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Research Article

Molecular Cancer Therapeutics

Silencing of Integrated Human Papillomavirus-16 Oncogenes by Small Interfering RNA–Mediated Heterochromatization

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Abstract

Double-stranded RNAs or small interfering RNAs (siRNA) targeting the promoters of genes are known to cause gene knockdown by a process known as transcriptional gene silencing (TGS). We screened multiple siRNAs homologous to one of the NF-1 binding sites in the human papillomavirus-16 (HPV-16) enhancer and identified one siRNA which causes specific TGS of the HPV-16 oncogenes E6 and E7 when transfected into two HPV-16–positive cell lines siHa and CaSki. This phenomenon was specific to the HPV-16 enhancer with no effect on the HPV-18 enhancer. TGS was associated with heterochromatization of the targeted region of the enhancer but no DNA methylation was noted during the time period studied. The choice of target in the enhancer was important as siRNAs differing by one or two bases showed no suppression of downstream gene expression. A low copy number enhancer-associated transcript was detected in the cell lines studied and its level decreased significantly after treatment with the siRNA that caused TGS. This supports the RNA:RNA model described previously for TGS. This siRNA which causes simultaneous silencing of E6 as well as E7 oncogenes by an epigenetic mechanism might be useful as a therapeutic modality for HPV-16–positive cervical and other epithelial cancers. *Mol Cancer Ther; 9(7); 2114–22. ©2010 AACR.*

Introduction

Cervical cancer is the second most common cancer among women (1) and is a significant cause of death among women in developing countries. It is the most common gynecologic malignancy in India with an incidence of 26.2 per 100,000 females and 74,118 annual deaths (2).

Human papillomavirus (HPV) is a causative factor in the etiology of cervical cancer. The HPVs are DNA viruses belonging to the papillomavirus family. Among the mucosotropic HPVs, around 18 subtypes are considered to be high-risk viruses because they induce lesions that could ultimately progress to cervical and other cancers. Among them, HPV-16 and HPV-18 are associated with nearly 70% of cervical cancers with HPV-16 commonly associated with squamous cell carcinomas and HPV-18 with adenocarcinomas (3–6). Persistent infection with the high-risk types of HPV is required for the development of cervical cancer (3, 4) with HPV-16 infection being more prevalent than HPV-18 (7). More and more epithelial cell malignancies such as anal carcinomas are also being identified in which integrated HPVs have a role in transformation (8).

E6 and E7 are the most important transforming factors of high-risk HPVs after integration. The E6 protein immortalizes cells by forming a complex with p53 and E6 associated protein (E6-AP), which results in rapid p53 ubiquitination and degradation (9). On the other hand, the E7 protein preferentially associates with the growth-suppressive, hypophosphorylated form of the protein product of the retinoblastoma gene and degrades it. This interaction sequesters pRb protein away from E2F-pRb complexes, resulting in the constitutive transcription of E2F-responsive S phase–specific genes (10). Suppression of E6 and E7 oncogenes in HPV-positive cell lines by overexpression of the E2 protein has already been shown to cause a reawakening of the dormant p53 and Rb tumor suppressor pathways, which in turn, lead to apoptosis and senescence (11–13).

Of the many experimental modalities of treatment being experimented with, numerous groups have reported silencing of the E6 gene or the E7 gene using double-stranded RNAs or small interfering RNAs (siRNA; post-transcriptional gene silencing or PTGS; refs. 14–17). However, PTGS of E6 alone has been reported to be insufficient for effective killing of HPV-16–integrated tumor cells and causes only a transient reactivation of p53 (18). Also, the nonheritable nature of PTGS also leads to a transient effect (19).

Promoter-targeted double-stranded RNAs have been shown to lead to CpG and/or histone methylation and decreased transcription (19–24). This is known as

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transcriptional gene silencing (TGS). Also, TGS is a more permanent method of gene knockdown because it can cause heritable chromatin modifications initially and DNA methylation later (24).

The transcription of the two oncoproteins of HPV is driven by a common long control region (LCR) which contains a strong distal enhancer followed by a weak proximal promoter (Fig. 1A). A part of the enhancer is enclosed in a nucleosome named nucleosome Ne and the promoter is enclosed in nucleosome Np16 (25).Within the nucleosome Ne are a number of activator protein-1 and NF-1 binding sites which strongly drive the transcription of E6 and E7 oncogenes after integration into the genome. We hypothesized that it would be useful to develop an siRNA targeting one of the NF-1 binding sites in the enhancer in order to simultaneously silence the expression of both the oncoproteins. This may then be developed as a gene therapy tool for cervical cancer in the future.

Materials and Methods

Cell culture

siHa, CaSki, and HeLa cells were obtained from the National Centre for Cell Sciences, Pune which is a cell banking repository involved in identification and distribution of cell lines for research purposes. The HPV status and the presence of E6 and E7 expression of all the three cell lines was verified using real-time PCR using type-specific primers. Cell lines were passaged for only 2 months after being received from the National Centre for Cell Sciences, Pune.

siHa is a HPV-16–positive cell line with one to two copies of HPV-16 per cell. The LCR region is fully unmethylated in both the copies. CaSki is a HPV-16 and HPV-18 positive cell line. It has 600 copies of HPV-16 per cell, of which only one has an unmethylated LCR. HeLa is a HPV-18–positive cell line with 10 to 50 copies of HPV-18 per cell. siHa and HeLa were maintained in DMEM

