Antiviral Research 98 (2013) 305–313

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Inhibition of cervical cancer cell growth in vitro and in vivo by lentiviral-vector mediated shRNA targeting the common promoter of HPV16 E6 and E7 oncogenes

Jiansong Zhou [a,c], Baohua Li [b], Chanjuan Peng [a,d], Fenfen Wang [b], Zhiqin Fu [a], Caiyun Zhou [a], Die Hong [b], Feng Ye [a], Weiguo Lü [a,b], Xing Xie [a,b,*]

[a] Women's Reproductive Health Laboratory of Zhejiang Province, China
[b] Department of Gynecologic Oncology, Women's Hospital, School of Medicine, Zhejiang University, Xueshi Rd#2, Hangzhou 310006, China
[c] Department of Gynecologic Oncology, Zhejiang Cancer Hospital, Banshan Bridge Guangji Rd#38, Hangzhou 310022, China
[d] Department of Ultrasonics, Zhejiang Cancer Hospital, Banshan Bridge Guangji Rd#38, Hangzhou 310022, China

ARTICLE INFO

Article history:
Received 27 September 2012
Revised 28 February 2013
Accepted 4 March 2013
Available online 21 March 2013

Keywords:
HPV
Cervical cancer
Monoclone
Promoter
Lentivirus

ABSTRACT

Deregulated expression of high-risk human papillomavirus oncogenes (E6 and E7) is a pivotal event for pathogenesis and progression in cervical cancer. Both viral oncogenes are therefore regarded as ideal therapeutic targets. Small interfering RNAs (siRNA) or double-stranded RNAs can knock down target genes effectively through siRNA-induced transcriptional gene silencing (TGS). Here, we established lentiviral-vector mediated shRNA (LV-shRNA) targeting common promoter of HPV16 E6/E7 and targeting E6 transcript, transduced the lentiviral construct into cervical HPV16-positive cell lines Siha and Caski, then selected and established stably transduced monoclonal cell lines. The results showed that LV-shRNA targeting promoter, as well as targeting E6 transcript, effectively knocked down E6 and E7 expression, resulted in accumulation of p53 and pRB protein and decrease of MCM7 and p16 protein, and consequently remarkably reduced the abilities of proliferation and invasiveness of cervical cancers cells in vitro. Then we inoculated subcutaneously those monoclonal cells into nude mice to establish the transplanted tumor animal models, and found dramatically inhibited tumorigenesis and growth, as well as prolonged survival time of mice incubated by cells with LV-shRNA targeting promoter and E6 transcript. Our results may provide evidence for application of LV-shRNA targeting HR-HPV key oncogenes, as a new treatment strategy, in cervical and other HPV-associated cancer therapy.

1. Introduction

Cervical cancer remains the third most common cancer in women worldwide (Jemal et al., 2011) and the leading malignancy in developing countries, accounting for 83% of whole cancer cases (Parkin et al., 2005). Although well organized screening and early therapeutic schedule have been carried out, the occurrence of invasive cervical cancer is still common, especially in developing areas. It is well known that persistent infection of high-risk human papillomavirus (HR-HPV) is a necessary causal event in cervical carcinogenesis. Among more than 200 hundred HPV genotypes (Fehrmann and Laimins, 2003), HPV-16 alone contributes to occurrence of more than 50% of the cervical cancer (Hong et al., 2008; Muñoz et al., 2003). Accumulated evidences have clearly proven that deregulated expression of two high-risk viral oncogenes, E6 and E7, is essential for malignant transformation and pivotal for maintenance of the malignant phenotype of cervical cancer cells (zur Hausen, 2000; Munger et al., 2004) by interacting with and eliminating key tumor-suppressive protein p53 and pRB in cell cycle checkpoint, respectively (DeFilippis et al., 2003). Since HR-HPV E6 and E7 play indispensible roles in tumorogenesis and progression of cervical cancer and exogenous viral gene has no striking homology to human genome, both viral oncogenes are regarded as the ideal targets for a novel block therapy for cervical cancers.

Among diverse techniques for gene silencing, RNA interference (RNAi) has been proved to be a powerful tool to inhibit specific gene expression, and holds a great promise for the treatment of viral diseases, genetic disorders, and cancers (Gu et al., 2006). Various in vitro and in vivo studies (Chang et al., 2010; Jonson et al., 2008) showed a potential application of HPV16-siRNAs targeting directly E6 or E7 gene for blocking the development of cervical cancers

Abbreviations: RNAi, RNA interference; siRNA, small interfering RNA; HPV, human papillomavirus.

* Corresponding author. Address: Department of Gynecologic Oncology, Women's Reproductive Health Key Laboratory of Zhejiang Province, Women's Hospital, School of Medicine, Zhejiang University, Xueshi Rd. no. 2, Hangzhou 310006, China. Tel.: +86 57187061501x1001.
E-mail address: xiex@mail.hz.zj.cn (X. Xie).

0166-3542/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.antiviral.2013.03.010

through apoptosis inducement and growth inhibition at the post-transcriptional level (PTGS). Recently, several studies demonstrated that siRNA was also able to knock down target genes effectively through siRNA-induced transcriptional gene silencing (TGS) both in plants and in mammalian cells (Morris et al., 2004; Ting et al., 2005; Mette et al., 2000), which offers an additional way, other than PTGS, to silence target genes. The involved mechanisms of siRNA TGS include epigenetic alterations, such as DNA methylation, histone modification, and chromatin conformation. Moreover, such epigenetic alterations are regarded to be heritable from the siRNA-transfected cells to the cells not transfected with siRNA (Richards, 2006; Jones et al., 2001), which can amplify the efficiency of siRNA.

HPV16 E6 and E7 expression is tightly controlled by a unique and common promoter (P97) that is located immediately upstream of the E6 gene (Smotkin and Wettstein, 1986). We previously found that siRNA targeting P97 via transient transfection definitely knocked down HPV16 E6/E7 in vitro and in vivo and first demonstrated that a promoter-targeting siRNA enabled to induce effectively an extraneous viral gene silencing via TGS (Hong et al., 2009). It is known that lentiviral vector (LV) possesses multiple advantages, such as high efficiency of infection to different cell types, relatively large packaging capacity, and stable transduction that is rather suitable for application in cancer therapy, compared with synthetic and vector-borne siRNA and other virus carriers. LV as a good gene delivery system has been explored for preclinical or clinical application and shown promising results (Humeau et al., 2004; Scherr and Eder, 2002). Here, we established LV-mediated shRNA targeting common promoter of HPV16 E6/E7 and targeting E6 transcript, transduced the lentiviral construct into cervical HPV16-positive cell lines Siha and Caski, selected and established stably transduced monoclonal cell lines, then inoculated subcutaneously those monoclonal cells into nude mice to established transplanted tumor animal models. We eventually observed the influences of LV-shRNA targeting common promoter of HPV16 E6 and E7, as well as targeting E6 transcript, on inhibition of proliferation and invasiveness of cervical cancer cells in vitro and retardation of transplanted tumor growth along with prolongation of survival time of mice with xenograft, to search a new therapy strategy for cervical and other HPV-associated cancers.

