E7* oncogene (psiRNAE7A, psiRNAE7B, psiRNAE7C and psiRNAE7D) were generated. The oligonucleotide sequences for DNA inserts are shown in Supplementary Table 1. To eliminate the probability of homology sequences with other human genes, the hairpin siRNAs-encoding sequences were analyzed by Blast. The integrity of all plasmid constructs was verified by DNA sequencing. The GenBank access number to HPV16 *E6* and *E7* cDNA oncogenes is: 29468132/AF402678.1.

Transfection assays with siRNA expression plasmids

SiHa cells were transiently transfected with psiRNAE6A, psiRNAE6B, psiRNAE6C, and psiRNAE6D plasmids to silence the *E6* oncogene and with psiRNAE7A, psiRNAE7B, psiRNAE7C and psiRNAE7D plasmids to silence the *E7* oncogene from HPV16, using lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Briefly, one day before transfection assay, the cells were plated at a density of 1×10^5 cells per well in a six-well plate containing 2 mL of DMEM with 10% FBS and penicillin/streptomycin. At the time of transfection, SiHa cells were rinsed and replenished with DMEM free FBS. The plasmids and lipofectamine were diluted in DMEM free FBS and incubated for 20 min at room temperature. The plasmid DNA concentration and lipofectamine reagent were normalized by GFP transfection and all assays were carried out with 0, 3 and 5 ug of plasmids. SiHa cells were incubated with plasmids and lipofectamine for 4 h, rinsed and replenished with DMEM containing 10% FBS. Forty eight h after transfection, cells were harvested and RNA isolation was carried out for semiquantitative end-point RT-PCR as well as for quantitative real-time RT-PCR assays. Cellular protein isolation was performed by Western Blot assays. The transfected SiHa cells were fixed in ethanol for flow cytometry assays and in paraformaldehyde for electron microscopy assays. All transfections were repeated at least four independent times.

Cellular viability assays

Cellular viability was measured using 3 -(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt MTS assay, which is a colorimetric method for determining the number of viable cells in a proliferation or cytotoxicity assay. Briefly, a total of 2×10^4 SiHa cells per well were plated in a 96-well plate. After twenty four h of plating, into each well containing the untreated cells and cells transfected with pSilencer1.0-U6, psiRNAE6 and psiRNAE7 plasmids in 100 uL DMEM, were added 20 uL of MTS reagent and these were incubated at 37°C during 4 h. MTS tetrazolium compound salt reagent is bioreduced by living cells into a colored formazan product that is soluble in tissue culture medium. After incubation, the absorbance values were measured at 490 nm in an automatic microplate reader. Cellular viability rate was calculated as the percentage of MTS adsorption as follows: % survival = (mean experimental absorbance/mean control absorbance) X 100. Each assay was carried out in three separate times.

Trasmission electron microscopy

Transfected SiHa cells (1×10^6 cells) were centrifuged and the pellet was fixed for 1 h at 4°C in 1 ml Karnovsky solution (10% paraformaldehyde, 10 mM sodium hydroxide, 25% glutaraldehyde) without $CaCl_2$ and sodium cacodylate buffer. Fixing solution was removed and the pellet was stored at 4°C in 0.1 M cacodylate buffer until sample processing. Samples were post-fixed in 1% (w/v) osmium tetroxide for 1 h, washed twice with distilled water, dehydrated in a graded series of ethanol solutions going from 70% to 100%, and resuspended in 100% propylene oxide. Cells were

embedded by successive 1 h incubations in epoxy resin of 1:1 and 2:1 resin:propylene oxide, and polymerized by incubating at 60°C for 48 h in 100% epoxy resin. 0.05-0.1 um sections were stained in 2.5% (w/v) uranyl acetate and 0.3% (w/v) lead citrate and viewed in an M-Jeo1 1010 electron microscope.

Semiquantitative end-point RT-PCR analysis

Transfected SiHa cells (1×10^6 cells) were harvested and processed for total RNA isolation using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Briefly, cells were washed with PBS and 1 ml Trizol was added. 200 uL chloroform was added and the cells were centrifuged. The aqueous phase was separated and the RNA was precipitated with isopropanol. The RNA was dissolved in DEPC-water and the concentration was measured. The mRNA was obtained using oligo dT (Invitrogen) and cDNA synthesis was performed by incubation with reverse transcriptase M-MLV (Invitrogen) at 37°C during 1 h. HPV16 *E6* and *E7* oncogene expression was measured by semiquantitative end-point RT-PCR using the sense 5'-TTA-CCA-CAG-TTA-TGC-ACA-GA-3' and antisense 5'-ACA-GTG-GCT-TTT-GAC-AGT-TA-3' primers for E6, and sense 5'-AGA-AAC-CCA-GCT-GTA-ATC-AT-3' and antisense 5'-TTA-TGG-TTT-CTG-AGA-ACA-GA-3' primers for E7, which have been previously reported.[45] The PCR reaction amplification conditions were 95°C for 10 min, 95°C for 1 min, 63°C for 1 min and 72°C for 1 min for 35 cycles followed by 72°C for 10 min. A 298 bp DNA fragment was obtained for *E6* oncogene and a 315 bp DNA fragment was obtained for *E7* oncogene. The GADPH (glyceraldehyde-3-phosphate dehydrogenase) housekeeping gene was used as a control using sense 5'-CAA-CAG-CCT-CAA-GAT-CAT-C-3'and antisense 5'-ACC-AGG-AAA-TGA-GCT-TGA-C-3' primers. The PCR reaction amplification conditions were 95°C for 10 min, 94°C for 1 min, 54°C for 1 min and 72°C for 1 min for 25 cycles followed by 72°C for 10 min. A 518 bp DNA fragment was obtained. For each PCR reaction, 1 ug cDNA, 2.5 mM dNTPs, 20 pmol each primer and 0.5 U Taq (Invitrogen, Carlsbad, CA.) were used in a 25 uL volume reaction. To ensure that amplification remained within the linear range, 1:5 serial dilutions of cDNA were made.

Quantitative real-time RT-PCR analysis

Total RNA isolation was carried-out from SiHa cells transfected as previously described. The cDNA synthesis was performed by incubation with 100 ng RNA, 1X RT buffer, 0.25 mM each dNTPs, 0.25 U/uL RNase-OUT inhibitor and 3.33 U/uL transcriptase reverse MultiSbribe, in a one step 7.5 uL volume reaction. The reaction was incubated in a 384 well plate at 37°C during 30 min in Applied Biosystems 7900 Real-Time PCR Instrument. The quantitative analysis of HPV16 *E6* and *E7* oncogene expression was carried out with TaqMan probes kit in Applied Biosystems 7900 Real-Time PCR Instrument. The dynamic ranges and PCR reaction amplification were normalized for HPV16 *E6* and *E7* oncogenes and GADPH housekeeping gene was used as control. Ct values were analyzed to determine the statistical significance of HPV16 *E6* and *E7* oncogene expression in SiHa cells transfected or non-transfected with siRNA expression plasmids.

