Effective gene silencing and inhibition of cervical cancer cell growth in vitro and in vivo with dual shRNAs

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Running title: Inhibiting cervical cancer cell growth with dual shRNAs

Abstract:

RNA interference holds great promise for the treatment of disease but delivery remains a key issue. Lentivirus vectors are widely used for stable transfer of short-hairpin RNA (shRNA) into cells and are expected to deliver a stable and durable interference. However, this does not appear to be the case. Here we show that lentiviral-delivered shRNAs directed against HPV E6/E7 oncogenes are effective for less than three weeks. This short-lived RNAi was not due to the loss of the vector in the host cells but was more likely to be related to shRNA expression or RNAi machinery itself. Using this vector to carry two copies of the same shRNA or two shRNAs targeting different genes (HPV E6 and VEGF) was more effectiveness at silencing the gene targets and inhibiting cell or tumor growth than their single shRNA counterparts. These results indicate that a multi-shRNA strategy is a more attractive approach than single-shRNA for developing a RNAi treatment for cancer.

**Key words:** Lentiviral vector, dual shRNA, RNAi; HPV E6/E7, cervical cancer, VEGF.
Introduction:

RNAi has been proved to be a powerful tool for gene function studies and shows great potential for the treatment of viral diseases, genetic disorders, and cancer (1-5). To date, at least 5 siRNA treatments have been tested in clinical trials (6-7) and many more are expected soon. For cancer treatment, cervical cancer represents an ideal model for testing RNAi treatment due to its association with human papillomaviruses (HPV) infection. It is also known that the viral early genes, E6 and E7, of high-risk HPV types are primarily responsible for the transformation of the epithelial cells and moreover their continuous expression is essential for cancer cell survival (8-10). Therefore, these two oncogenes are regarded as ideal targets for developing and testing RNAi-based therapeutic treatments. Previously, studies, including our own, have demonstrated that silencing HPV E6/E7, with siRNA or shRNA, leads to cervical cancer cells undergoing apoptosis or senescence (11-14) and inhibition of tumor growth in vivo (14-17).

These results suggest that RNAi therapy could be developed for cervical cancer treatment. However, for this treatment to become a reality a number of issues need to be addressed including achieving stable and durable RNAi. Previously, we showed that lentiviral-delivered shRNA triggers effective silencing of E6 and E7 and leads to cervical cancer cell growth inhibition in vitro and in vivo (14). However, while lentiviral-mediated silencing is thought to be permanent we had observed reduced suppression of E7 protein two weeks after treatment. Others have found similarity, albeit using a slightly different vector, that lentiviral delivery did not result in a lasting and stable shRNA suppression (18).

In addition to this problem, cervical cancer cell lines CaSki and SiHa have been reported to be able to develop resistance to RNAi via the expression of a previously unknown 50kDa cytoplasmic protein that unwound the antisense strand of E7 siRNA from the RISC (21). The resistance was not
only seen in siRNA treatment but may also be seen in shRNA (22). Such resistance in cervical cancer cells could be an obstacle to the development of RNAi-based treatment. One potential solution to overcome these issues is developing a multiple shRNA strategy or combining RNAi treatment with chemical-based cancer drugs (23-24). The multiple shRNA strategy has already been tried in the context of HIV-1 treatment and shown to successfully overcome viral escape and mutation (25-26). For example, Brake et al showed that HIV-1 could escape from a single shRNA attack but not from four shRNAs that were simultaneously expressed in a cell (27). A multiple shRNA strategy was also used to silence hepatitis C or B virus and superior inhibition of viral replication was observed (28-29). However, using shRNAs to target multiple genes has not been previously investigated for cervical cancer and is all the more important as this may overcome RNAi resistance.

Here we investigated the durability of lentiviral-delivered RNAi treatment as well as a multiple shRNA strategy in cervical cancer HeLa cells, targeting HPV 18E6E7 and VEGF, a well-known angiogenesis factor important for tumor formation and growth. We show that both shRNAs worked well at slicing their target genes in the dual shRNA construct and dual shRNA therapy was more effective than single shRNA in gene silencing and inhibition of cell and tumor growth.