2. Materials and methods

2.1. Cell culture and grouping

The human cervical carcinoma cell lines Caski, Siha, HeLa, and C33A (purchased from American Type Culture Collection, USA) were cultured at 37 °C and 5% CO2 in RPMI 1640 (GIBCO, invitrogen) supplemented with 10% (vol/vol) FBS and 1% penicillin/streptomycin. SiHa is an HPV16-positive cell line and contains about one to two copies of integrated HPV16 genome. Caski is also an HPV16-positive cell line which contains about 600 copies. HPV18-positive cell line Hela and HPV-negative cell line C33A were used as control cell lines.

Five groups were included in the experiments: the LV-shRNA targeting promoter and E6 transcript were applied to the experimental group, two related scrambled LV-shRNAs were used as negative controls, and the cells without LV-shRNA transfection were used as the blank controls.

2.2. Production of lentivirus expressing shRNA

Lentivirus production was done using the BLOCK-iT™ Lentiviral RNAi Expression System (Invitrogen). Briefly, we first designed and synthesized complementary DNA oligos (Invitrogen), each containing four nucleotide overhangs necessary for directional cloning. Complementary sequences of shRNA corresponding to promoter and E6 transcript siRNA were described in Supplementary Fig. 1. E6 transcript sequence was described by Yamato (Yamato et al., 2008). After annealing to generate double-stranded oligos, they were connected into the pENTER/U6 vector (Invitrogen), then took the sequenced and purified entry clones encoding promoter and E6 shRNA connected to plenti6/BLOCK-iT™-DEST using an LR recombination reaction to generate the plenti6/BLOCK-iT™-DEST expression construct. Lentivirus was produced by co-transfecting 9 μg of the ViraPower™ Packaging Mix and 3 μg of plenti6/BLOCK-iT-DEST expression plasmid DNA into the 293FT Producer Cell Line using Lipofectamine 2000 (Invitrogen), and then harvested and titered by HT1080 cell line. The lentiviral stock titer was 2.2×10^6 TU/ml.

2.3. Transduction and monoclonal cell selection

The optimal MOI for Caski and Siha cell lines was obtained by determining the expression of laminA/C (BD Biosciences) using western blotting after plenti6-GW/U6-lamin^{shRNA} lentiviral construct transduction. Stably transduced cervical carcinoma cells with the suitable MOI were separately selected by placing cells under blasticidin presence. The stably transduced monoclonal cells were chosen by infinite dilution method as follows: LV stably transduced cells were trypsinated, numbered, and 130 cells were added into 6.5 ml of complete medium, then 100 μl of cell suspension per well were seeded in the first three rows of the 96-well plates. Then 2.9 ml of complete medium were added into the remain suspension, 100 μl of cell suspension per well were seeded in the next three rows. Finally, 2.2 ml of complete medium were added into the remain suspension, in the same way, 100 μl of cell suspension per well were seeded in the remaining two rows of the 96-well plates.

2.4. Analysis of E6 and E7 mRNA levels by real-time quantitative RT-PCR

The stably transduced Caski and Siha monoclonal cells, as well as HeLa and C33A non-monoclonal cells, were seeded in each well of the 6-well plates. Total RNA was extracted with Trizol reagent (Invitrogen), then 1 μg of RNA was reverse-transcribed and amplified by real-time PCR (Takara) to detect E6/E7 mRNA expression. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference. The PCR primers were E6 forward, 5′-GAGCGACCCAGAAAGTTACCA-3′; and reverse, 5′-AAATCCCGAAAAGCAAAGTCA -3′; E7 forward, 5′-CATGGAGATACACCTACATTGC-3′; and reverse, 5′-CACAACCGAAGCGTAGAGTC-3′; GAPDH forward, 5′-GAAGGTGAAGGTCGGAGTC-3′; and reverse, 5′-GAAGATGGTGATGGGATTTC-3′.

2.5. Detection of E6, MCM7, p53, E7, pRB, and p16 protein expression

Protein extracts for each group of stably transduced Caski and Siha monoclonal cells were prepared, blotting was performed as described previously (Sunaga et al., 2004). Primary antibodies used were mouse monoclonal anti-E6 (CHEMICON), rabbit monoclonal anti-MCM7 (Epitomics), mouse monoclonal anti-p53 (Santa Cruz Biotechnologies), mouse monoclonal anti-E7 (Santa Cruz Biotechnologies), goat polyclonal anti-pRB (Santa Cruz Biotechnologies), and mouse monoclonal anti-p16 (Santa Cruz Biotechnologies). The GAPDH (Santa Cruz Biotechnologies) was used as internal control for protein loading and analysis.

2.6. Cell viability assay

The stably transduced Caski and Siha monoclonal cells and stably transduced HeLa and C33A cells were trypsinated and then seeded into the 96-well plates. Growth of each cell line was evaluated by using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT, Amresco) assay. Briefly, cells were incubated with 20 μl of MTT (5 mg/ml) at 37 °C for 4 h at different time points. After the medium containing MTT was aspirated, the formazan crystals were dissolved in 150 μL of dimethyl sulfoxide (DMSO, Sigma). The absorbance was recorded using a Universal Microplate Reader at a wavelength of 490 nm.

2.7. Apoptosis assay

Apoptosis was quantified by flow cytometric analysis of Annexin V-FITC (BD Pharmingen) staining in combination with propidium iodide (PI). Annexin V (+) and PI (−) cells were considered to be in early apoptosis and the percentage of this kind of cells was calculated.

2.8. Cell invasion assay

The invasiveness of LV-shRNA transduced cells in vitro was measured using transwell insert. Briefly, the 24-well transwell inserts (pore size, 8 μm; Costar, Cambridge, MA) were coated with 150 μl of Matrigel (BD Biosciences, Franklin Lakes, NJ) with a dilution rate of 1:6 and dried at 37 °C for 30 min. After removing the solution, 8×10^4 cells, suspended in 200 μl of Opti-MEM® I Reduced Serum Medium (Invitrogen), were placed in the upper compartment of Cell Culture Insert. And 500 μl of RPMI 1640 supplemented with 10% FBS were added to the lower compartment of Cell Culture Insert. After 48 h incubation at 37 °C in 5% CO_2, cells on the upper surface of the transwell insert were completely removed by wiping with a cotton swab. The invaded cells that remained on the lower surface of the filter were fixed and stained with 1% crystal violet (Sigma) solution and 10% ethanol. The stained cells in five randomly selected microscopic fields per filter were counted.