Western blot assays

Forty eight h after transfection assays, SiHa cells were harvested and protein isolation was carried out for Western Blot assays. Briefly, the cells were washed with PBS and incubated for 30 min at 4°C with lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 0.5% SDS, 1% NP40, 0.5 mM AEBSF, 10 ug/μL Antipain, 10 ug/uL aprotinin, 10 μg/μL khymostatin, 10 ug/uL leupeptin, 10 μg/μL pepstatin, 1 mM EDTA, 100 mM PMSF and 0.5 mM DTT (Sigma Aldrich, NJ, USA).The lysates were centrifuged at 11,000 rpm for 15 min. Total proteins from supernatants were determined using the BCA kit (Pierce). 30 μg of proteins were electrophoresed on 12% SDS-PAGE, transferred into nitrocellulose membranes and incubated for antibodies detection. Biotinilated and prestained molecular weight marker was included. IgG mouse monoclonal antibody DO-1/sc-126 was used to detect human p53 protein, IgG mouse monoclonal antibody Ser 807-811/sc-16670 was used to detect human pRb protein, IgG mouse monoclonal antibody C1P5/sc-460 was used to detect HPV16 *E6* oncoprotein and IgG mouse monoclonal antibody ED17/sc-6981was used to detect HPV16 *E7* oncoprotein. Human beta-actin protein was detected using polyclonal antibody sc-1616-HRP (all antibodies were purchased from Santa Cruz, Biotechnology, Santa Cruz, CA, USA). After that peroxidase coupled secondary goat antibody mouse anti-IgG was added, the visualization of bound antibodies and protein were detected by enhanced chemiluminiscence using the renaissance Western Blot kit (Pierce). The membranes were subjected to autoradiography at -70°C with an intensifier screen.

Flow cytometry assays

Transfected SiHa cells (1×10^6 cells) were harvested, centrifuged, fixed in cold 70% ethanol and stored at -20°C. Prior to analysis, ethanol was removed and cells were incubated at room temperature for 10 min in 1 mL buffer A (1 mg/mL citric acid, 0.1% Nonidet P40, 1.16 mg/mL spermine tetrahydrochloride, 60.5 ug/mL trizma hydrochloride ph 7.6) containing 30 ug/ml porcine pancreatic trypsin. After this, the SiHa cells were incubated at room temperature for 10 min with 1 mL of 30 ug/mL trypsinogen and 100 ug/mL ribonuclease A dissolved in buffer A. Then, SiHa cells were incubated at 4°C for 10 min in 1 mL of 500 ug/ml propidium iodide and 1.16 mg/mL spermine tetrahydrochloride dissolved in buffer A. During each incubation, samples were vortexed intermittently every 2 min. Approximately 10,000 nuclei were processed in FACS Sort Becton Dickinson (Ar laser, 488 nm and 620 nm excitation and emission wavelengths, respectively) and results were analyzed with ModFit LT (Verity) software. Instrument settings were fixed using non-transfected SiHa cells.

Statistical analysis

All experiments were performed at least three times. The data were analyzed and χ^2 test was carried out to compare frequencies between the different experimental groups. P-values less than 0.01 were considered to be statistically significant and were indicated with an asterisk (*).

Results

Selection of siRNA sequences for silencing of HPV16 E6 and E7 oncogenes

The most effective siRNA sequences were selected using an algorithm based on new guidelines developed by Ui-Tei and are shown in Supplementary Table 1.[26] This algorithm was used as a helpfuL web based tool to design siRNAs targeting against HPV16 *E6* and *E7* oncogene encoding region from HPV16 cDNA sequence (supplementary Figures 1a and b). Also from this web site, sequences and conditions required for proper transcription initiation and termination for designing siRNAs, as well as, GC content and other relevant parameters, were selected. In addition, to minimize the off-target silencing effects for the selection of siRNAs sequences that are guaranteed to have some mismatches to all unrelated sequences, we used this software because it has a rigorous specificity measure called the mismatch tolerance (this means the minimum number of mismatches between the siRNAs sequence and any non-targeted sequence). A higher mismatch tolerance of a siRNA sequence indicates its high specificity in the presence of some mismatches. Furthermore, neither of the siRNA sequences for *E6* and *E7* oncogenes share homology with exons of known human genes. Each DNA insert that encode the siRNAs was cloned in the

pSilencer1.0-U6 siRNA expression vector. Four plasmid constructs for HPV16 *E6* oncogene (psiRNAE6A, psiRNAE6B, psiRNAE6C and psiRNAE6D) and four for HPV16 *E7* oncogene (psiRNAE7A, psiRNAE7B, psiRNAE7C and psiRNAE7D) were generated.

siRNAs for HPV16 E6 and E7 oncogenes induce differential silencing of viral oncogene expression

siRNAs effects can be influenced by secondary mRNA structure and positioning of the cognate sequence within the mRNA molecule. To analyze the individual effect of each siRNA expression plasmid, we first determined if the U6 promoter-driven siRNAs could induce a specific silencing effect at mRNA expression by transient transfection of psiRNAE6 and psiRNAE7 plasmids. For this purpose, SiHa cells were transfected with the four siRNA expression plasmids related to *E6* and with the four siRNA expression plasmids related to *E7* in an independent manner. As shown in Figures 1a and b, each plasmid had different effects on the HPV16 *E6* and *E7* mRNA expression level. In particular, we identified a significant decrease in mRNA expression in both *E6* and *E7* oncogenes when cells were transfected with psiRNAE6B and psiRNAE7B plasmids. Forty eight h after transfection, the *E6* and *E7* mRNA expression level decreased at 75% and 80% respectively, compared with cells transfected with pSilencer1.0-U6 (empty vector) as well as with negative control pSilencer2.0-U6 neo (data not show). In addition, we did not observed differences in *E6* and *E7* mRNA expression levels between SiHa cells treated with the empty vector and negative control pSilencer2.0-U6 neo vector, which expresses a hairpin siRNA with limited homology to any known sequences in human genome, compared with untreated SiHa cells. In a group of parallel experiments, we analyzed the HPV16 *E6* and *E7* oncogene expression in SiHa cells by effect of psiRNAE6B and psiRNAE7B transfection plasmids by real-time RT-PCR. In Figure 1c we determined that mRNA expression level decreased at 65% for *E6* oncogene and at 60% for *E7* oncogene, these data were in agreement with end-point semiquantitative RT-PCR results. The GAPDH mRNA expression level did not show any changes. Due to the fact that the silencing of HPV16 *E6* and *E7* oncogene expression was more efficient during the treatment with psiRNAE6B and psiRNAE7B plasmids, the next assays were performed in SiHa cells transfected with these same plasmids. These data suggest that psiRNAE6B and psiRNAE7B are siRNA expression plasmid specifics for HPV16 *E6* and *E7* oncogenes, and these have the ability to generate a selective and specific mRNA silencing of viral oncogenes in human cervical cancer cells transformed by HPV16.