**Materials and Methods:**

**shRNA design and cloning:**
The shRNA targeting at HPV 18E6 (18E6-1) was as previously described (14). This shRNA targets at a common site of all three alternative spliced mRNA classes of HPV 18 and was shown to be effective at simultaneously knockdown of both E6 and E7 expression as they are bi-cistronically transcribed (14). The shRNA targeting human VEGF-A (shVEGF-2) was adopted from previous publication and was shown to be effective at silencing VEGF (30). For dual shRNA design, we used two simple methods:
direct synthesis and linking method. In the first case, the two shRNA expression cassette and their complementary strands were synthesized as one DNA fragment (PROLIGO, Lismore, Australia) and annealed in the annealing buffer (100mM K-acetate, 30mM HEPES-KOH pH7.4, 2mM Mg-acetate) by heating to 95°C for 5 min followed by cooling to room temperature. The two cassettes were spaced by Bam H I site (for further cloning or restriction digesting check) and the first shRNA Xho1 site was replaced by the Bam H I site. For the linking method, each shRNA expression cassette and its complementary strand were synthesized and annealed as above. The first shRNA expression cassette ended with Bam H I site rather than Xho1 site and the second one started with Bam H I site rather than Hpa I site. The annealed two shRNA expression cassettes were linked together using the method previously described (31). Above annealed oligo DNA was cloned into plasmid pLentiLox3.7 (pLL3.7, transfer vector) as described previously (14). The insert was confirmed by both restriction enzyme digestion and DNA sequencing. Plasmid pLL3.7 has a self inactivating LRT (32) for the bio-safety consideration and also contains an eGFP gene under CMV promoter, this enables monitoring the infection of lentiviruses by eGFP expression. Other lentiviral packaging plasmids were as described in detail by Dull et al (33) and were used for third generation lentiviral vector production.

**LV-shRNA production**

Lentiviral production and titration were as previously described (14). Briefly, the packing plasmids and pLL3.7 were amplified in *E. coli* and purified using W/Endo-free Qiagen Maxi-Prep Kits (Promega, Sydney Australia) according to the manufacturer’s instructions. Packing cell line 293T cells were transfected with 6.6µg pLL3.7 (-/+ insert) and 3.3µg of each packaging plasmid in 133µl 1.25M CaCl₂, 0.5ml H₂O, and 0.66ml 2x HBS (140mM NaCl, 1.5mM Na₂HPO₄, 50mM HEPES, pH 7.05) in a T₇₅ flask. The viral supernatant was harvested and concentrated using Vivaspin 20ml Concentrators (100 MW, VivaScience Sartorius Group, Sydney Australia). The lentiviral stocks were stored in small aliquot at -80°C for titration and cell infection.
Cell transduction and flow cytometry

HeLa cells were maintained in DMEM supplemented with 10% heat-inactivated foetal calf serum, 100 units/ml penicillin G, 100µg/ml streptomycin sulphate and 0.29mg/ml of L-Glutamine (Gibco-Invitrogen). For transduction, cells were plated in 6-well plates (1x10^5 cells /well) or in T25 flasks (2x10^5 /flask) and were cultured overnight. LV-shRNAs diluted in medium containing 8µg/ml polybrene were added to the cells in 0.5ml (6-well plate) or 1ml (flask) polybrene-medium for incubation for 1 hour at 37°C. Fresh polybrene DMEM medium was added and incubation continued for 48 hours. Transduced HeLa cells were continuously cultured and harvested by trypsin and washed with PBS before resuspending in 0.5ml 1% paraformaldehyde/PBS for flow cytometry analysis using FACS Calibur or FACS Canto.

Colony forming assay

Transduced HeLa cells were harvested and counted. One hundred cells were seeded in each well of 12-well plates with 1 ml complete DMEM, each group has 3 or 4 repeats. The cells were cultured for 7 days and then fixed with 95% ethanol for 30 mins at room temperature and stained with 0.1% crystal violet for 5 mins. The colony was counted and the data was presented in mean of the 3 repeats.