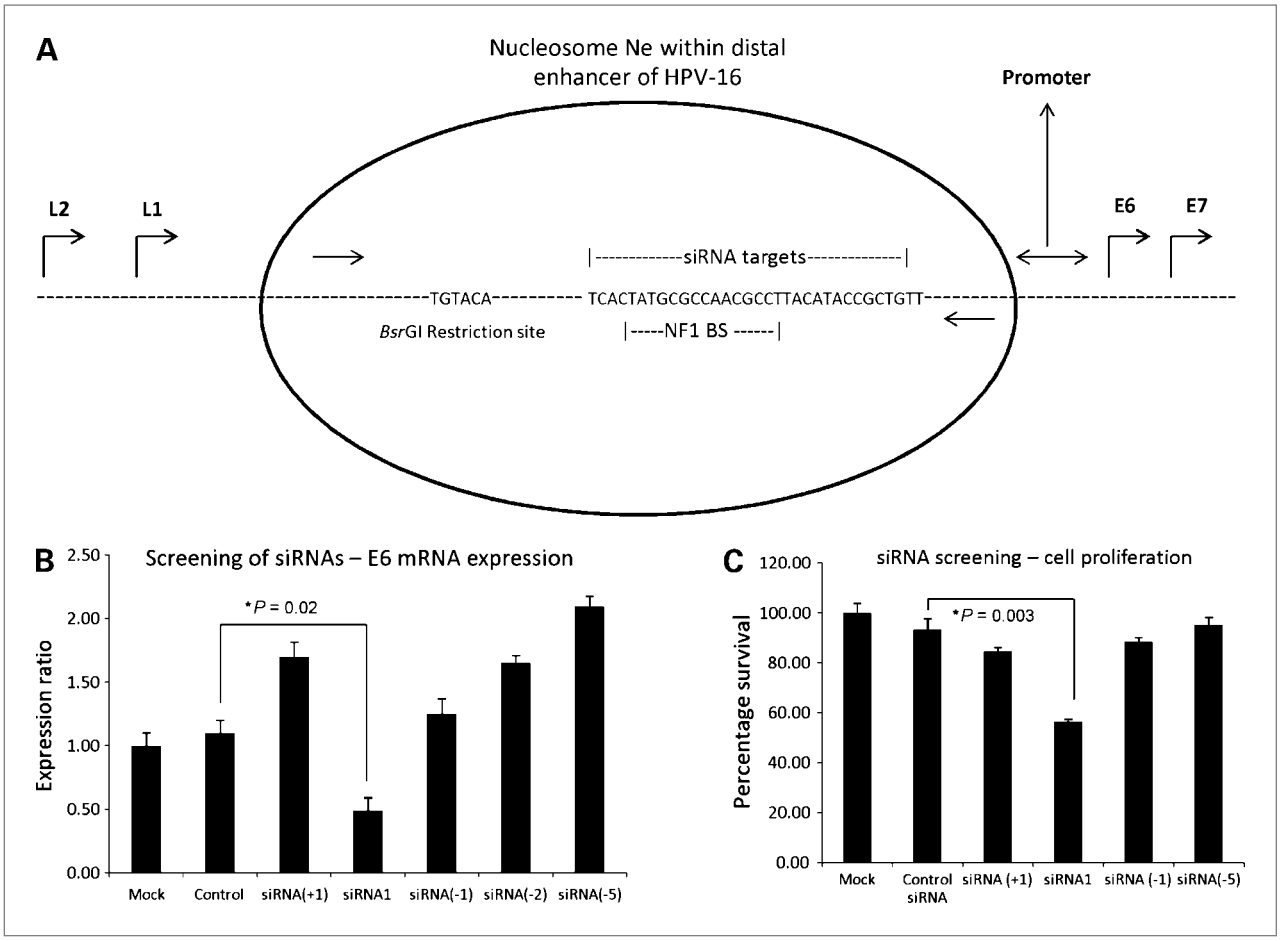

Figure 1. Screening of five siRNAs identifies an siRNA causing TGS. A, a schematic representation of the HPV-16 enhancer. The oval represents the approximate positioning of nucleosome Ne. The horizontal arrows within the nucleosome mark the binding sites for the primers used in H3K9 ChIP and restriction enzyme accessibility assays. The locations of the early and late genes have been marked as indicated. siRNA1 target is marked in gray. B, real-time PCR shows a significant decrease in E6 oncogene expression ($P = 0.02$) after siRNA1 transfection in siHa cells. Seven reference genes were used for accurate quantitation. C, siHa cells show a significant decrease in cell proliferation 24 h after siRNA1 transfection ($P = 0.003$). Minimal change was seen in other siRNA-treated cells.

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whereas CaSki was maintained in RPMI 1640. Photomicrographs were taken on an Eclipse-TE inverted phase contrast microscope (Nikon, Japan).

siRNA designing and synthesis

The characterization of transcription factors binding to the HPV-16 enhancer was done using Transcription Element Search System. siRNA synthesis was done by *in vitro* transcription as described previously (26). siRNAs were quantified using RiboGreen RNA binding dye (Invitrogen).

siRNA transfection

Cells were plated at 4,000 cells per well in a 96-well plate, 10^5 cells per well in a six-well plate, 3×10^5 cells per 25 cm^2 flask, or 10^6 cells per 75 cm^2 flask. Twenty-four hours later, they were transfected with 10 nmol/L of specific or control siRNA using OligofectAMINE (Invitrogen) as per the manufacturer's protocol. The sequences of siRNAs used are given in Supplementary Table S1.