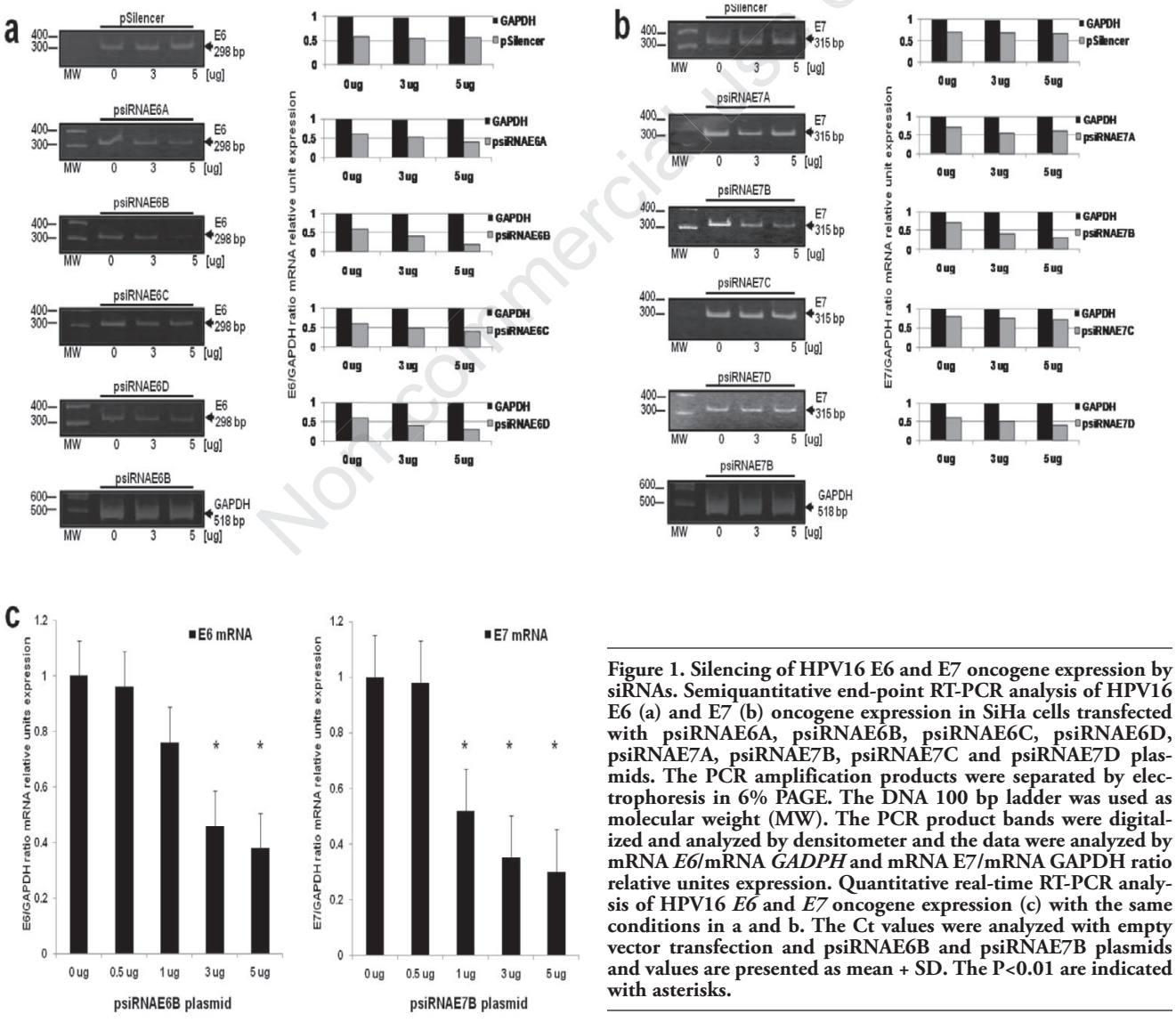

Figure 1. Silencing of HPV16 E6 and E7 oncogene expression by siRNAs. Semiquantitative end-point RT-PCR analysis of HPV16 E6 (a) and E7 (b) oncogene expression in SiHa cells transfected with psiRNAE6A, psiRNAE6B, psiRNAE6C, psiRNAE6D, psiRNAE7A, psiRNAE7B, psiRNAE7C and psiRNAE7D plasmids. The PCR amplification products were separated by electrophoresis in 6% PAGE. The DNA 100 bp ladder was used as molecular weight (MW). The PCR product bands were digitalized and analyzed by densitometer and the data were analyzed by mRNA *E6*/mRNA *GADPH* and mRNA E7/mRNA GAPDH ratio relative unites expression. Quantitative real-time RT-PCR analysis of HPV16 *E6* and *E7* oncogene expression (c) with the same conditions in a and b. The Ct values were analyzed with empty vector transfection and psiRNAE6B and psiRNAE7B plasmids and values are presented as mean + SD. The P<0.01 are indicated with asterisks.

Silencing of HPV16 E6 and E7 oncogene expression by siRNAs induces autophagy and cellular proliferation alterations in tumor cells