Western blotting

Western blotting was conducted as previously described (14). HeLa cell lysates were collected after lentiviral transduction or at intervals of two weeks during continuous culture. Anti-HPV 18E7 polyclonal antibody was purchased from Santa Cruz Biotechnology. Anti-human Bax polyclonal antibody was from Cell Signaling Technology. Anti-human β-tubulin antibody was from Sigma.
**PCR and Real-time PCR**

Freshly transduced HeLa cells were harvested for total RNA and DNA extraction using TRIzol® reagent (Invitrogen) as instructed by the manufacturer. Reverse transcription reactions were performed with oligo-dT primer. The primers for RT-PCR were as following: 18E7; Forward 5’-AAAATGAAATTCCGGTTGA-3’; Reverse 5’-GGCTGGTAAATGGTGATGAT-3’; VEGF: Forward 5’-TCTACCTCCACCATGCCAAAGT-3’; Reverse 5’-GATGATTCTGCCCTCCTCCTT-3’; Human β-actin: Forward 5’-AGCCTCGCCTTTGCCGA-3’; Reverse: 5’-CTGGTGCCGCAGGCGGCG-3’. Real-time PCR was carried out with SYBgreen master mixture (Promega) using a Rotor-Gene RG-3000 (Corbett Research, Australia) with the following program: pre-heating 95°C 10min; then 40 cycles of 94 °C 15sec; 58 °C 30sec; and 72 °C 30sec. The results were analyzed using Rotor-Gene6 software and presented as mean ± SEM of relative gene expression, which were normalized by actin expression as previously described (34).

PCR was performed for pLL3.7 vector and 18s rDNA in transduced HeLa cells. The primers for pLL3.7 were (Forward 5’-CAGTGCAGGGGAAAGAATAGT-3’; Reverse 5’-ATGGGCTATGAACTATAATGAC-3’) and human 18S rDNA (Forward 5’-CCA TCG AAC GTC TGC CCT A-3’; Reverse 5’-TCA CCC GTG GTC ACC ATG-3’) were as described previously (35). 18s rDNA was a single copy gene and was used as DNA loading control. The PCR was performed in 20 µl volume with 2.5 µl of reverse transcription product. PCR program was pre-heating 95°C for 5 min; the cycle of 94 °C for 45sec; 56 °C for 1min; and 72 °C for 2 min. Twenty seven cycles were used for detecting E6/E7 mRNA and 40 cycles for short form mRNA detection.

**Cell Titer-blue assay for cell viability:**

Transduced HeLa cells were cultured and counted, 3 x 10^4 cells were seed in each well of 96-well plates. Each transduced cell line has 4-6 repeats. DMEM medium were used as blanks. The cells were cultured overnight and then 15µl CellTiter-Blue™ reagent (Promega) was added to each well and the
cells were incubated for 30-40 minutes at 37°C. The reaction was stopped by transferring the supernatant to another plate and the plates were read by Taqman 7700 (AB Applied Biosystems) for fluorescence.

Animal experiment
Female Rag<sup>−/−</sup> mice of 6 weeks were used in this study. Transduced HeLa cells were harvested and resuspended in PBS at 1x 10^7 cells/ml. For tumor transplant model, the mice were subcutaneously injected (5 mice/group) with 50 µl cell suspension to the neck scruff and the tumors was monitored by size and were collected and weighted at day 35 after injection. All animal experiments were approved by the University of Queensland animal ethics committee.

Data analysis
Data was collected and expressed as mean ± S.D for each group and unpaired t-test was used to analyze the differences and discriminate the significant differences (two-tails, P<0.05) between two groups.