Cell proliferation assay

Cell proliferation was measured using the CellTiter 96 AQueous One solution cell proliferation assay kit (Promega).

Cell viability assay

Cells were plated in 96-well plates and transfected with control or specific siRNA in triplicates. Viable cells were counted on the 1st and 2nd day posttransfection after trypan blue staining.

Flow cytometry

Cells were trypsinized, washed with PBS, and fixed overnight in 70% ethanol. Cells were then stained with propidium iodide (Sigma-Aldrich) and fluorescence was acquired using a BD FACS Flow Cytometer. Data was analyzed using WinMDI software available at http://pingu.salk.edu/software.html.

Real-time PCR

RNA was isolated from cells at appropriate time points by Trizol reagent (Sigma-Aldrich), DNase (MBI Fermentas) treated and quantified using NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). RNA (500–1,000 ng) was converted to cDNA using random decamers as primers and Moloney murine leukemia virus reverse transcriptase (MBI Fermentas). Real-time PCR was done on a RotorGene 6000 real-time PCR machine (Corbett Research, Australia).

Accurate quantitation of gene expression by real-time PCR is possible by averaging the geometric mean of multiple internal control genes and using them as reference (27). Therefore, seven reference genes, i.e., 18S, PPIA, POL2RA, B2M, GAPDH, β-actin, and tubulin were used. Relative quantitation was done using the Relative Expression Software Tool (28). The list of primers used for all experiments is given in Supplementary Table S2.

Western blot analysis

Cultured cells were washed with PBS and lysed with triple lysis buffer [50 mmol/L Tris–Cl (pH 7.4), 150 mmol/L NaCl, 0.02% sodium azide, 0.1% SDS, 1% NP40, and 0.5% sodium deoxycholate]. Cell lysates were centrifuged for 10 minutes and supernatants were collected. Equivalent amounts of protein were resolved on 5% to 12% SDS-PAGE gels and transferred to nitrocellulose membranes. The resulting blots were blocked for 1 hour at room temperature with 4% bovine serum albumin. Immunoblotting antibodies used were anti-actin (sc-8432), anti-GADD45α (sc-796), anti-p53 (sc-126; all from Santa Cruz Biotechnology), and anti-p21 and anti-PUMA (both from Cell Signaling Technology). Specific proteins were detected with the appropriate secondary antibodies labeled with alkaline phosphatase by using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium detection system (Promega).

DNA isolation

Genomic DNA from cultured cells was isolated using GenElute Mammalian genomic DNA Miniprep Kit (Sigma-Aldrich).

Bisulfite sequencing

Five hundred nanograms of genomic DNA was bisulfite-treated using EpiTect Bisulfite Kit (Qiagen). Bisulfite primers were designed from http://bisearch.enzim.hu/. Bisulfite primers were M13-tagged and sequencing was done using universal M13 primers. Sequencing was done by professional agencies using the dideoxy method.

Methylation-sensitive high-resolution melting

Methylation-sensitive high-resolution melting was done as described previously (29). The enhancer region of siHa cells is 0% methylated (0 of 2 copies methylated) and CaSki cells is 99.8% methylated (599 of 600 copies methylated). They were taken as 0% and 100% methylated controls, respectively. High-resolution melting was done from 65°C to 85°C and fluorescence was acquired every 0.2°C on a RotorGene 6000 machine. Normalization of melt profiles was done using the RotorGene software.

Restriction enzyme accessibility assay

Restriction enzyme accessibility assay was done as described earlier (30). Briefly cells were pelleted by centrifugation at 2,000 rpm at 4°C. Cells (10^5) were washed in ice-cold PBS and resuspended in 200 μL of cold NP40 lysis buffer [10 mmol/L Tris-Cl (pH 7.4), 10 mmol/L NaCl, 3 mmol/L $MgCl_2$, 0.5% NP40, 0.15 mmol/L spermine, and 0.5 mmol/L spermidine]. Cells were incubated on ice for 5 minutes and nuclei were pelleted down by centrifuging at 3,000 rpm for 5 minutes. Nuclei were resuspended in 200 μL of digestion buffer. One hundred microliters was kept as undigested and 100 μL was digested with 10 units of *Bsr*GI restriction enzyme for 30 minutes. DNA was isolated from undigested and

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digested samples using RBC Genomic DNA isolation kit (Real Genomics, Taiwan). The targeted LCR region was amplified on a RotorGene 6000 real-time PCR machine and the ratio of digested to undigested DNA was calculated using the ΔΔCt method.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay for dimethylated H3K9 tails was done using the H3K9 Methyl ChIP kit (Epigentek). Input DNA, dimethylated H3K9 antibody immunoprecipitated DNA and mouse IgG antibody immunoprecipitated DNA were amplified using primers specific for the targeted region in the LCR on a RotorGene 6000 real-time machine. The percentage of immunoprecipitation was calculated as described earlier (31). The centrosome of chromosome 16 has 100% methylated histone tails and the PCR primers amplifying this region were used as positive controls.

Statistical analyses

All real-time PCR experiments were repeated at least thrice and the expression ratios of the genes of interest in siRNA1 and control siRNA-treated cells were compared for statistical significance. In the survival and growth curves experiments, the percentage or number of cells in siRNA1 and control siRNA-treated samples were compared for statistical significance. The Western blots, methylation, and chromatin studies were repeated at least twice.