2.9. Mouse tumor model studies

To investigate the effects of LV-shRNA targetting the promoter and E6 transcript on the tumorigenicity of xenograft and the influence on survival of tumor-burdened animals, ninety female BALB/c-nu mice, aged 4–6 weeks, were purchased from Shanghai Laboratory Animal Center (slac, Shanghai, China) and housed within a dedicated SPF facility at Laboratory Animal Center of Zhejiang University. They were subcutaneously inoculated with the stably transduced Caski (8×10^6) and Siha (1×10^7) monoclonal cells, respectively. Mice were divided into four experiment groups (ten mice/group), and one blank control group (five mice/group), in which the same volume of PBS was subcutaneously injected.

Tumor volume was continuously blindly measured by periodic caliper every 3–4 days for Caski groups and one week for Siha group. Some of the mice were sacrificed under anesthesia and each tumor was excised and weighed. Parts of each tumor tissue were wax embedded, sliced up for 4 μm thick, and H&E stained. The remaining mice were used for further study to obtain survival time data.

2.10. Statistical analysis

All experiments were performed at least in triplicate and results were recorded. The SPSS16.0 software package was used for statistical analysis. Data were presented as means plus standard deviation, Student's *t* test was used to analyze the significance between groups. Statistical significance was determined at $P < 0.05$ level.

3. Results

3.1. Efficiencies of E6/E7 mRNA knockdown by LV-shRNA targeting the promoter and the E6 transcript

After Siha and Caski cells were transduced with LV-shRNA at optimal MOI (2 for Siha and 5 for Caski) separately, then using infinite dilution method, the stably transduced monoclonal cells transduced by promoter-targeting and E6 targeting LV-shRNA were obtained. Real-time quantitative RT-PCR detection showed that E6 and E7 mRNA expression in SiHa cells transduced by the promoter-targeting LV-shRNA were reduced by 94.72% and 92.51% compared with the scramble control, respectively. Meanwhile, E6 and E7 mRNA expression in SiHa cells transduced by the E6-targeting LV-shRNA were reduced by 92.01% and 90.74% compared with the corresponding scramble control, respectively. Similarly, E6 and E7 mRNA expression in Caski cells transduced by the promoter-targeting LV-shRNA were reduced by 86.02% and 69.82% compared with the relevant scramble control, respectively. E6 and E7 mRNA expression in Caski cells transduced by the E6-targeting LV-shRNA were reduced by 76.41% and 66.93% compared with the related scramble control, respectively (Supplementary Fig. 2).

3.2. Effects of LV-shRNA targeting the promoter and the E6 transcript on the expressions of E6/E7 and related proteins

As shown in Fig.1, levels of both p53 and pRB proteins were increased in Siha cells transduced by the promoter-targeting LV-shRNA ($p = 0.046$ and 0.012, respectively) and by the E6 transcript-targeting LV-shRNA ($p = 0.021$ and 0.035, respectively) compared with the related scramble control. While the levels of E6, E7, MCM7, and p16 protein were decreased in Siha cells transduced by the promoter-targeting LV-shRNA ($p = 0.026$, 0.045, 0.007, and 0.023, respectively) and by the E6-transcript targeting LV-shRNA ($p = 0.028$, 6.10E-04, 0.021, and 0.012, respectively). Similarly, levels of both p53 and pRB protein were upregulated in Caski cells transduced by the promoter-targeting LV-shRNA ($p = 7.58$E-04 and 0.0059, respectively) and by the E6-transcript targeting LV-shRNA ($p = 0.026$ and 0.001, respectively) compared with the relevant scramble control. While the levels of E6, E7, MCM7, and p16 proteins were downregulated in Caski cells transduced by the promoter-targeting LV-shRNA ($p = 0.013$, 0.029, 0.0017, and 3.79E-04, respectively) and by the E6-transcript targeting LV-shRNA ($p = 0.0056$, 0.0081, 0.0043, and 2.39E-04, respectively). (Fig. 2).

3.3. The proliferation and apoptosis of cells transduced by the promoter-targeting and E6-transcript targeting LV-shRNAs

After 72 h of culture, the cellular proliferation was reduced by 52.6% in SiHa cells transduced by the promoter-targeting LV-shRNA and 50.2% in cells transduced by the E6-transcript targeting LV-shRNA compared with the relevant scramble control (Fig. 3A). At the same time, the cellular proliferation was reduced by 36.85% in Caski cells transduced by the promoter-targeting LV-shRNA and 31.21% in transduced by the E6-transcript targeting LV-shRNA compared with the relevant scramble control. No significant differences existed between the scramble and the blank control groups in both SiHa and Caski cells (Fig. 3B). In addition, the cellular proliferation also did not show any significant differences in C33A (Fig. 3C) and HeLa (Fig. 3D) cells transduced with and without LV-shRNA.

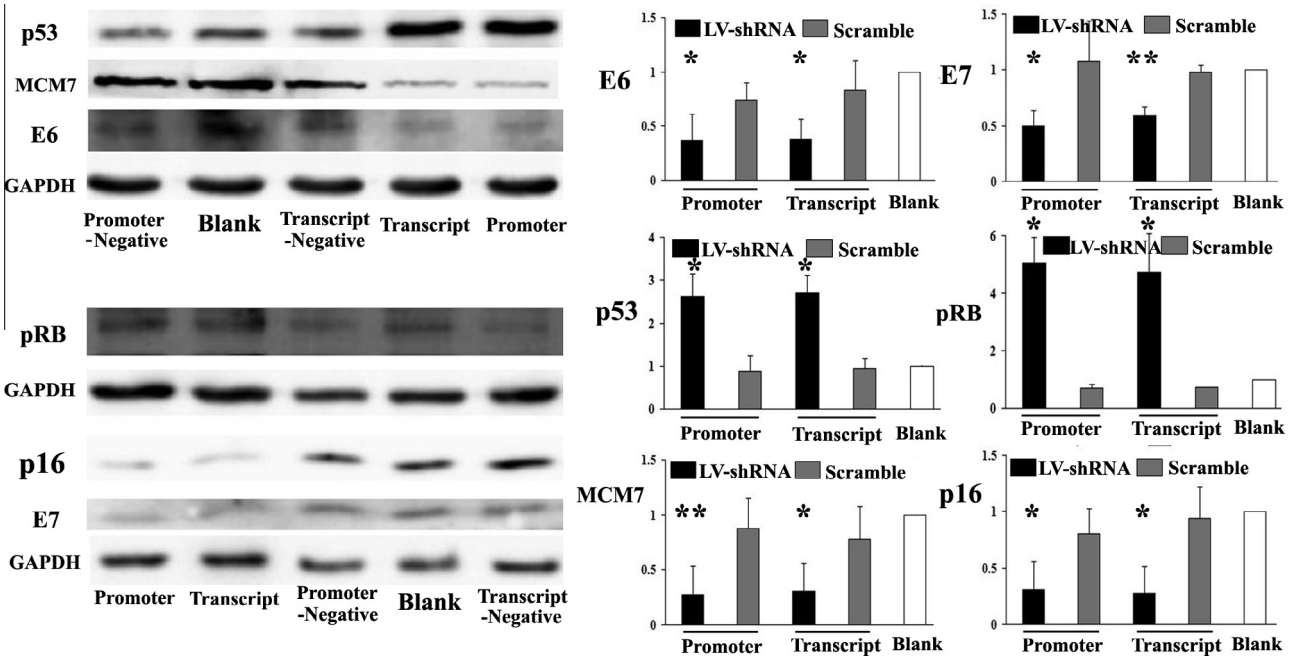

Fig. 1. Effects of the HPV16 promoter and E6-transcript targeting LV-shRNA on E6, E7 and related protein expression in Siha cells, respectively, compared with relevant scramble LV-shRNA. The extracts from stably transduced Siha monoclonal cells were analyzed by Western blotting. Increased expressions of p53 and pRB protein, and decreased expressions of E6, E7, MCM7, and p16 protein were observed in Siha monoclonal cells transduced by the LV-shRNAs that target HPV16 promoter and E6 transcript. $^{*}p < 0.05$, $^{**}p < 0.01$.