Results:

1. RNAi is Short-lived in HeLa cells

We had previously observed that shRNA-mediated suppression of E7 protein appeared to become reduced over a two week time period (14) and wished to explore this over a longer period of time asking if a stable and lasting gene silencing can be achieved in cervical cancer cells with the lentiviral vector. Therefore we analyzed E7 protein levels over a 16 week period using HeLa cells transduced with a lentivirally delivery shRNA to HPV 18 E6 (18E6-1). We have previously shown this shRNA to be effective at reducing E7 protein expression as E7 is expressed from the same mRNA as E6 (14).
HPV 18E7 protein level expression was low at week 1 but back to normal expression levels by week 3 (Fig 1A). This loss of suppression continued for 4 months. A control lentiviral shRNA against another HPV type, HPV 16E7 (16E7-2) was used as a negative control (36), and we observed no changes of E7 protein levels during the same period. To further confirm the loss of shRNA suppression we carried out colony forming assays on shRNA-treated cells. It can be seen that by week three, colony forming ability was restored to control levels (Fig 1B), a result consistent with E7 protein level (Fig 1A). These results show that gene silencing mediated by the shRNA 18E6-1 lasted less than 3 weeks, after which the E7 protein level and cell growth ability were no different from non-transduced HeLa cells.

One explanation for these results is that the loss of gene silencing is due to the loss of the vector in host genomic DNA or the outgrowth of non-transfected cells. To address this we examined transduced cells for the presence of our lentiviral transfer plasmid, pLL3.7, using PCR. The results show that in the detection period of 14 weeks, the plasmid was persistently present in cells without any obvious loss or decrease (Fig 1C). The vector, pLL3.7, encodes not only the shRNA but also eGFP driven from a CMV promoter which is used as the basis for selection of transfected cells. Moreover, we had previously shown to eGFP expression correlated with gene copy number and silencing efficacy (14). Therefore, to further confirm cells contained the vector we examined eGFP expression using FACS. We observed that eGFP expression did not decline for at least 5 weeks (Fig 1D). From the above data we concluded that the vector was present in the host genome and that the loss of suppression was not due to a population of non-transfected cells.

2. **Construction of twin and dual-target shRNAs in a lentiviral vector**

Cervical cancer cells can develop resistance to RNAi triggered by synthetic siRNA (21) and our data above demonstrates that shRNA delivered by lentiviral is also short-lived. One strategy to overcome this is the multi-copy or multi-target approach which allows additive or synergistic effects to induce
rapid cell death before resistance occurs. To investigate this possibility in cervical cancer we designed two, dual shRNA constructs; one contained two copies of the 18E6-1 shRNA (twin 18E6-1) and the other contained shRNAs targeting HPV 18E6/E7 and human VEGF-A (dual-target shRNA). Fig 2A shows the predicted RNA fold structure. Figure 2B shows the confirmation of inserts by restriction enzyme digestion. The inserts were further confirmed by DNA sequencing.

3. **Dual-shRNA constructs silence their target genes more effectively than single shRNA**

To test if dual shRNA constructs can effectively silence their target genes, we produced lentiviruses expressing each of them. HeLa cells were transduced with lentiviruses at a 2x dose, that is twice the dose required to give 100% transduction efficiency, and RNA and protein samples were collected for real-time RT-PCR and Western blot analysis at various times following incubation. The single copy vectors expressing shRNA against just 18E6-1 or VEGF were also produced and used to compare with the dual shRNA constructs. A 16E7-2 shRNA was used as a negative control. Both mRNA and protein levels of HPV 18E7 (Fig 3A and C) and VEGF (Fig 3B) expression indicated that the dual target shRNA construct could effectively silence their target genes. As expected, Twin 18E6-1 was more effective than single copy 18E6-1 shRNA at silencing E6/E7 expression (Fig 3A). Interestingly, the dual-target shRNA was more effective at silencing E7 and VEGF than the single shRNA constructs even though one would predict they expressed the same level shRNA. This result suggests an interaction between HPV E6/E7 and VEGF. A few studies have shown that HPV E6/E7 could regulate VEGF expression (37-39). However, the current result indicates that VEGF may also modulate HPV E7 expression.