Paired *t* tests and Wilcoxon signed rank tests were used to calculate the significance in all experiments. Two-tailed *P* values were calculated and $P < 0.05$ was considered significant. The results of the parametric and nonparametric tests corroborated with each other and the *P* values mentioned in the text and figures as well as in the legends are those calculated by paired *t* tests.

The data has not been corrected for multiple comparisons. However, the results presented in the study are those which were significant over all biological and technical replicates.

Results

Screening of HPV-16 enhancer–targeted siRNAs identifies one siRNA causing a decrease in E6 and E7 expression

The NF-1 and activator protein-1 binding sites in the HPV-16 enhancer act as strong transcriptional activators of the E6 and E7 oncogenes. Hence, we decided to target the NF-1 binding site containing three CpG sites using siRNAs. Five siRNAs differing by a few bases and homologous to one of the NF-1 binding sites in the HPV-16 enhancer (Fig. 1A; Supplementary Table S1) were designed along with a control siRNA with a randomized sequence. The test and control siRNAs were confirmed to have no homology to any other human sequence according to National Center for Biotechnology Information BLAST software. The siRNAs also showed no significant miRNA-like targets or immunostimulatory motifs following analysis by the siRNA specificity server (32). These siRNAs were then synthesized by *in vitro* transcription and quantified.

siRNAs were screened using real-time PCR for E6 and E7 oncogene expression and cell proliferation assays after transfection into the HPV-16–positive siHa cell line. At a dose of 10 nmol/L, siRNA1 was found to decrease the E6 and E7 oncogene expression significantly ($P = 0.02$) by 60–80% in the siHa cell line at 48 hours post transfection (Fig. 1B). Also, there was a 50% to 60% reduction ($P = 0.003$) in cell proliferation at 24 hours posttransfection (Fig. 1C). However, other siRNAs targeting nearby regions showed no significant drop in the levels of E6 and E7 or cell proliferation.

Decrease in HPV-16 E6 and E7 expression by siRNA1 is specific to the HPV-16 enhancer

There was a significant decrease in E6 and E7 mRNA levels after siRNA1 treatment at 24, 48, and 72 hours when compared with that of control siRNA–treated cells at those time points (Fig. 2A). The slight increase in E6 and E7 expression at 72 hours when compared with 48 hours was found to be nonsignificant ($P = 0.35$). Similarly, there was a significant reduction in the E6 and E7 ($P < 0.001$ in both cases) mRNA levels in CaSki (HPV-16/18 positive) cells 48 hours after transfection with siRNA1 (Fig. 2B). However, siRNA1 caused no diminution in E6 or E7 levels in HPV-18–positive HeLa cells 48 hours posttransfection (Fig. 2C). These findings established the specificity of siRNA1 for the HPV-16 enhancer.

siRNA1 transfection in HPV-16–positive siHa cervical carcinoma cell line causes apoptosis

Transfection of siRNA1 caused apoptotic morphology in siHa and CaSki cells at 48 hours posttransfection (Supplementary Fig. S1), although no such changes were observed in HeLa cells (data not shown). siRNA1-transfected siHa cells showed reduced proliferation rate (Fig. 3A) when compared with control siRNA–treated cells as seen by cell counting after trypan blue staining ($P = 0.01$ at day 1 and $P = 0.04$ at day 2). Flow cytometry of siRNA1-treated and propidium iodide–stained siHa cells showed a 2-fold increase in the sub-G_1 fraction of cells when compared with control siRNA–treated cells ($P = 0.01$; Supplementary Fig. S2; Fig. 3B). siRNA1 caused a significant decrease in cell proliferation ($P < 0.05$ at all doses) in a dose-dependent manner (with 50% decrease at 12 nmol/L dose) in siHa cells as seen by a cell proliferation assay done at 24 hours posttransfection (Fig. 3C).

Downregulation of E6 and E7 expression by siRNA1 is related with increased expression of p53 targets and decrease in E2F1 targets in siHa and CaSki cell lines

Downregulation of the E6 protein was associated with activation of wild-type p53 pathway which was

