Inhibition of cervical cancer cell growth *in vitro* and *in vivo* with dual shRNAs

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Abbreviations: RNAi: RNA interference; shRNA: short hairpin RNA; HPV: human papillomavirus; siRNA: short interfering RNA; VEGF: vascular endothelial growth factor; LV: lentiviral vector.

Abstract:

RNA interference (RNAi)-based gene silencing is widely used in laboratories for gene function studies and also holds a great promise for developing treatments for diseases. However, *in vivo* delivery of RNAi therapy remains a key issue. Lentiviral vectors have been employed for stable gene transfer and gene therapy and therefore are expected to deliver a stable and durable RNAi therapy. But this does not seem to be true in some disease models. Here we showed that lentivirus delivered short-hairpin RNA (shRNA) against human papillomavirus (HPV) E6/E7 oncogenes were effective for only two weeks in cervical cancer model. However, using this vector to carry two copies of the same shRNA or two shRNAs targeting at two different but closely related genes (HPV E6 and VEGF) was more effective at silencing the gene targets and inhibiting cell or even tumor growth than their single shRNA counterparts. The cancer cells treated with dual shRNA were also more sensitive to chemotherapeutic drugs than single shRNA treated cells. These results suggest that a multi-shRNA strategy may be a more attractive approach for developing a RNAi therapy for this cancer.

Key words: Lentiviral vector, Dual shRNA, RNAi; HPV E6/E7, Cervical cancer, VEGF.

Introduction:

RNAi triggered by small RNA has proved to be a powerful tool for gene function studies and also shows great potential for the treatment of viral diseases, genetic disorders, and cancers (1-5). To date, several siRNA treatments have been tested in clinical trials (6-7). For cancer treatment, cervical cancer is an ideal model because it is highly associated (99%) with HPV infection and viral early genes, E6 and E7, of high-risk HPV types are primarily responsible for the transformation of the epithelial cells and 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 lentivirus-delivered shRNA triggered effective silencing of E6 and E7 and led to cervical cancer cell growth inhibition *in vitro* and *in vivo* (14). However, while lentiviral-mediated gene silencing is thought to be stable and long lasting there were reports on the short-lived delivery of lentiviral vector (18, 40).

In addition to the above issue, 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 interacted with the unwound antisense strand of E7 siRNA from the RISC (21). The resistance was not only seen in siRNA treatment but was also seen in shRNA (22). Such resistance in cervical cancer cells could be an obstacle for the development of RNAi-based treatment. One potential solution to overcome these issues is to develop a multiple shRNA strategy or combine RNAi treatment with chemical-based cancer drugs (23-24). The multiple shRNA strategy has been investigated 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, multiple shRNA strategy has not been well studied in cancers and not investigated for cervical cancer and this strategy may be used to overcome RNAi resistance in cervical cancer.

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, which is a well-known angiogenesis factor important for tumor formation and growth. We show that both shRNAs worked well at silencing their target genes in the dual shRNA construct. Dual shRNA therapy was more effective than single shRNA in gene silencing and inhibition of cell and even 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 simultaneous 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 $CaCl_2$, 0.5ml H_2O, and 0.66ml 2x HBS (140mM NaCl, 1.5mM Na_2HPO_4, 50mM HEPES, pH 7.05) in a T_{75} 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^{o}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, 100ug/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 ($2x 10^5$ /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'-GGCTGGTAAATGTTGATGAT-3'; VEGF: Forward

5'-TCTACCTCCACCATGCCAAGT-3'; Reverse 5'-GATGATTCTGCCCTCCTCCTT-3'; Human β-actin: Forward 5'-AGCCTCGCCTTTGCCGA-3'; Reverse: 5'-CTGGTGCCTGGGGCG-3'. Real-time PCR was carried out with SYB green master mixture (Promega) using a Rotor-Gene RG-3000 (Corbett Research, Australia) with the following program: pre-heating 95^{o}C 10min; then 40 cycles of 94 ^{o}C 15sec; 58 ^{o}C 30sec; and 72 ^{o}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'-ATGGGCTATGAA CTAATGAC-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^{o}C for 5 min; the cycle of 94 ^{o}C for 45sec; 56 ^{o}C for 1min; and 72 ^{o}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 and drug treatment:

Transduced HeLa cells were cultured and counted, 3 x 10^4 cells in 100 μl medium 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^{TM} reagent (Promega) was added to each well and the cells were incubated for 30-40 minutes at 37^{o}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. For drug sensitivity assay, the transduced cells were seeded in 96-well plates as above and 50 μl fresh medium containing 3 times of the final concentrations (Rapamycin 20nM and SAHA 5μM) of drugs were added to each well. The following Cell Titer-blue assay was same as above.

Animal experiments

Female $Rag^{-/-}$ mice of 6 weeks were used in this study. Transduced HeLa cells were harvested and re-suspended in PBS at 1x 10^7cells /ml. For tumor xeno-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. The animal experiment was 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. **Short-lived RNAi in HeLa cells**