4. **Dual shRNA constructs are more effective at inhibiting cancer cell growth in vitro**

To further confirm that twin 18E6-1 and dual-target shRNA have more effect on cervical cancer cell growth, we tested cell viability and colony forming ability of the transduced HeLa cells. Consistent
with E7 protein level, HeLa cells transduced with twin 18E6-1 shRNA and dual-target shRNA were much less viable and formed fewer colonies compared with the negative controls in both assays (Fig 4). Whereas VEGF shRNA alone did no significantly affect HeLa cells, cells transduced with the single 18E6-1 were significantly less viable (Fig 4 A) and formed fewer colonies (Fig 4B, C) than the control. These data further have proved that the dual-target shRNA has an additive or synergistic effect on target gene silencing.

5. **Dual-target shRNA is more effective at inhibiting tumor growth *in vivo* and caused more apoptosis**

To verify our *in vitro* results, we carried out tumor transplant studies using RAG knockout mice. Transduced HeLa cells (2x dose) were subcutaneously injected to mice and tumors were collected 35 days after injection and weighted. Compared with untreated control (HeLa) and LV-shRNA control 16E7-2, the average tumor weights of 18E6-1, twin 18E6-1, VEGF, and dual-target shRNA groups were all reduced (Fig 5 A). However, only the dual-target shRNA group was significantly reduced (P<0.05) compared with the controls. This result is consistent with *in vitro* data, and further confirms that the dual-target shRNA is more effective at not only silencing the target genes but also inhibiting tumor growth *in vivo*. To investigate if the dual target shRNA was more effective at inhibiting cell growth was due to apoptosis we detected Bax protein level in the transduced HeLa cells, a downstream component of the p53-related apoptosis pathway. The dual-target shRNA triggered more Bax expression (apoptosis) in the cancer cells than other single or even twin shRNAs (Fig 5B) perhaps explaining why the dual-target shRNA was more effective than other shRNAs in inhibiting tumor growth.

6. **Short-lived RNAi for dual shRNA constructs**
To investigate if RNAi triggered by our dual shRNA constructs would last longer than single shRNAs, we examined the expression of VEGF and E7 following transduction (Fig 6). We observed that the suppression of both VEGF (Fig 6A) and E7 (Fig 6B) were lost at the same rate as our single 18E6-1 shRNA vectors, confirming that lentiviral delivered shRNA (single or dual) is short-lived and only lasts about 2-3 weeks.

**Discussion:**

The lentiviral vector has been explored as a vehicle for stable and durable gene therapy in preclinical treatment and clinical trials (19; 20) therefore we hoped it could do so for RNAi in cervical cancer cells. However, our data indicate the gene silence delivered by this vector can last only about two weeks. This result is consistent with a previous observation though in different cells (18). The reason for this seems not due to the loss of the shRNA expressing vector as the pLL3.7 plasmid had been present in the cells for 3.5 months after transduction. In addition, the high percentage of eGFP positive cell population lasted at least 5 weeks, suggesting that the plasmid can still actively expressed the carried genes after 3 weeks and that the overgrowth of the negative cell population did not take place. One previously reported explanation is that the U6 promoter was less stable than H1 promoter in lentiviral transduced, in this case in human primary lymphocytes (40). Our vectors contain the U6 promoter and indeed one could replace it with an H1 promoter but this is not without problems as H1 is less effective than U6 at gene silencing (40). Another potential explanation is that the transduced HeLa cells have developed resistance to RNAi, as previously described in cervical cancer cells (21). Whatever the reason is, the use of current lentiviral vectors for RNAi treatment does not result in long lasting suppression and therefore introduces the possibility of cancer cell escape.
A alternative strategy using multiple shRNA was tested to overcome this issue, an approach that has the added benefit of targeting multiple genes or pathways central to cancer cell maintenance. Other advantages of this strategy include being able to simultaneously target isotypes of a gene and using one vector to carry multiple copies of shRNA targeting at different sites of a gene. In the current study, we explored both a two-copy and two-target shRNA strategy. Although the two dual shRNA constructs did not offer longer RNAi, the data showed that targeting HPV 18E6 and VEGF-A together gave enhanced gene silencing of each target gene compared to single targeted shRNA suggesting an additive or synergistic gene silencing was occurring. In vitro, the dual-target shRNA seemed almost equally effective to twin 18E6-1. However, in vivo, only the 18E6-1vegf group had significantly smaller tumors than the controls, suggesting that silencing 18E6 together with VEGF in vivo is more efficient at inhibiting tumor growth in vivo than the twin 18E6-1 which inhibited only E6/E7 genes. Several studies have shown that silencing VEGF can slow or stop blood vessels formation (angiogenesis) in few different cancer types (30; 41; 42). A recent study also showed that silencing VEGF, hTERT, and Bcl-xl simultaneously was more effective at blocking human laryngeal squamous carcinoma (Hep-2) cell growth and suggested this was a more attractive approach for treating human cancer (43). In addition, it has been shown that silencing both VEGF isotypes A and C was beneficial for inhibiting lymph node and lung metastases (42). These data demonstrate that a multiple shRNA strategy combing E7 and VEGF is a good treatment approach. To extend this concept, the multiple-target strategy will be a better option for other viral vectors that would stimulate immune response in the host and the repeat treatments are not possible (eg. Adenovirus).