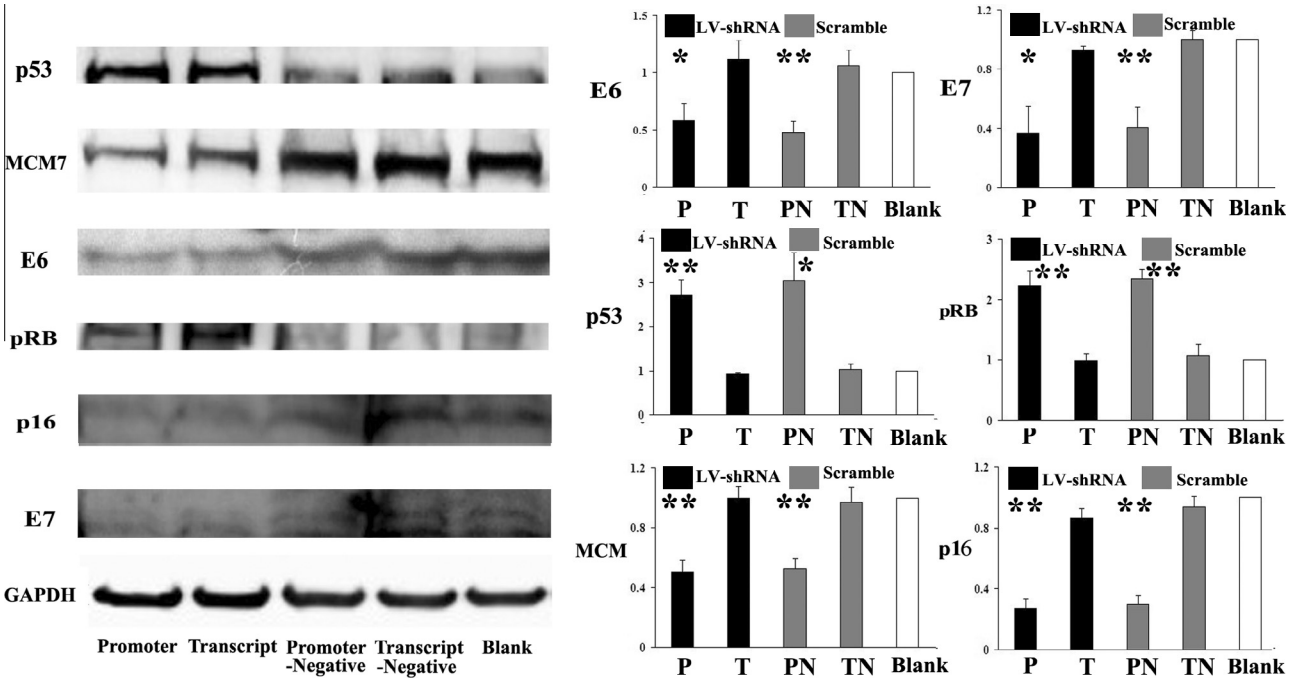

Fig. 2. Effects of the HPV16 promoter and E6-transcript targeting LV-shRNA on E6, E7 and related protein expression in Caski cells, respectively, compared with relevant scramble LV-shRNA. The extracts from stably transduced Caski monoclonal cells were analyzed by Western blotting. Increased expressions of p53 and pRB protein, and decreased expressions of E6, E7, MCM7, and p16 protein were observed in Caski monoclonal cells transduced by the LV-shRNAs that target HPV16 promoter and E6 transcript. $^{*}p < 0.05$, $^{**}p < 0.01$.

However, the apoptotic rates did not present any significant changes in Caski cells transduced by the promoter-targeting LV-shRNA compared with the relevant scramble control both in 48 h (12.23 ± 1.05 vs 10.52 ± 0.58 $p = 0.068$) and 72 h (16.55 ± 1.48 vs 14.68 ± 2.78 $p = 0.362$).

3.4. The invasive potential of cells transduced by LV-shRNA targeting the promoter and E6 transcript

The invasive capability of Siha and Caski cells transduced by the promoter-targeting and E6 targeting LV-shRNAs was assessed