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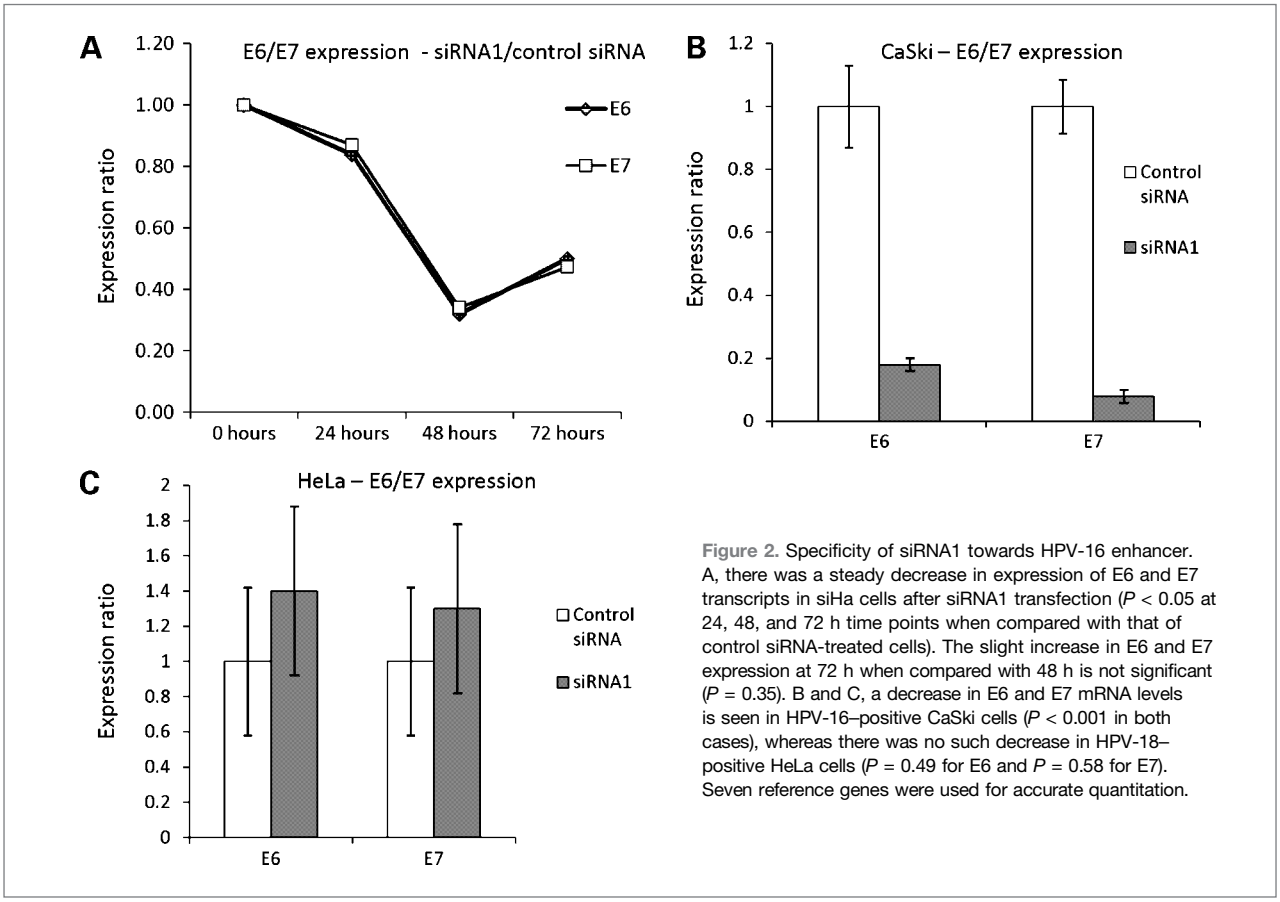

Figure 2. Specificity of siRNA1 towards HPV-16 enhancer. A, there was a steady decrease in expression of E6 and E7 transcripts in siHa cells after siRNA1 transfection ($P < 0.05$ at 24, 48, and 72 h time points when compared with that of control siRNA-treated cells). The slight increase in E6 and E7 expression at 72 h when compared with 48 h is not significant ($P = 0.35$). B and C, a decrease in E6 and E7 mRNA levels is seen in HPV-16–positive CaSki cells ($P < 0.001$ in both cases), whereas there was no such decrease in HPV-18–positive HeLa cells ($P = 0.49$ for E6 and $P = 0.58$ for E7). Seven reference genes were used for accurate quantitation.

seen by an increase in the mRNA expression levels of the p53 target gene, p21, by real-time PCR ($P < 0.001$) at 48 and 72 hours posttransfection in siHa cells (Fig. 4A). mRNA levels of other p53 target genes, i.e., CD95 ($P = 0.02$), Noxa ($P < 0.001$), and Puma ($P = 0.001$) also increased significantly at 24 hours posttransfection (Fig. 4B). This was corroborated by a Western blot, done 72 hours after siRNA1 transfection which showed a significant increase in the level of p53 protein and its downstream targets, i.e., p21, PUMA, and GADD45α ($P < 0.001$ in all cases; Supplementary Fig. S3). Similarly, downregulation of E7 was related to a significant decrease in the expression level of the E2F1-responsive gene cyclin A2 ($P < 0.001$). Another E2F1 candidate gene cyclin E showed a downward trend in expression.

Analysis of downstream genes in CaSki also showed a significant increase in expression levels of p53-dependent genes Puma ($P = 0.03$) and Noxa ($P = 0.04$), and a decrease in cyclin A2 levels ($P = 0.003$; Fig. 4C). However, there was no induction of p21 in CaSki cells which could be due to the presence of multiple copies of HPV-16 or due to the presence of HPV-18 sequences.

siRNA1 does not trigger IFN response

siRNAs could cause a nonspecific IFN response when transfected into eukaryotic cells (33). This is a type of innate antiviral response and involves the activation of the PKR/RNaseL pathway. This can be confirmed by a 50- to 500-fold induction of 2′,5′-oligoadenylate synthetase 1, a classic IFN response marker (34). There was no significant induction of 2′,5′-oligoadenylate synthetase 1 in siHa and CaSki cells after siRNA1 transfection indicating the absence of an IFN response (Fig. 4D).

siRNA1 causes TGS by H3K9 dimethylation and chromatin condensation and does not involve CpG methylation

The mechanism of TGS involves either DNA methylation or chromatin condensation of the targeted locus or blockage of the formation of a preinitiation complex (20–24, 35). Bisulfite treatment of genomic DNA followed by high-resolution melting analysis (Supplementary Fig. S4), as well as sequencing (Fig. 5A), showed no change in the methylation status of the targeted region after siRNA1 transfection, which is normally completely unmethylated.