An interesting observation in this study was the relationship between HPV viral oncogene E6/E7 and angiogenesis factor VEGF. Few studies have shown that HPV 16 E6 onco-protein can increase VEGF level in cervical cancer cells by activating its promoter (44) or expression of HPV 16 E6/E7 in human primary keratinocytes increased the VEGF level in these cells (39). These reports suggest that there is a relationship between oncogene E6/E7 and angiogenesis factor VEGF by the former modulates the later
expression. However, in this study, we showed that silencing VEGF could also affect E7 expression. As far as we are aware, this observation has not been reported. This observation may explain why silencing both genes had significant effect on E7 expression (mRNA and protein levels) and cell growth in vitro. As how VEGF affect E7 expression or if this was through the similar pathway as E6/E7 modulate VEGF is not clear and worthy further investigation. However, this result suggests that using this strategy as a tool we can investigate the biological function relationship for the two genes or more genes.

Our data have proved that our dual shRNA constructs work well at silencing their target genes, indicating that the simple way we constructed the dual shRNA expression cassettes in lentiviral vectors is useful. These constructs can be easily modified to add another one or two shRNAs (two synthesized or linked shRNA with BamH I site) using the BamH I cloning site to make a multiple shRNA construct. However, care must be taken to space each shRNA expression cassette because the simple combination of the expression cassettes and the repeat sequence may result in reduced activity of each shRNA or even deletion of some shRNA (45). In addition, from our data it is obvious that although the multi-shRNA strategy has some advantages over single shRNA, the short-lived gene silencing is not avoidable with dual-shRNA constructs. Therefore, the multiple shRNA strategy should emphasize more effective and simultaneous hits to cancer cells to trigger apoptosis in the cells before they develop resistance to RNAi.

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Conflict of interest:

Authors declare that there is no competing financial interest in relation to the work described.

References:

10. von Knebel Doeberitz M, Oltersdorf T, Schwarz E, Gissmann L. Correlation of modified


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**Titles and legends to figures:**
Fig 1. Short lived RNAi in HeLa cells transduced with lentiviral 18E6-1 shRNA. HeLa cells transduced with lentiviral shRNA 18E6-1 against HPV 18 E6 had low level of E7 protein by Western blot assay at week 1 (1W) after transduction (A). However, the E7 protein level came back to the level of HeLa cells before transduction (H) and lasted for 16 weeks (A). As a negative control, HeLa cells transduced with ineffective lentiviral shRNA 16E7-2 were not observed any changes (A). In both cases, human beta-tubulin (Tub) was also assayed as loading controls. B represents the result of colony forming assay from HeLa cells transduced with 18E6-1 and the control 16E7-2 during a period of 9 weeks. Each time point has at least three repeats. C. the PCR results of the expression of pLL3.7 (PLL) in the LV-18E6-1 (18E6-1) and lentiviral vector carrying only pLL3.7 plasmid (PLL) transduced HeLa cells during the period of 14 weeks. Human 18s DNA was similarly assayed as loading control. D. FACS result of eGFP expression of 18E6-1 and PLL transduced HeLa cells during the period of 5 weeks. Negative control was the HeLa cells with mock transductions. *: P<0.05.