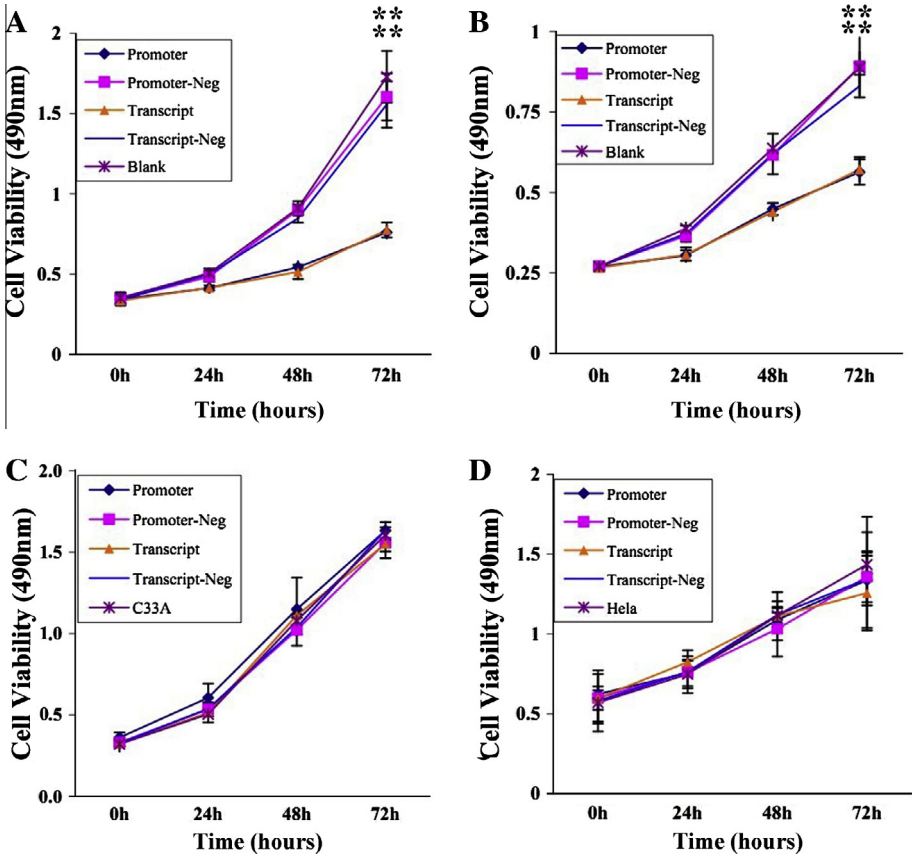

Fig. 3. Effects of the HPV16 promoter and E6 transcript targeting LV-shRNA on cellular proliferation. (A and B) MTT assay showed that proliferation was inhibited in Siha and CaSki monoclonal cells stably transduced by LV-shRNA targeting HPV16 promoter and E6 transcript, respectively, compared with the related scramble LV-shRNA, but no similar differences were observed in HPV-negative C33A (C) and HPV18-positive Hela cells (D). $^{*}p < 0.05$, $^{**}p < 0.01$.

using transwell insert in vitro. As shown in Fig. 4A, many of Siha cells untransduced by the promoter and E6 transcript scramble targeting LV-shRNA could invade through basement membrane easily (236.67 ± 23.86, 227.67 ± 13.65, and 231.33 ± 22.01 cells per field, respectively), However, only a few of Siha cells transducted by the promoter-targeting or the E6-transcript targeting LV-shRNA could migrate through basement membrane at 48 h (63.67 ± 8.08 and 73.67 ± 12.34 cells per field, respectively). The similar phenomenon was observed in Caski cells (Fig. 4B).

3.5. Influences of the promoter and E6 transcript targeting LV-shRNA on growth of the transplanted tumors and survival of mice

All nude mice were alive after inoculation with monoclonal cells and presented 100% tumorigenesis. The H&E staining of xenograft inoculated with Siha and Caski cells transducted by different LV-shRNAs and control cells showed the appearance of typical cervical carcinomas with allotypic nuclear division and tumor giant cells (Fig. 5). The growth of transplanted tumors in mice inoculated by Siha cells with targeting promoter and targeting E6 transcript LV-shRNA was decreased by average 66.88% and 68.4% at day 28, and average 81.36% and 77.87% at day 70, compared to the related scramble controls (Fig. 6)B1. Weights of excised tumor inoculated by Siha cells with the promoter and E6-transcript targeting scramble LV-shRNA were 7.29-fold and 9.11-fold heavier than those inoculated by cells transduced with LV-shRNA targeting the promoter and E6 transcript (Fig. 6C1). Similar situation was observed in Caski cells (Fig. 6B2, 6C2). In addition, survival time was significantly prolonged in mice inoculated with Siha cells ($p = 0.008$ and $p = 0.007$) and Caski cells ($p = 0.01$ and $p = 0.01$) with targeting promoter and targeting E6 transcript LV-shRNA compared with the related scramble groups (Supplementary Fig. 3).

4. Discussion

Invasive cervical cancer is still a common malignancy in women though well organized screening has been expanded. Clinical data show that the prognosis of cervical cancer patients remains unoptimistic up to date, with a 5-year survival rate of 9.3–65.8% in those with advanced stage diseases (Quinn et al., 2006), despite the progress of surgery, radiation, and chemotherapy. The development of novel therapeutic strategies is urgently required. It is known that overexpression of HR-HPV E6 and E7 oncogene is essential for malignant transformation and the maintenance of the malignant phenotype of cervical cancer cells. As well-characterized targets, p53 and retinoblastoma protein are directly inactivated by E6 and E7 respectively, consequently, leading to a series of signaling pathway dysfunction. Additionally, recent studies showed that the alteration of additional pathways was equally important for malignant transformation and maintenance in cervical cancer. These additional factors include crucial regulators of cell cycle progression, telomere maintenance, apoptosis as well as chromosomal stability (Moody and Laimins, 2010). Thus, both viral proteins, E6 and E7, are believed as therapeutic targets in blocking cervical cancer progress through proliferation inhibition and death inducement of tumor cells. Various studies have shown that E6 and E7 expression can be effectively silenced by RNAi directly targeting E6 or E7 transcript, which consequently reduces cell growth and colony formation, induces cell senescence and/or apoptosis, improves chemotherapeutic cytoxicity to cervical cancer cells

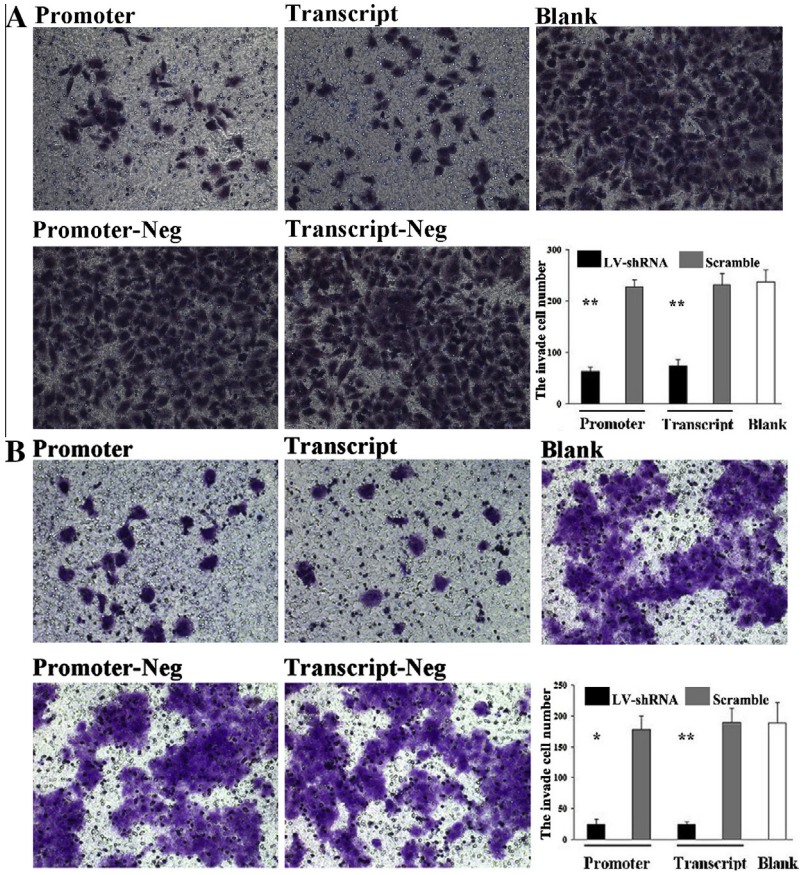

Fig. 4. Effects of HPV16 promoter and E6 transcript targeting LV-shRNA on the invasiveness of SiHa and Caski cells (×400). The invasive potential were suppressed in Siha (A) and Caski (B) monoclonal cells stably transduced by LV-shRNA targeting HPV16 promoter and E6 transcript, respectively, compared with related scramble LV-shRNA (mean ± sd).