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Restriction enzyme accessibility assay using *Bsr*GI, the restriction site of which is present inside the nucleosome Ne, was carried out on control and siRNA1-treated samples in siHa. A 4-fold decrease ($P < 0.001$) in accessibility of the locus in the siRNA1-treated samples (Supplementary Fig. S5A) implied chromatin condensation which is associated with the repression of transcription.

To further characterize the chromatin modification induced by siRNA1 targeting, ChIP assays were carried out using antimethylated H3K9 antibodies. Dimethylation of H3K9 histone tails is a silent histone mark associated with heterochromatization of and reduced gene expression from a locus (36). ChIP assays showed a 3.5-fold enrichment ($P < 0.001$) at the targeted enhancer in siRNA1-treated siHa cells (Supplementary Fig. S5B).

siRNA1 causes a decrease in enhancer-associated transcript levels

Transcription across promoters has recently been reported (37–39) and such RNAs have been named as promoter upstream transcripts. Although their exact function is not known, they are postulated to be a reservoir for RNA polymerase II molecules which might help in the rapid activation of downstream genes.

Some groups working on TGS have identified a low copy number promoter–associated sense transcript and have postulated that these transcripts guide the antisense strand of the promoter targeted siRNA to its target to cause epigenetic modifications and TGS (22, 24, 35). This is referred to as the RNA:RNA model of TGS (19).

We used primers specific to the targeted region of the enhancer to determine the presence of any enhancer-associated transcript and to correlate its levels with the TGS observed. A low copy number, enhancer-associated transcript was found in both siHa and CaSki cell lines. The ratio of the mRNA/enhancer-associated transcript level was around 1,000:1 as seen by real-time PCR (data not shown). After treatment by siRNA1, the levels of this enhancer-associated transcript decreased significantly, both in CaSki ($P = 0.04$) and siHa ($P = 0.03$) cell lines (Fig. 5B).

Discussion

TGS has the potential to cause long-term gene silencing because it involves epigenetic mechanisms which are heritable (24). It would be an ideal tool to use in the case of HPV-positive cervical cancers which have two transforming factors, E6 and E7, driven by a common enhancer.

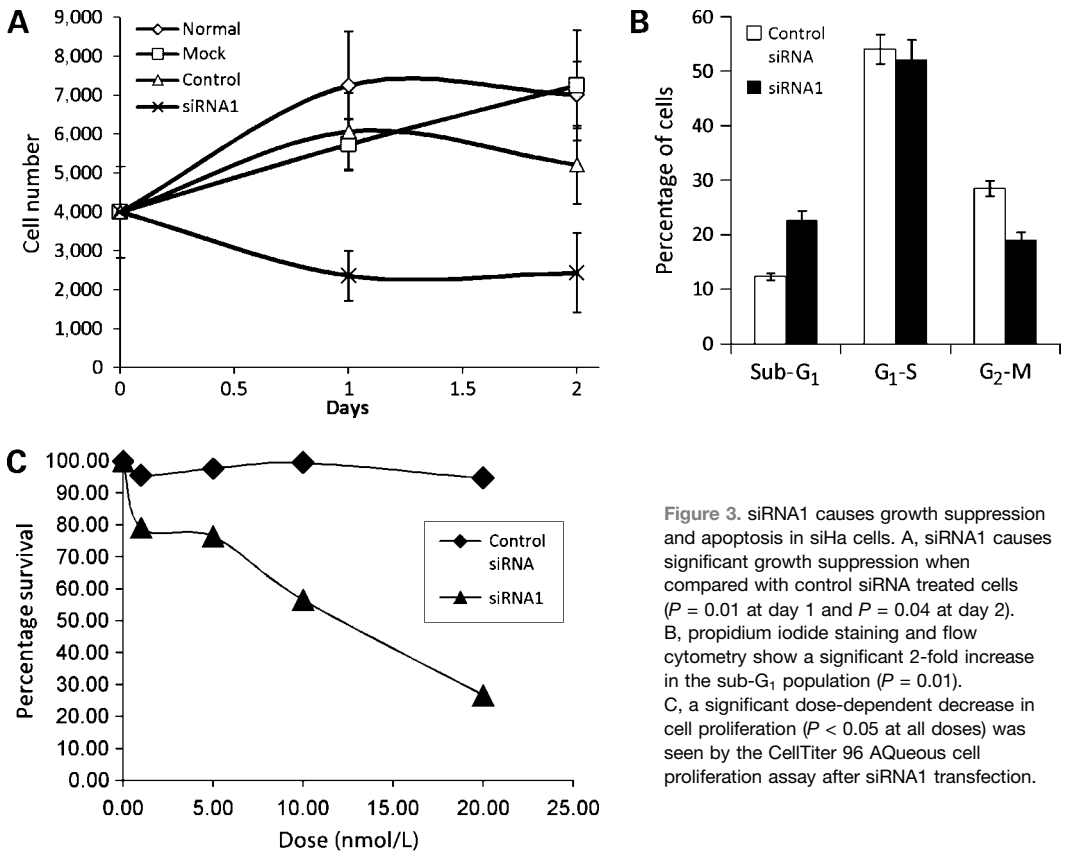

Figure 3. siRNA1 causes growth suppression and apoptosis in siHa cells. A, siRNA1 causes significant growth suppression when compared with control siRNA treated cells ($P = 0.01$ at day 1 and $P = 0.04$ at day 2). B, propidium iodide staining and flow cytometry show a significant 2-fold increase in the sub-G_1 population ($P = 0.01$). C, a significant dose-dependent decrease in cell proliferation ($P < 0.05$ at all doses) was seen by the CellTiter 96 AQueous cell proliferation assay after siRNA1 transfection.