To examine if silencing of HPV16 *E6* and *E7* oncogene expression by siRNAs would affect cellular viability, MTS assays were carried-out on days 0, 1, 2, 3, 4 and 5 after transfection; using equal amount of SiHa cells transfected with psiRNAE6B and psiRNAE7B plasmids. Figures 2e-h and 2i-l show that silencing of HPV16 *E6* and *E7* oncogenes by siRNAs decreased the viability of SiHa cells compared with non-transfected or transfected cells with empty vector (Figures 2a-d). A marked decrease in cellular viability was observed from day 2 to 5 after transfection with siRNA expression plasmids. To evaluate if silencing of HPV16 *E6* and *E7* oncogene expression induced by siRNAs expressed in plasmids has cellular effects, we analyzed by transmission electron microscopy the morphology features of SiHa cells transfected with psiRNAE6B and psiRNAE7B plasmids (Figure 3). Viable SiHa cells non-transfected or transfected with the empty vector (Figures 3a-c) presented regular spots of condensed chromatin and well-defined nucleolus (nl) appeared distributed throughout the nucleus (n), as normal mitochondria with tubular cristae. SiHa cells transfected with psiRNAE6B and psiRNAE7B plasmids are show in Figures 3d-i, respectively. SiHa cells show autophagy features, such as a high content of autophagic vacuoles (black arrows) and swollen mitochondria can be seen in SiHa cells during silencing of HPV16 E6 and E7 oncogene expression by siRNAs treatment using different amounts of plasmids. In some cells, a swollen endoplasmic reticulum and Golgi apparatus forming structures resembling small vacuoles, which are apoptotic features, were also detected although chromatin condensation was not observed. In regard whether the simultaneous silencing of *E6* and *E7* increases the effects on autophagy with respect to the single silencing of *E6* or *E7* only, in first instance, we evaluated by end-point RT-PCR the HPV16 *E6* and *E7* oncogene expression by simultaneous transfection with siRNAs for *E6* and E7. The result indicated that HPV16 *E6* and *E7* mRNA level expression were more efficient by simultaneous silencing of *E6* and *E7* oncogenes that with single silencing of *E6* and *E7* of independent manner (data not show). Therefore, we also evaluated the influence of simultaneous silencing of both viral oncogenes on the autophagy process induction. We found that simultaneous silencing of HPV16 *E6* and *E7* oncogenes by siRNAs increases the biological effects of autophagy in human cancer cells, and we observed morpho-

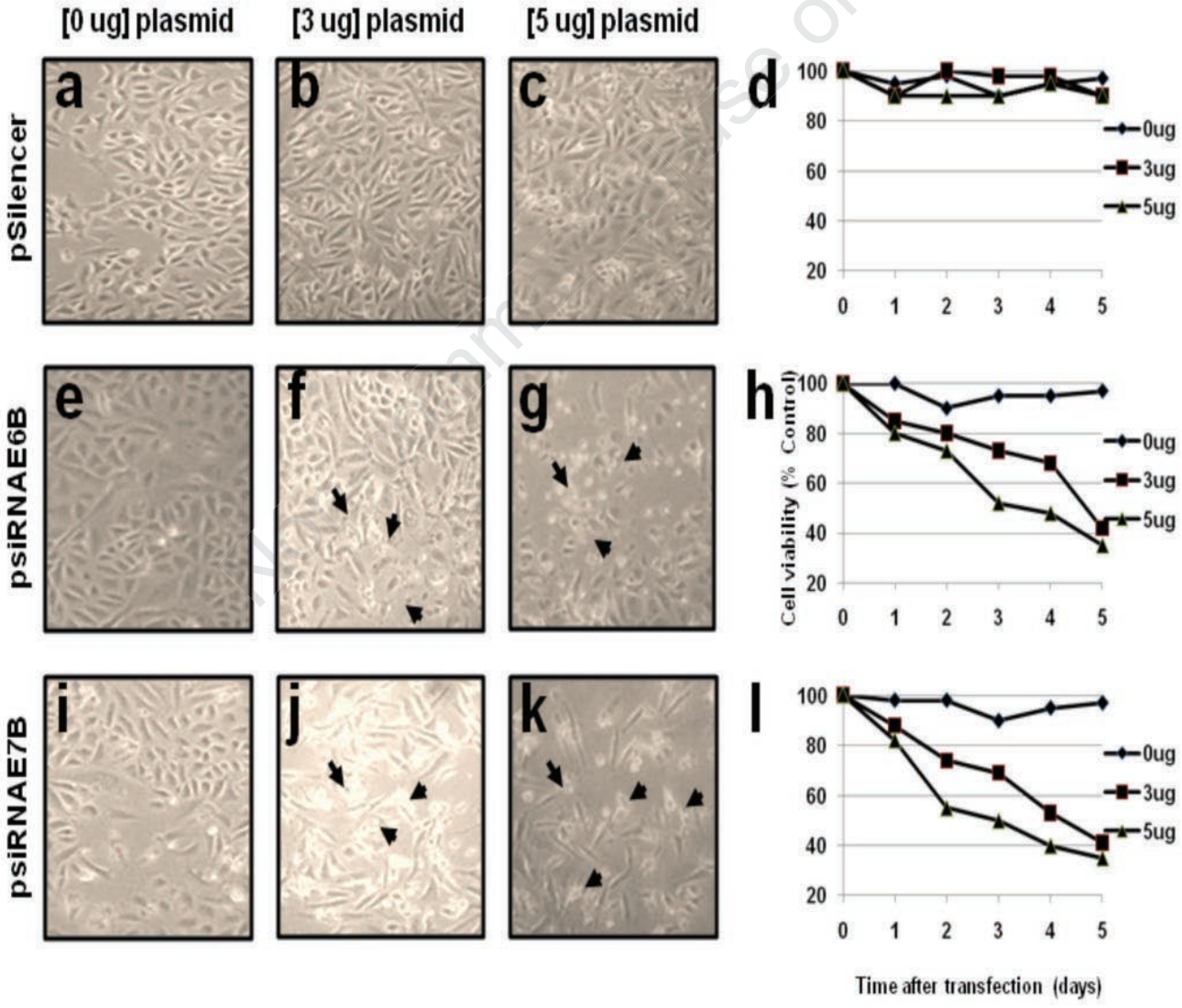

Figure 2. Tumor cell viability for silencing of HPV16 E6 and E7 oncogene expression by siRNAs. SiHa cells were analyzed by white light microscopy (20X) 48 h after transfection with pSilencer plasmid (a-c), with psiRNAE6B plasmid (e-g) and with psiRNAE7B plasmid (i-k). The arrow blanks indicate the dead cells. Cellular viability was measured using MTS assay kit (d, h, l).