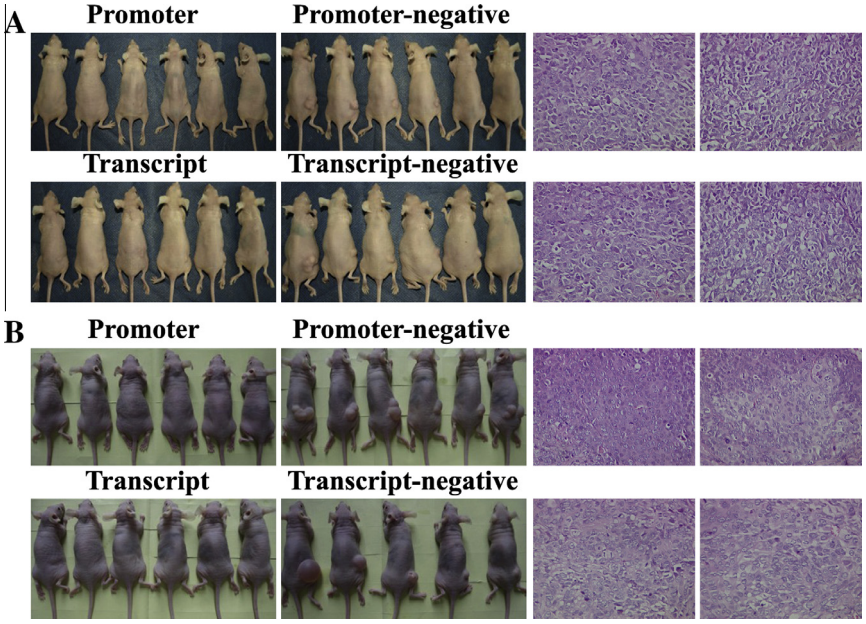

Fig. 5. Photographs of mice and the H&E staining of excised xenografts from BALB/c-nu mice. SiHa (A) and Caski (B) cells were tranduced in vitro by LV-shRNA targeting the HPV16 promoter and E6 transcript and related scrambles. And the H&E staining of xenograft tissues showed the appearance of typical cervical carcinomas with allotypic nuclear division and tumor giant cells (×400).

in vitro, promotes xenograft retardation, and even results in complete loss of tumor growth in tumor-burdened animal models (Gu et al., 2006; Chang et al., 2010; Liu et al., 2009; Courtete et al., 2007). However, there still are disadvantages for directly targeting viral oncogene transcript, such as various virus intragenotype variants and the off-target effect.

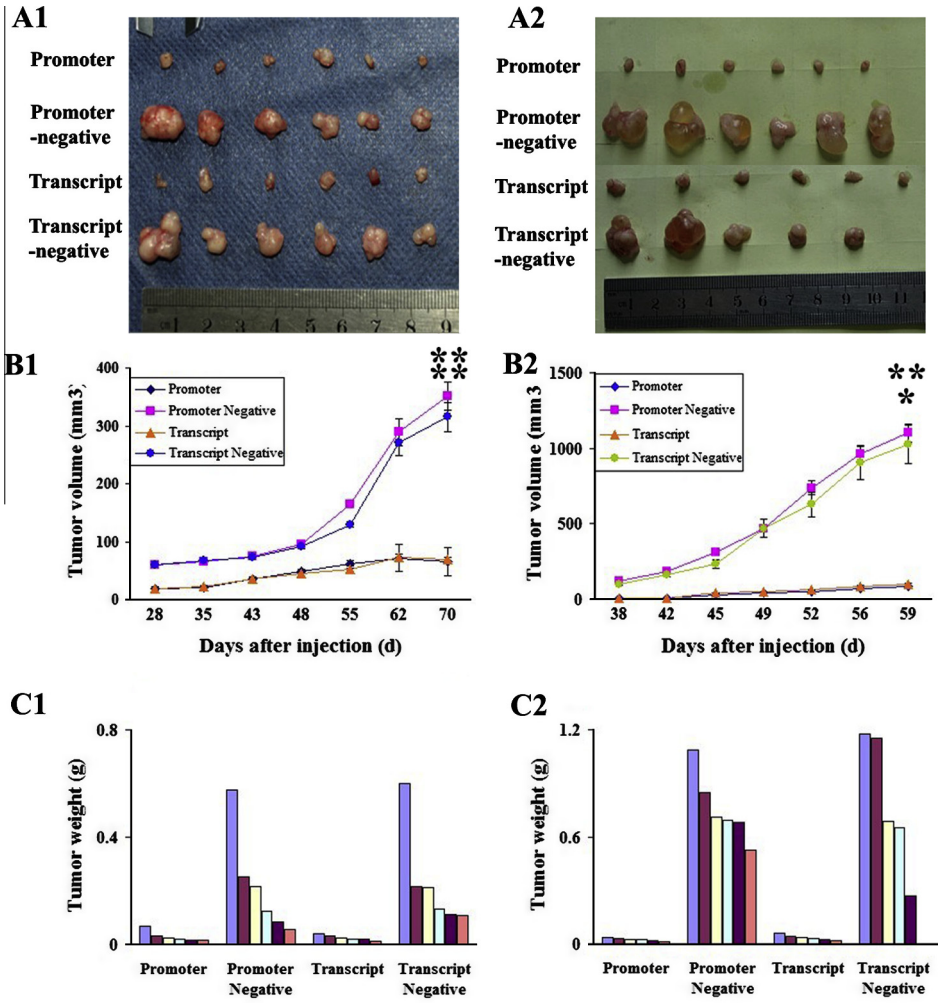

Fig. 6. Inhibition of transplanted tumor growth in BALB/c-nu mice inoculated by Siha and Caski cells with LV-shRNA targeting the HPV16 promoter and E6 transcript. (A1, A2), Photographs of excised xenografts from inoculated mice. (B1, B2), comparison among the volumes of tumors inoculated by Siha and Caski cells treated with different LV-shRNAs. (C1, C2), comparison among the weights of excised tumors inoculated by Siha and Caski cells treated with different LV-shRNAs.