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After screening and validation, we have identified one such siRNA which could cause simultaneous downregulation of both these oncogenes (Fig. 1B). It is specific to the HPV-16 enhancer and does not cause any nonspecific IFN response (Fig. 4D).

Loss of both transforming factors also caused the HPV-16–positive cells to undergo apoptosis, whereas there was no such morphologic change in HPV-18–positive HeLa cells. This is a result of the reactivation of p53 pathways as shown in this study (Fig. 4B and C). This phenomenon of TGS is highly sequence-specific, as other siRNAs differing by only a few bases upstream or downstream of the region failed to elicit similar levels of silencing (Fig. 1B).

siRNA1-mediated TGS was associated with an epigenetic modification, i.e., H3K9 dimethylation around the targeted region which led to chromatin condensation (Supplementary Fig. S5A and B). However, there was no DNA methylation (Fig. 5A) at the targeted region in the time period studied (72 hours). This is in conformation with previous work which suggests that histone modifications occur initially in TGS followed by DNA methylation (24).

Two models for the mechanism of TGS have been proposed, i.e., the RNA:DNA model and the RNA:RNA model (19). We identified a low copy number, enhancer-associated transcript similar to the promoter-associated transcripts identified by other researchers (22, 24, 35). The levels of this enhancer-associated transcript decreased significantly after TGS in our study, as it did in other studies (Fig. 5B). This finding substantiates the RNA:RNA model of TGS in siRNA-mediated HPV-16 LCR suppression.

Current research also suggests that promoter-associated transcripts are the result of the stalling of RNA polymerase II before the initiation of transcription and actively transcribed genes have a higher number of promoter-associated transcripts (37–39). Therefore, the