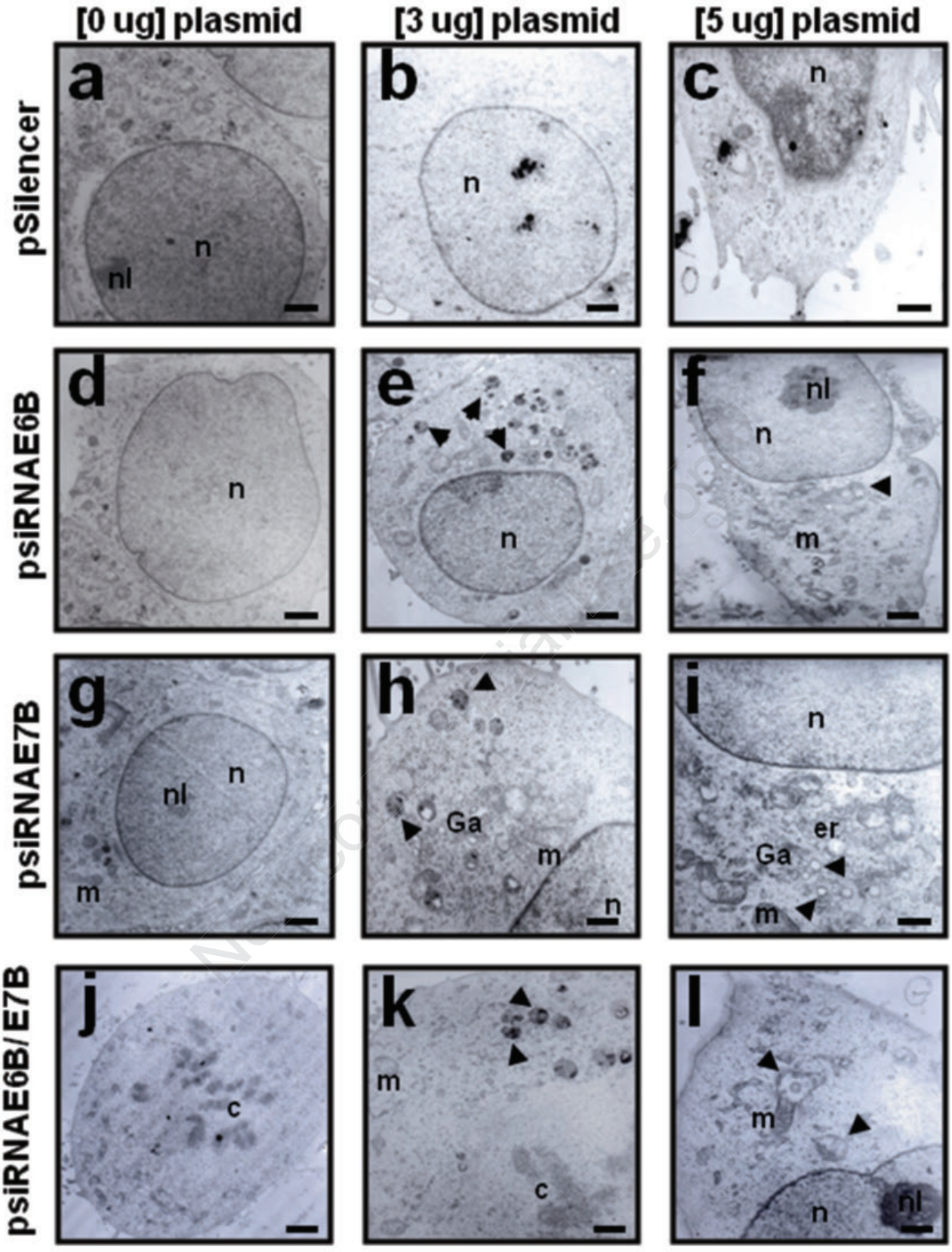

Figure 3. Morphologycal features of tumor cells for silencing of HPV16 E6 and E7 oncogene expression by siRNAs. SiHa cells were analyzed by transmission electron microscopy 48 h after transfection with pSilencer plasmid (a-c), with psiRNAE6B plasmid (d-f), with psiRNAE7B plasmid (g-i) and co-transfection with psiRNAE6B/psiRNAE7B plasmids (j-l). Autophagic features (black arrows), chromatin (c), endoplasmic reticulum (er), Golgi apparatus (Ga), mitochondria (m), nucleus (n) and nucleolus (nl), are indicated. Scale bar: a-g and j = 1.0 um; h, i, k and l = 500 nm.

logic features like as autophagy (Figure 3k-l). SiHa cells that showed these morphologic features, but still maintained the plasma membrane integrity, were identified as early autophagic-like cells. In a later death phase, designated late autophagic-like of Transfected SiHa cells with large amount of plasmids, plasma membrane integrity was not lost and the main cytoplasmic contents were leaked out. Very swollen mitochondria along with autophagosomes remained within the cell, and the nuclear envelope remained intact. Nonetheless, condensed chromatin and nuclear fragmentation, a typical characteristic of apoptosis, were not observed even in the most advanced phases of cellular death. These data suggest that silencing of HPV16 *E6* and *E7* oncogene expression by siRNAs can induce cellular death by autophagy mechanism.

Silencing of HPV16 E6 and E7 oncogene expression by siRNAs has effect upon p53 and pRb protein expression

In normal conditions, when cells have cellular stress or genotoxic damage, cellular death by autophagy and apoptosis are mediated by gene tumor suppressor products p53 and pRb, which are main regulators of cellular cycle. In SiHa cells which express the HPV16 *E6* and *E7* oncoproteins, autophagy and apoptosis induction is blocked. To evaluate if the cellular effects of cellular death were a consequence of silencing of HPV16 *E6* and *E7* oncogene expression by siRNA treatment, we analyzed *E6* and *E7* oncoprotein expression, as well as, the cellular target p53 and pRb protein expression (Figure 4). Using Western Blot assay, we identified the inhibition of *E6* oncoprotein expression and the reestablishment of p53 cellular protein expression after treatment of SiHa cells with different amounts of psiRNAE6B plasmid (Figure 4a). Regarding E7, this oncoprotein binds to pRb cellular protein family resulting in the generation of hypophosphorylated pRb isoform. Thus, the treatment of SiHa cells with different amounts of psiRNAE7B plasmid induced inhibition of *E7* oncoprotein expression and the decrease or loss of pRb upper band, which corresponds to hyperphosphorylated pRb isoform, while pRb lower band remained expressed, which corresponds to hypophosphorylated pRb isoform and is the active form associated with E2F (Figure 4b). We used beta-actin protein as a control and we did not observed any alteration in cellular protein expression when SiHa cells were transfected with the same siRNA expression plasmids. Thus, our results confirm that repression of HPV16 *E6* and *E7* oncoprotein expression and reestablishment of p53 protein expression, as well as, the loss of hyperphosphorylated pRb isoform were induced in SiHa cells after treatment with psiRNAE6B and psiRNAE7B plasmids. These effects were specific for silencing of HPV16 *E6* and *E7* oncogene expression induced by siRNAs. These data allow us to demonstrate that silencing of HPV16 E6 and *E7* oncogenes expression has a similar effect to cellular stress response or generalized genotoxic damage with reestablishment of p53 and pRb expression and function.