It has been recognized that HPV16 expresses E6 and E7 proteins from a single bicistronic mRNA, but still little is known about how each of both viral proteins are translated from a native mRNA. It seems quite thorny that we even don't know in most cases which transcripts are true mRNAs used for protein translation during papillomavirus infection. The principle that one gene encodes one mRNA with one ORF for translation of one protein does not apply to any genotypes of papillomavirus. So alternative RNA splicing plays a critical role in determining which ORFs will be translated from these bicistronic transcripts (Zheng and Baker, 2006) , and a specific siRNA for E6 or E7 alone could not be used. Fortunately, the HPV-16 genome contains two major promoters. The P97 promoter is located upstream of the E6 ORF and is responsible for almost all early gene expression. The P670 promoter is located within the E7 ORF region and is responsible for late gene expression. Thus, P97 is the only promoter for E6 and E7 transcription and the RNAi targeting the promoter P97 may result in abolition of both E6 and E7 protein. We have confirmed in the previous studies that transiently transfected siRNA targeting P97 effectively knocks down HPV16 E6/E7 expression and consequently induces growth inhibition of cervical cancer cells in vitro and transpalanted tumors in vivo (Hong et al., 2009; Zhou et al., 2012). Considering the limitation of transiently tranfected siRNA, here we used LV-shRNA targeting the common promoter of HPV16 E6 and E7, as well as targeting E6 transcript. It was found that E6 and E7 mRNA and protein expression were effectively reduced in HPV16-positive Siha and Caski monoclonal cells that were stably transduced by promoter-targeting and E6 targeting LV-shRNA, leading to accumulation of p53 and pRB, and reduction of MCM7 and p16 proteins, and eventually resulting in significant decreased proliferating and invasive ability in cervical cancer cells. Our findings suggest that LV-shRNA specific to HR-HPV oncogenes enables to effectively decrease the malignancy of cervical cancer cells. Moreover, we observed that such inhibition of E6/E7 mRNA expression and cell growth by LV-shRNA did not appear in stably transduced HPV18-positive and HPV-negative cells. Thus, the suppressive effect induced by LV-shRNA is HPV genotype-specific. Although such specific blocking may have more effectiveness, it would be needed to design a LV-shRNA targeting multiple genotypes before this technique is clinically applied.

In addition, our previous studies showed that transiently transfected siRNA induced more obvious apoptosis in cervical cancer cells, especially in transplanted tumors (Zhou et al., 2012). In the present study, however, we did not found such a difference in apoptosis between LV-shRNA transfected cells and the controls in vitro. The reason is not completely clear, but may be related

with the LV-shRNA stably transduced monoclonal cells that were selected under blasticidin presence and chosen by infinite dilution method, and cultured for one month before monoclone formed. Thus, it is probably difficult to induce apoptosis by shRNA in such monoclonal cells that may possess a potential resistance to apoptotic inducement.

To explore further the therapeutic potential of LV-shRNA, we determined the suppressive effect of LV-shRNA on tumorigenesis and xenograft growth in mouse models. Our results showed that both the promoter-targeting and E6 transcript-targeting LV-shRNA effectively inhibited the formation and growth of transplanted tumors inoculated with LV-shRNA stably transduced Siha and Caski monoclonal cells. Furthermore, we observed the survival time in tumor burdened mice inoculated by the promoter and E6 transcript targeting LV-shRNA was much longer than those inoculated by related scramble LV-shRNAs, suggesting that the promoter-targeting LV-shRNA, like the E6 transcript-targeting one, possesses the therapeutic potential for cervical cancer patients.

Lentiviral vector has been shown as a vehicle for stable and durable gene therapy in preclinical treatment and clinical trials (Humeau et al., 2004; Scherr and Eder, 2002; Check, 2005). However, nonspecific integration can be problematic because of the possibility of undesirable events such as insertional mutagenesis (Kohn et al., 2003; Lewinski et al., 2005). Several studies proposed to use integration-defective lentiviral vector (IDLV) instead of normal LV (Karwacz et al., 2009; Yáñez-Muñoz et al., 2006; Negri et al., 2010). But, the biggest disadvantage for such IDLV is that virus only persists as circular episomes. That is, these episomes may be lost and transfected shRNA no longer functions during target cell division. Thus, to search more available viral vectors for gene therapy in cancers seems still to be an arduous task before LV-shRNA enables to be efficiently and safely provided in a clinical setting.

6. Conclusion

Taken together, LV-shRNA specific to HPV16 oncogenes including targeting the promoter and the E6-transcript effectively knocks down E6 and E7 expression, along with accumulation of p53 and pRB protein and decrease of MCM7 and p16 protein, and results in remarkably reduced abilities of proliferation and invasiveness of cervical cancers cells in vitro. Consequently, tumorigenesis and growth of transplanted tumors in mouse models were effectively suppressed and survival of tumor burdened mice was prolonged. Our findings may provide important evidences for application of LV-shRNA targeting HR-HPV key oncogenes, as a new treatment strategy, in cervical and other HPV-associated cancer therapy.

Disclosure of potential conflicts of interest

No potential conflict of interest relevant to this article was reported.

Acknowledgments

This work was supported by grant from the Special Research Fund for Health Nonprofit Industry (200802109) and Zhejiang Province Nature Foundation (Y2100322).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2013.03.010.