Figure 2. Dual shRNA secondary structures and the cloning result. A and B show the computational RNA folding images of twin 18E6-1 shRNA (A) and dual-target shRNA (B). C, the agarose gel image showing the DNA fragment sizes of inserts in pLL3.7 after restriction enzyme digestion reaction (with Xba I and Not 1). 1, DNA ladders; 2, pLL3.7 plasmid alone; 3, 18E6-1 plasmid; 4, twin 18E6-1 plasmid; 5, dual-target shRNA plasmid.

Figure 3. Gene silencing efficacy of dual shRNA constructs. Real-time RT-PCR results of HPV 18E7 (A) and human VEGF-A (B) gene expression in HeLa cells after transduction of lentiviral shRNA. Each column represents at least 3 repeats. C represents the Western blotting results of 18E7 protein levels knocking down by dual shRNAs. E6-1: 18E6-1; Tw: twin 18E6-1; E6-1VE: dual-target shRNA of 18E6-1 and VEGF; 16E7: 16E7-2. **: P<0.01; ***: P<0.001.
Figure 4. The effect of dual lentiviral-shRNA on cell viability and cell growth. A shows the cell viability of the transduced HeLa cells assayed using cell-titer blue assay. Each group has 4 repeats. B shows the colony formation of transduced HeLa cells in a 24-well plate and C shows the colony counting results of each group. Each treatment has at least 4 repeats. **: P<0.01; ***: P<0.001.

Figure 5. Apoptosis analysis and tumor growth of transduced HeLa cells. A. the result of tumor growth (weight) experiment in xeno-transplant Rag⁻/⁻ mouse model, each group represents 5 mice. B. Western blotting result of Bax protein, one of the downstream components of p53 apoptosis pathway.

Figure 6. HPV 18E7 and VEGF expression after 3 weeks transduction of dual shRNA in HeLa cells. A. the real-time PCR result of VEGF expression at 3 weeks after transduction. B. Western blotting result of 18E7 protein at 3 weeks after transduction. C shows the densitometry analysis of B.
Fig 1.

A.

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18E6-1

| E7 |     |    |    |    |    |
| Tub|     |    |    |    |    |

16E7-2

B.

Colony No.

Time (weeks)

![Graph showing the number of colonies over time for 18E6-1 and 16E7-2.](image-url)
**C.**

Negative control

![Histograms for different time points with MFI values](image)

PLL

- **1W**: MFI: 3708
- **3W**: MFI: 3637
- **5W**: MFI: 1585

18E6-1

- **1W**: MFI: 3700
- **3W**: MFI: 1928
- **5W**: MFI: 1130
Fig 2.

A.  

B.  

C.  

1 2 3 4 5

500bp →
Fig 3.

A. 18E7 Expression

B. VEGF Expression

C. E7 and Tubulins

D. Densitometry:
Fig 4.

A.

![Graph showing CTB counts for different samples with a p-value of 0.309.]

B.

![Image showing colony counts for different samples.]

C.

![Graph showing colony counts for different samples with a p-value of 0.062.]

16E7-2, VEGF, 16E6-1, T-18E6-1, 18E6-1VEGF.

Colony No.

CTB counts

P=0.309

P=0.062
Fig. 5.

A

Rapamycin

![Graph showing CTB counts for different conditions: HeLa, HeLa+, PLL, PLL+, 18E6-1, 18E6-1+, T-18E6-1, T-18E6-1+. The graph indicates a significance level of P<0.05.]

B

SAHA

![Graph showing CTB counts for different conditions: HeLa, HeLa+, PLL, PLL+, 18E6-1, 18E6-1+, TWH, TWH+, 18E6-1vegf, 18E6-1vegf+. The graph includes significance levels: *, **, and ***.]
Fig 6.

A.

B.

16E7  vegf  E6-1  Tw  E6-1vegf

Bax

Tub