siRNAs for HPV16 E6 and E7 oncogenes induce apoptosis of cervical cancer cells

The ideal effect of a therapeutic agent during treatment of cancer consists in induction of selective death of tumor cells without affecting surrounding normal cells. For cervical cancer it is known that HPV16 *E6* and *E7* oncogenes are directly related with apoptosis suppression of cancer cells.[1] We previously demonstrated that HPV16 *E6* and *E7* oncogene expression is silenced by specific siRNAs expressed in our plasmids. Furthermore, we demonstrated that this silencing resulted in the reestablishment of p53 and hypophosphorylated pRb isoform protein expression, which are the main cellular protein targets of *E6* and *E7* oncoproteins. We also evidenced that viral oncogene silencing induces the cellular death by autophagy process. Therefore, we decided to analyze if silencing of HPV16 *E6* and *E7* oncogene expression using these siRNAs, can also induce the selective death of cancer cells by apoptosis process. Determination of DNA content by flow cytometry at different phases of the cell cycle had been a successful method to find out whether cells are in proliferation or going through cellular death by apoptosis. We determined the DNA content by flow cytomet-

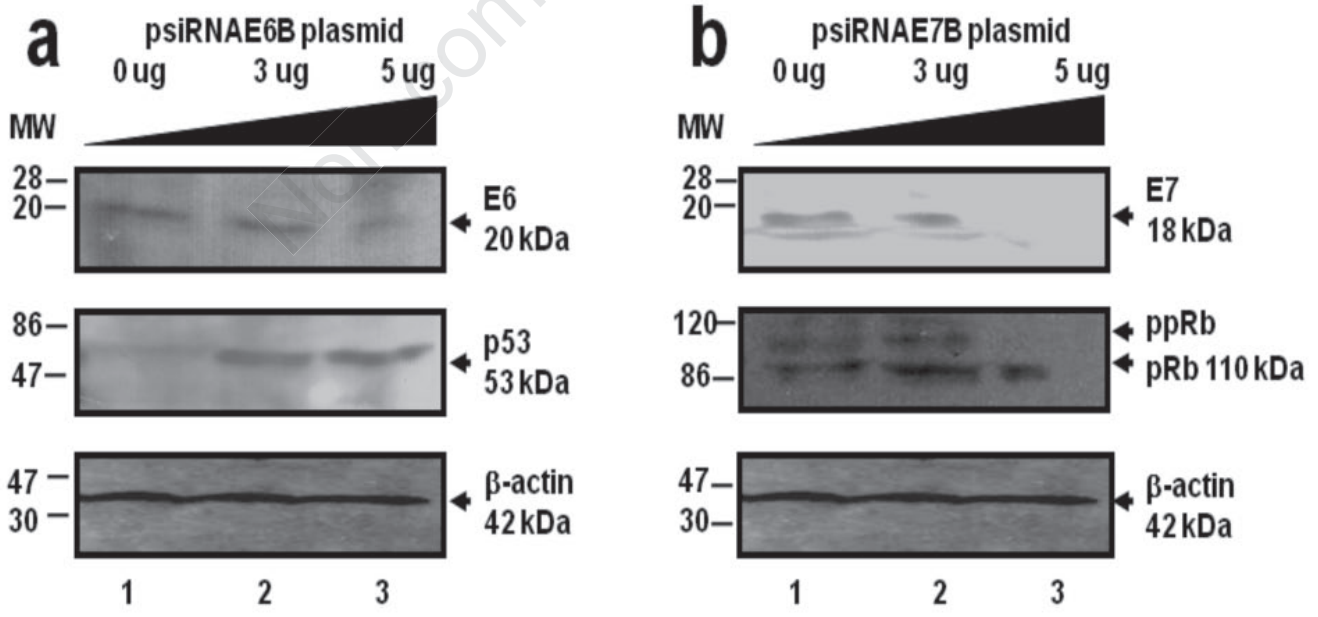

Figure 4. HPV16 E6, E7, p53 and pRb protein expression for silencing of E6 and E7 oncogenes by siRNAs for silencing of e6 and e7 oncogenes by siRNAs. Total cellular proteins were obtained from SiHa cells after 48 h transfection with psiRNAE6B and psiRNAE7B plasmids. The proteins were separated in 12% SDS-PAGE and transferred to nitrocellulose membranes which were incubated with each antibody. HPV16 E6 oncoprotein and p53 cellular protein detection (a). HPV16 E7 oncoprotein and pRb cellular protein detection (b). Similar amounts proteins were analyzed in the immunoblots and the anti-beta-actin antibody was included as control.