References

Chang, J.T., Kuo, T.F., Chen, Y.J., Chiu, C.C., Lu, Y.C., Li, H.F., Shen, C.R., Cheng, A.J., 2010. Highly potent and specific siRNAs against E6 or E7 genes of HPV16- or HPV18-infected cervical cancers. Cancer Gene Ther. 17, 827–836.
Check, E., 2005. Pioneering HIV treatment would use interference and gene therapy. Nature 437, 601.
Courtete, J., Sibler, A.P., Zeder-Lutz, G., Dalkara, D., Oulad-Abdelghani, M., Zuber, G., Weiss, E., 2007. Suppression of cervical carcinoma cell growth by intracytoplasmic codelivery of anti-oncoprotein E6 antibody and small interfering RNA. Mol. Cancer Ther. 6, 1728–1735.
DeFilippis, R.A., Goodwin, E.C., Wu, L., DiMaio, D., 2003. Endogenous human papillomavirus E6 and E7 proteins differentially regulate proliferation, senescence, and apoptosis in HeLa cervical carcinoma cells. J. Virol. 77, 1551–1563.
Fehrmann, F., Laimins, L.A., 2003. Human papillomaviruses: targeting differentiating epithelial cells for malignant transformation. Oncogene 22, 5201–5207.
Gu, W., Putral, L., Hengst, K., Minto, K., Saunders, N.A., Leggatt, G., McMillan, N.A., 2006. Inhibition of cervical cancer cell growth in vitro and in vivo with lentiviral-vector delivered short hairpin RNA targeting human papillomavirus E6 and E7 oncogenes. Cancer Gene Ther. 13, 1023–1032.
Hong, D., Ye, F., Chen, H., Lü, W., Cheng, Q., Hu, Y., Xie, X., 2008. Distribution of human papillomavirus genotypes in the patients with cervical carcinoma and its precursors in Zhejiang Province China. Int. J. Gynecol. Cancer 18, 104–109.
Hong, D., Lu, W., Ye, F.Hu.Y., Xie, X., 2009. Gene silencing of HPV16 E6/E7 induced by promoter-targeting siRNA in SiHa cells. Br. J. Cancer 101, 1798–1804.
Humeau, L.M., Binder, G.K., Lu, X., Slepushkin, V., Merling, R., Echeagaray, P., Pereira, M., Slepushkina, T., Barnett, S., Dropulic, L.K., Carroll, R., Levine, B.L., June, C.H., Dropulic, B., 2004. Efficient lentiviral vector-mediated control of HIV-1 replication in CD4 lymphocytes from diverse HIV+ infected patients grouped according to CD4 count and viral load. Mol. Ther. 9, 902–913.
Jemal, A., Bray, F., Center, M.M., Ferlay, J., Ward, E., Forman, D., 2011. Global cancer statistics. CA Cancer J. Clin. 61, 69–90.
Jones, L., Ratcliff, F., Baulcombe, D.C., 2001. RNA-directed transcriptional gene silencing in plants can be inherited independently of the RNA trigger and requires Met1 for maintenance. Curr. Biol. 11, 747–757.
Jonson, A.L., Rogers, L.M., Ramakrishnan, S., Downs Jr., L.S., 2008. Gene silencing with siRNA targeting E6/E7 as a therapeutic intervention in a mouse model of cervical cancer. Gynecol. Oncol. 111, 356–364.
Karwacz, K., Mukherjee, S., Apolonia, L., Blundell, M.P., Bouma, G., Escors, D., Collins, M.K., Thrasher, A.J., 2009. Nonintegrating lentivector vaccines stimulate prolonged T-cell and antibody responses and are effective in tumor therapy. J. Virol. 83, 3094–3103.
Kohn, D.B., Sadelain, M., Glorioso, J.C., 2003. Occurrence of leukaemia following gene therapy of X-linked SCID. Nat. Rev. Cancer 3, 477–488.
Lewinski, M.K., Bisgrove, D., Shinn, P., Chen, H., Hoffmann, C., Hannenhalli, S., Verdin, E., Berry, C.C., Ecker, J.R., Bushman, F.D., 2005. Genome-wide analysis of chromosomal features repressing human immunodeficiency virus transcription. J. Virol. 79, 6610–6619.
Liu, W.L., Green, N., Seymour, L.W., Stevenson, M., 2009. Paclitaxel combined with siRNA targeting HPV16 oncogenes improves cytotoxicity for cervical carcinoma. Cancer Gene Ther. 16, 764–775.
Mette, M.F., Aufsatz, W., van der Winden, J., Matzke, M.A., Matzke, A.J., 2000. Transcriptional silencing and promoter methylation triggered by double-stranded RNA. EMBO J. 19, 5194–5201.
Moody, C.A., Laimins, L.A., 2010. Human papillomavirus oncoproteins: pathways to transformation. Nat. Rev. Cancer 10, 550–560.
Morris, K.V., Chan, S.W., Jacobsen, S.E., Looney, D.J., 2004. Small interfering RNA-induced transcriptional gene silencing in human cells. Science 305, 1289–1292.
Munger, K., Baldwin, A., Edwards, K.M., Hayakawa, H., Nguyen, C.L., Owens, M., Grace, M., Huh, K., 2004. Mechanisms of human papillomavirus-induced oncogenesis. J. Virol. 78, 11451–11460.
Muñoz, N., Bosch, F.X., de Sanjosé, S., Herrero, R., Castellsagué, X., Shah, K.V., Snijders, P.J., Meijer, C.J., 2003. International Agency for Research on Cancer Multicenter Cervical Cancer Study Group. Epidemiologic classification of human papillomavirus types associated with cervical cancer. N. Engl. J. Med. 348, 518–527.
Negri, D.R., Bona, R., Michelini, Z., Leone, P., Macchia, I., Klotman, M.E., Salvatore, M., Cara, A., 2010. Transduction of human antigen-presenting cells with integrase-defective lentiviral vector enables functional expansion of primed antigen-specific CD8(+) T cells. Hum. Gene Ther. 21, 1029–1035.
Parkin, D.M., Bray, F., Ferlay, J., Pisani, P., 2005. Global cancer statistics, 2002. CA Cancer J. Clin. 55, 74–108.
Quinn, M.A., Benedet, J.L., Odicino, F., Maisonneuve, P., Beller, U., Creasman, W.T., Heintz, A.P., Ngan, H.Y., Pecorelli, S., 2006. Carcinoma of the cervix uteri. FIGO 26th Annual Report on the Results of Treatment in Gynecological Cancer. Int. J. Gynaecol. Obstet. 95 (Suppl. 1), S43–S103.
Richards, E.J., 2006. Inherited epigenetic variation – revisiting soft inheritance. Nat. Rev. Genet. 7, 395–401.
Scherr, M., Eder, M., 2002. Gene transfer into hematopoietic stem cells using lentiviral vectors. Curr. Gene Ther. 2, 45–55.
Smotkin, D., Wettstein, F.O., 1986. Transcription of human papillomavirus type 16 early genes in a cervical cancer and a cancer-derived cell line and identification of the E7 protein. Proc. Natl. Acad. Sci. USA 83, 4680–4684.

Sunaga, N., Miyajima, K., Suzuki, M., Sato, M., White, M.A., Ramirez, R.D., Shay, J.W., Gazdar, A.F., Minna, J.D., 2004. Different roles for caveolin-1 in the development of non-small cell lung cancer versus small cell lung cancer. Cancer Res. 64, 4277–4285.

Ting, A.H., Schuebel, K.E., Herman, J.G., Baylin, S.B., 2005. Short double-stranded RNA induces transcriptional gene silencing in human cancer cells in the absence of DNA methylation. Nat. Genet. 37, 906–910.

Yamato, K., Yamada, T., Kizaki, M., Ui-Tei, K., Natori, Y., Fujino, M., Nishihara, T., Ikeda, Y., Nasu, Y., Saigo, K., Yoshinouchi, M., 2008. New highly potent and specific E6 and E7 siRNAs for treatment of HPV16 positive cervical cancer. Cancer Gene Ther. 15, 140–153.

Yáñez-Muñoz, R.J., Balaggan, K.S., MacNeil, A., Howe, S.J., Schmidt, M., Smith, A.J., Buch, P., MacLaren, R.E., Anderson, P.N., Barker, S.E., Duran, Y., Bartholomae, C., von Kalle, C., Heckenlively, J.R., Kinnon, C., Ali, R.R., Thrasher, A.J., 2006. Effective gene therapy with nonintegrating lentiviral vectors. Nat. Med. 12, 348–353.

Zheng, Z.M., Baker, C.C., 2006. Papillomavirus genome structure, expression, and post-transcriptional regulation. Front. Biosci. 11, 2286–2302.

Zhou, J., Peng, C., Li, B., Wang, F., Zhou, C., Hong, D., Ye, F., Cheng, X., Lü, W., Xie, X., 2012. Transcriptional gene silencing of HPV16 E6/E7 induces growth inhibition via apoptosis in vitro and in vivo. Gynecol. Oncol. 124, 296–302.

zur Hausen, H., 2000. Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis. In: Natl. Cancer Inst. 92, 690–698.