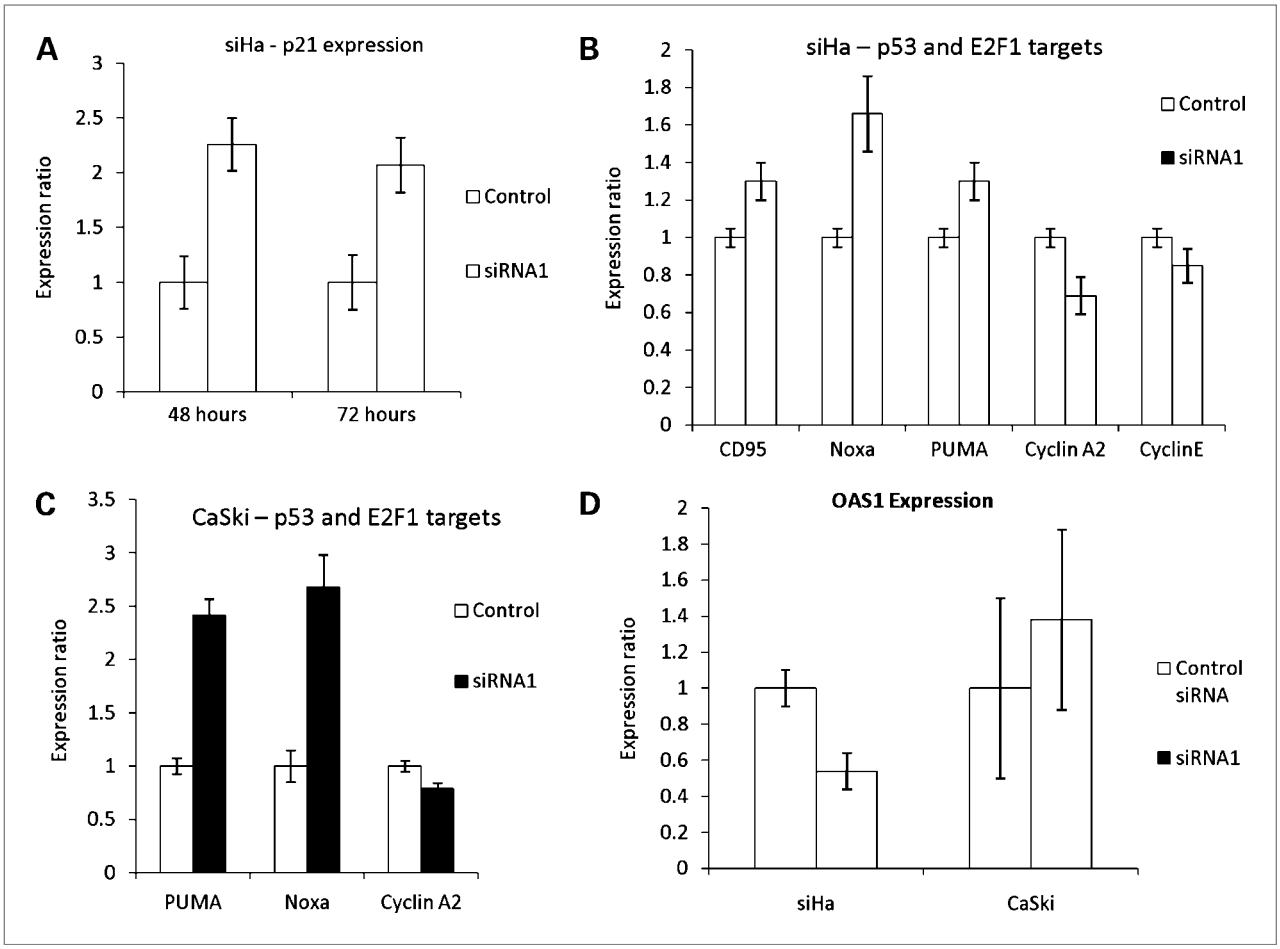

Figure 4. Effect of siRNA1 on downstream genes. A, p21 mRNA levels increase significantly ($P < 0.001$ at both time points) after siRNA1 transfection in siHa cells. B, there was a significant increase in expression levels of p53 target genes, i.e., CD95 ($P = 0.02$), Noxa ($P < 0.001$), and Puma ($P = 0.001$) in siHa cells. A decrease in the levels of E2F1 candidate genes, i.e., cyclin A2 ($P < 0.001$) and cyclin E, is also seen. C, transfection of siRNA1 causes an increase in the levels of PUMA ($P = 0.03$) and Noxa mRNA ($P = 0.04$) and a decrease in cyclin A2 levels ($P < 0.001$) in CaSki cells. D, no significant increase in 2′,5′-oligoadenylate synthetase 1 mRNA levels, an IFN response marker, was observed in siRNA1-treated siHa or CaSki cells.

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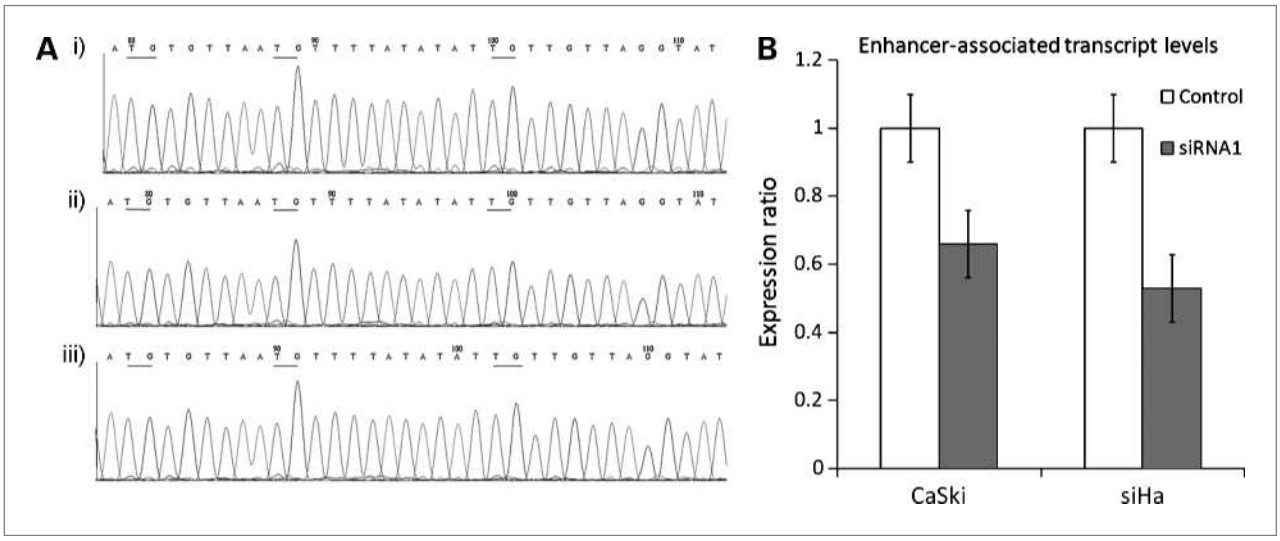

Figure 5. siRNA1 does not cause DNA methylation. A, bisulfite sequencing of targeted region in siHa after mock (i), control siRNA (ii), and siRNA1 transfection (iii) shows no change in DNA methylation. The CpG sites are underlined. B, identification of a low copy enhancer–associated transcript, the levels of which decrease significantly after siRNA1 transfection in CaSki (P = 0.04) and siHa (P = 0.03) cell lines.

decrease in enhancer-associated transcript levels could be due to the TGS itself which causes a decrease in E6/E7 gene expression.

In a recent study using siRNA targeted against the HPV-16 promoter in one of the cell lines, siHa, it was shown that such a treatment could inhibit E6 and E7 gene expression simultaneously in those cells (40). However, the targeted region was different and the dose used for suppression was 1,000-fold higher (10 μmol/L versus 10 nmol/L) than that used conventionally and also in our study. This might have lead to the induction of nonspecific responses in the reported study which were not looked at. Hence, the targets and doses of the siRNAs used in both studies are different, with that of ours being much more efficient. The reason for this may lie in the fact that the promoter of HPV-16 is much weaker than the enhancer in driving the expression of E6 and E7 oncogene expression (25). Therefore, it would be more desirable to target the HPV-16 enhancer, as in our study, to shut down the transcription of both oncogenes.

Thus, we have identified an siRNA which was confirmed to cause heterochromatin formation of the integrated HPV-16 enhancer loci integrated in the genomes of siHa and CaSki cell lines after transfection at a low dose of 10 nmol/L, leading to simultaneous downregulation of E6 and E7 oncogene expression. This leads to the reactivation of downstream tumor suppressor pathways (p53 and pRb), enhanced apoptosis, and decreased cell proliferation and might therefore be useful in HPV-16–positive cervical cancers as well as in other epithelial carcinomas. Such an approach also has a broader application in the silencing of other deleterious integrated viral genes. However, the effect of siRNA1 should be validated in animal models as well as in cancer cell lines with multiple active copies of HPV-16 integrated in the genome before considering it as a therapeutic modality in cervical cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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