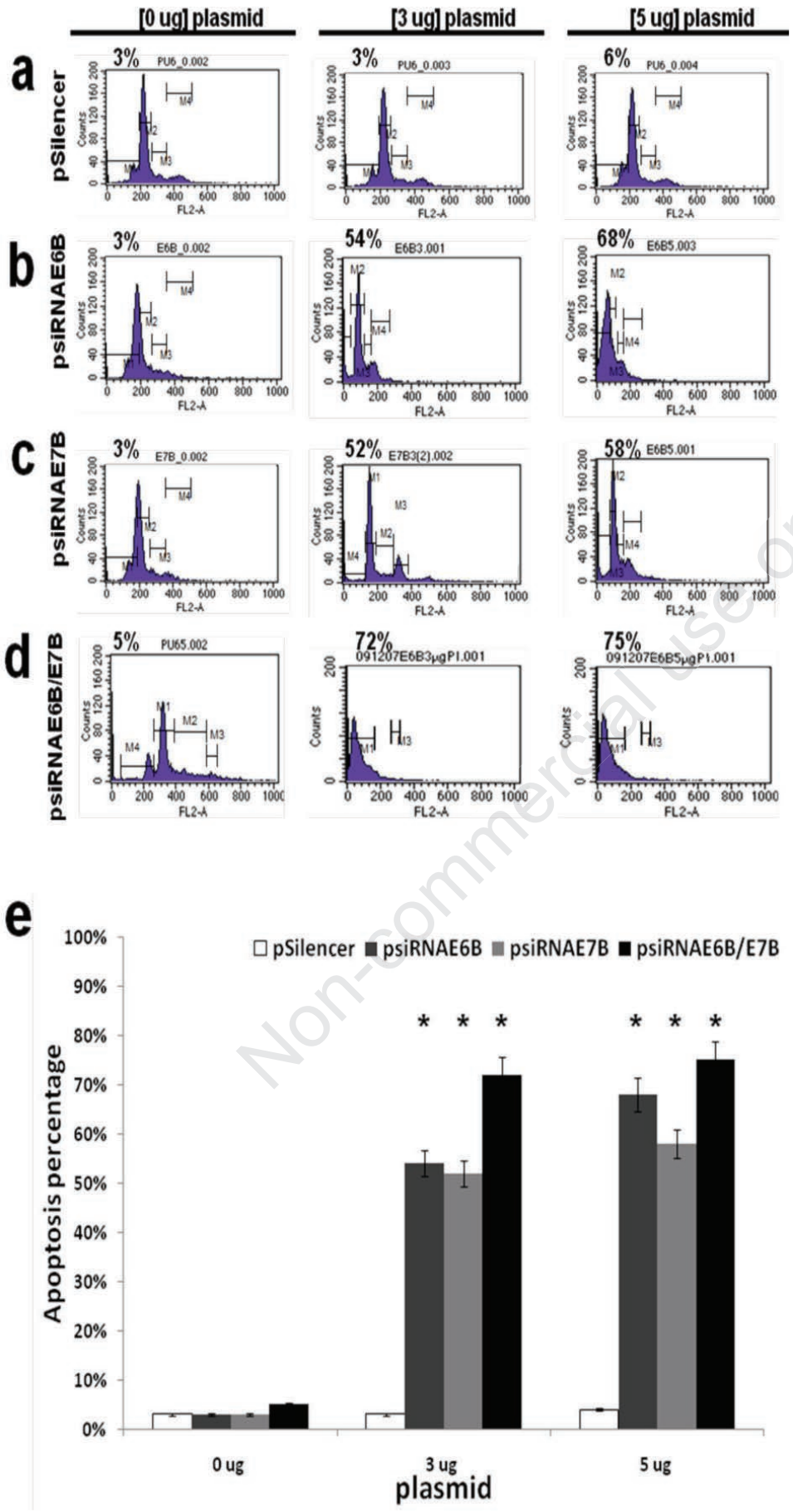

Figure 5. Apoptosis analysis of cancer cells by treatment with psirnae6b and psiRNAE6B plasmids. SiHa cells were transfected with pSilencer plasmid (a), with psiRNAE6B plasmid (b), with psiRNAE7B plasmid (c), with psiRNAE6B/E7B plasmids (d), and were harvested and fixed in 70% ethanol. Cells were treated with propidium iodide and approximately 10,000 nuclei were processed by FASC-Sort. The data were analyzed with ModFitLT software and the assays were normalized with non-transfected SiHa cells. Percentage of apoptosis was evaluated introducing propidium iodide into cells after 48 h transfection (e). The P<0.01 are indicated with asterisks.

ric analysis in SiHa cells transfected with psiRNAE6B and psiRNAE7B plasmids (Figure 5). We found cellular death by apoptosis in 52% to 68% of transfected SiHa cells, while 3% to 6% of SiHa cells presented cellular death when they were non-transfected or transfected with the empty vector (Figures 5a-c). In regard whether the simultaneous silencing of *E6* and *E7* increases the effects on apoptosis with respect to the single silencing of *E6* or *E7* only, in a group of parallel experiments we evaluated the influence of simultaneous silencing of both viral oncogenes on the apoptosis process induction. We found that simultaneous silencing of HPV16 *E6* and *E7* oncogenes by siRNAs increases in 72% to 75% of cellular death by apoptosis in human cancer cells (Figure 5d). The Figure 5e represents the apoptotic percentage of SiHa cells transfected with psiRNAE6B and psiRNAE7B plasmids and the rise in apoptotic percentage was statistically significant compared to transfection with the empty vector ($P<0.01$). These findings suggest that induction of cellular death by apoptosis in human cervical cancer cells transfected with psiRNAE6B and psiRNAE7B plasmids, which produce the specific siRNAs for HPV16 *E6* and *E7* oncogenes, is mediated by silencing of the viral oncogene expression and is selective to carcinoma cells transformed by HPV16.

Taken together, these evidences support the notion that administration of siRNA expression plasmids for HPV16 *E6* and *E7* oncogenes, is able to induce the silencing of viral oncogene expression and to reestablish the activity of the tumor suppressor p53 and hypophosphorylated pRb cellular proteins. All these together could have biological influence on human cervical cancer cells transformed by HPV16, by induction of cellular death by autophagy and apoptosis. At this time, we do not know which are the specific molecular pathways involved, but it is being studied by our group.

Discussion

Since their discovery, the role of siRNAs has revolutionized studies in molecular biology. The ability to selectively silence mammalian gene expression using siRNAs offers new and exciting perspectives to the understanding of mammalian cell biology and its pathology. However, it cannot be assumed that all genes will prove equally susceptible to the RNA interference mechanism. The use of siRNAs has been shown to knock-down a large number of genes expressed in mammalian cells and it has been used to characterize mammalian gene function.[27-29] Nevertheless, a potential application of siRNAs is in specific gene therapy against cancer. The siRNAs are non-coding RNAs of 21-25 nucleotides in length that mimic endogenous microRNAs, which can effectively reduce the translation of target mRNAs by binding to their 3'-UTR. The process is dependent upon mRNA accessibility and within the target mRNA molecule, upon accessibility of the short internal nucleotide sequences that are homologous to the siRNA transcripts. Therefore, various factors can influence the vulnerability of a given mRNA to siRNA-mediated degradation, including secondary structures of the mRNA, and proteins which package mRNA for translocation within the cell. Other protein-mRNA interactions are also relevant, including proteins which can direct a given mRNA to a specific sub-cellular locus, and those mRNAs which can be bound by the proteins they encode. As a consequence, the generation of biofunctional siRNAs must be carefully and robustly designed to get highly efficient siRNAs for the silencing of specific target genes.

There are at least two strategies to generate siRNAs: the first is by chemical synthesis and the second is by cloning in molecular vectors. The siRNAs can be chemically synthesized and transfected into mammalian cells by cationic lipofection.[30] With this strategy it is possible to efficiently induce silencing of genes with siRNA synthetic duplex. Nevertheless, this approach has disadvantages such as the high costs of production and sometimes several doses are required *in vivo* animal models. The cloning of DNA inserts in a molecular vector that will be transcribed in the corresponding siRNAs is a tool that has been developed using siRNA expression plasmids.[31,32] These vectors contain DNA inserts designed with software to generate highly efficient siRNAs. When these plasmids are transfected into mammalian cells, the DNA inserts are transcribed as siRNAs under the control of the RNA Pol-III promoter, and form secondary structures of the stem-loop type which are processed by RISC and are assembled with target mRNA. In the present study, DNA inserts that transcribe specific siRNAs for HPV16 *E6* and *E7* oncogenes were designed whit software from Applied Biosystems-Ambion. These DNA inserts were cloned in pSilencer1.0-U6 siRNA expression plasmid, which contains the U6 RNA Pol-III promoter, in order to generate siRNA transcripts. With these plasmid constructs we identified a differential effect in silencing of HPV16 *E6* and *E7* oncogene expression. When we analyzed the exact position of siRNAs in *E6* and *E7* cDNA we observed that siRNAs of psiRNAE6B and psiRNAE7B plasmids had complementarity with nucleotide sequences of 3'-UTR from both oncogenes. This peculiar complementary nucleotide of siRNAs, can partly explain the higher silencing effect of psiRNAE6B and psiRNAE7B plasmids on *E6* and *E7* oncogene expression comparing to the other siRNA expression plasmid constructs. These data are agreement with the evidences reported on differential regulation of siRNAs.[33] Thus, our findings support the fact that specific sequence siRNAs for HPV16 *E6* and *E7* oncogenes were carefully designed to ensure a specific down-regulation of *E6* and *E7* mRNA. As a consequence, with this strategy we got biofunctional siRNAs and generated a pool of plasmid constructs, which can be used to analyze the expression and function of relevant cellular molecules, related to HPV16 *E6* and *E7* oncogene expression. Furthermore, these siRNA expression plasmids can be cloned in other molecular vectors such as adenovirus that would be used in the next step of analysis *in vivo*.

In addition, we attempted to determine if these siRNA expression plasmids could be used in HPV16-transformed cancer cells. In our study, we demonstrated that siRNAs can silence HPV16 *E6* and *E7* oncogene expression by a decrease in mRNA expression level in human cervical cancer cells. To understand the mechanism by which siRNAs for *E6* and *E7* inhibit the growth of SiHa cells, we examined their effects on p53 and pRb protein expression, which are the most relevant cellular target molecules of high-risk HPV *E6* and *E7* oncogenes. In our model, we demonstrated that psiRNAE6B and psiRNAE7B plasmids were able to cause a specific decrease of *E6* and *E7* oncoprotein expression as well as a reestablishing of p53 and pRb cellular proteins, and induction of cellular death. Under physiological circumstances, p53 protein levels are controlled by Mdm2-mediated ubiquitination followed by proteasomal degradation. In response to stress or DNA damage, p53 is subjected to post-translational modifications that allow it to avoid Mdm2-mediated degradation and hence to accumulate.[34] Within the nucleus, active p53 tetramers can bind to the promoter region of multiple genes that code for apoptotic and autophagic modulators, including AMP-activated protein kinase (AMPK) b1 and b2 subunits, death associated protein kinase 1 (DAPK-1), damage-regulated autophagy modulator (DRAM), pro-apoptotic Bcl-2 proteins (Bad, Bax, Bnip3, and Puma), sestrin 2, and tuberous sclerosis protein 2 (TSC2), as well as genes encoding for lysosomal proteins.[34] Alternatively, p53-dependent induction of autophagy has been documented in response to DNA damage.[35] In cervical cancer cells, high-risk HPV *E6* oncoprotein binds to several cellular proteins including E6AP, a protein-ligase of the proteolysis ubiquitin pathway. Then, E6/E6AP complexes target p53 tumor suppressor protein for rapid degradation by the proteasome.[4] Our results demonstrated that the silencing of *E6* oncogene expression by siRNAs has effects over the target p53 protein expression, as well as on growth suppression and cellular death induc-

tion by autophagy and apoptosis. Thus, biological effects of siRNAs on cervical cancer cells can be explained, at least in part, by p53 reactivation pathways to regulate the autophagy and apoptosis mechanisms.

Several groups have found that administration of siRNAs for HPV *E6* and *E7* oncogenes induces cellular death by apoptosis, cellular senescence, or an increase of cellular cytotoxicity by drugs used in chemotherapy for cervical cancer. These findings show that siRNAs have an effect on the inhibition of cancer cell ability to induce tumor growth *in vivo*.[36-48] However, in our study for first time we report that silencing of HPV16 *E6* and *E7* oncogene expression is able to induce cellular death by autophagy and apoptosis. Autophagy is an evolutionarily conserved catabolic pathway that consists of the lysosomal degradation of intracellular components and leads to the generation of new metabolic substrates that favor adaptation to stress and cell survival. It is involved in numerous physiological processes and in multiple pathological conditions including cancer. Under physiological circumstances, baseline levels of autophagy occur in most tissues to ensure the turnover of long lived proteins and the elimination of old or damaged organelles. The autophagic flow is consistently intensified in situations of cell stress, favoring the adaptation and survival of cells.[49] Several gene expression disruptions that are associated with oncogenesis regulate autophagy, suggesting that basal autophagy may function as a tumor suppressor mechanism, especially in the first steps of oncogenesis and tumor progression. Conversely, stress-induced autophagy may represent an important prosurvival mechanism for tumors exposed to a hypoxic microenvironment or subjected to chemotherapy. Thus, autophagy may exert either antitumor or pro-tumor functions.[49] In our study, using electron microscopy we observed several cellular features such as cell shrinkage, mitochondria with tubular cristae, scarce cisternae of rough endoplasmic reticulum, conspicuous Golgi apparatus and a variable number of autophagic vacuoles. These evidences suggest that cellular death induced by siRNAs can be mediated by autophagy. How these effects are achieved in mechanistic terms and which are the molecular activation pathways involved in cellular death by autophagy and apoptosis, are not clear yet, but are being studied by our group.

In conclusion, the findings reported here have the follow implications: i) The administration of specific siRNA expression plasmids for HPV16 *E6* and *E7* oncogenes induces the silencing of these viral oncogenes in human cervical cancer cells transformed by HPV16; ii) The silencing of *E6* and *E7* viral oncogene expression inhibits *E6* and *E7* oncoprotein expression and induces the reestablishment of p53 and pRb cellular protein expression; iii) The reestablishment of p53 and pRb activation pathways can explain the biological effects of induction of cellular death by autophagic and apoptotic mechanisms in cervical cancer cells; iv) The silencing of HPV16 *E6* and *E7* oncogene expression by siRNAs and its effects upon p53 and pRb does not exclude that other molecular pathways can be influenced by silencing of *E6* and *E7* oncogene expression during the cervical carcinogenesis process, and this offers excellent opportunities to identify new therapeutic molecular targets; v) Since most HPV positive cervical cancers lack wild-type p53 and pRb protein, our findings suggest that a siRNA therapy strategy could effectively inhibit the growth of virus-related cancers. The relevance of the silencing properties of HPV *E6* and *E7* oncogene expression by siRNAs will be better appreciated once they are applied in clinical protocols. This goal will require an adequate analysis of siRNA sequence design to induce the silencing of the E6-E7 bicistron, the adequate selection of cloning vectors of siRNAs; the selection of transport molecular vehicles for siRNAs to administrate at the tumor site, as well as the design of treatment schemes such as chemotherapy, radiotherapy or immunotherapy treatment, combined with the use of siRNAs. Thus, our results show that the use of siRNAs has a great potential for the treatment of neoplasias that have a viral etiology, such as cervical cancer. Therefore, there is no doubt that the use of siRNA technology platform is a real gene therapy strategy against the development of cervical cancer. The challenge is now to develop more efficient strategies for the application of this technology in clinical trials.

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