We have previously observed that, comparing with E7 protein reduction in week one after transduction with LV-shRNA, the reduction of E7 protein level at week two was moderated (14). Our first task was therefore to measure shRNA-mediated suppression of E7 over a longer time course to observe if stable and lasting gene silencing can be achieved in cervical cancer cells with the lentiviral vector. Therefore we analyzed E7 protein levels over a 9 week period using HeLa cells transduced with lentivirus-delivered shRNA (targeted to HPV 18 E6 and known as 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 was suppressed at week 1 but returned to baseline by week 3 (Fig 1A). Baseline expression was then observed for the entire 2 months of monitoring. To further confirm

the loss of shRNA suppression after week 1, 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 the levels of E7 protein (Fig 1A). A control lentiviral shRNA against another HPV type, HPV 16E7 (16E7-2), was used as a negative control (36); we observed no changes in E7 protein levels during the same period. These results indicate 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 the loss of gene silencing is the absence of the lentiviral vector in the host cells. To confirm this we examined the expression of the eGFP gene, carried on the lentiviral vector, using FACS. We observed that the mean fluorescent intensity declined remarkably at 3 weeks in 18E6-1 transduced cells and further declined moderately at week 5 though these cells were still eGFP positive compared with negative control (Fig 1C). We had previously shown that eGFP expression was correlated with lentiviral vector copy number and silencing efficacy (14). From this data we assumed that the vector was still present in the host genome and that the loss of suppression was probably related to the cell population with lower eGFP expression after the death of higher eGFP expressing cells through apoptosis (14).

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 demonstrated that shRNA delivered by lentiviral was also short-lived. One strategy to overcome this issue is the multi-copy or multi-target approach which allows additive or even synergistic effects to induce rapid cell death before resistance occurs. To investigate this possibility in cervical cancer we designed two shRNA constructs; one contained two copies of the 18E6-1 shRNA (twin 18E6-1) and the other contained the 18E6-1 shRNA and a shRNA targeting human VEGF-A (dual-target shRNA). VEGF is an angiogenesis factor and plays an important role in tumor formation and growth, therefore

suppression of both genes will in principle result in inhibition of HeLa cell growth and tumor formation and growth. 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 our dual shRNA constructs effectively silence 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 (14), and RNA and protein samples were collected for real-time RT-PCR and Western blot analysis at various times following incubation. 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 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 not 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 have further proved that the dual-target shRNA has an additive or synergistic effect on target gene silencing.

5. Dual shRNA transduced cells increase sensitivity to drug treatments

We previously showed that HeLa cells treated with shRNA against HPV 18 E6/E7 were 4-fold more sensitive to chemotherapeutic drug cisplatin (13). To investigate whether dual shRNA treated HeLa cells would be more sensitive to anti-cancer drugs than single shRNA treated cells, we firstly chose an Akt pathway inhibitor rapamycin to treat the 18E6-1 and twin-18E6-1 transduced cells. The results showed that the increased sensitivity to the drug treatment was observed in only two-copy shRNA treated cells (Fig 5A). To further confirm this result, we used another anti-cancer drug in clinical trials, histone deacetylase inhibitor SAHA which was shown to be able to induce apoptosis in cancer cells (46, 47). The results showed that though single 18E6-1 transduced HeLa cells were sensitive to SAHA treatment, dual shRNA treated cells of both twin and two-target shRNAs were even more sensitive to SAHA treatment (Fig 5B). These data suggest that twin and two-target shRNA have more profound impact on cell survival than their single shRNA counterparts and that for developing combinational therapy twin or two-target shRNA should be considered first.

6. 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 (Fig5 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.

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 would also produce a long-lived 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 to be related to the presence of a cell population with lower eGFP expression (indicating low copy number of lentiviral vector) after the death of higher eGFP expressing cells (high copy numbers of lentiviral vector) through apoptosis as we previously described (14). However, there is another possible explanation from a previous report; that the U6 promoter was not stable in lentivirus-transduced cells, in this case in human primary lymphocytes (40). In our lentiviral vector, the shRNA expression cassette was under the control of a U6 promoter. The U6 promoter can be replaced by the more stable H1 promoter however H1 was shown less effective at gene silencing than U6 (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, the data indicates that in cervical cancer, lentiviral vector can only deliver a short-lived RNAi therapy of about two weeks.

To overcome this issue of lentiviral vector, a multiple shRNA strategy was tested, 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 the twin copy and dual-target shRNA strategy. It is probably not surprising to observe a better suppression from twin 18E6-1 compared with single 18E6-1 as we previously showed that suppression of E7 expression was dose-dependent on the lentiviral shRNA copy numbers. Therefore, one possible argument will be that twin shRNA is replaced by twice transductions with the single shRNA. However, the vector itself can cause side effects on the cells especially when the dose is high (14). Therefore the multi-shRNA strategy has its advantage for clinical application.

In addition, 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 additive or synergistic gene silencing was occurring. *In vitro*, the dual-target shRNA seemed almost equal to or even more effective than twin 18E6-1. *In vivo,* only the 18E6-1-VEGF group had significantly smaller tumors than the controls, suggesting that silencing 18E6 and E7 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 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). It has been also 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 combining target E6/E7 and VEGF is an attractive treatment approach for cervical cancer or other HPV related cancers.

An interesting observation in this study was the relationship between HPV viral oncogene E6/E7 and angiogenesis factor VEGF. Studies have shown that HPV 16 E6 onco-protein can increase VEGF level in cervical cancer cells by activating its promoter (44). It is also known that 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 with the former modulates the latters expression. However, in this study, we showed that silencing VEGF could also affect E7 expression. As far as we are aware, this has not been reported previously. This observation may explain why silencing both genes had significant effect on E7 expression (mRNA and protein levels) and cell growth *in vitro* or even tumor growth *in vivo*. Whether VEGF affects E7 expression through a similar pathway to the E6/E7 modulation of VEGF expression is unclear and would be interesting to investigate further. However, this result suggests that the multiple gene knockdown strategy can be used as a tool to investigate the functional relationship for two or more genes.

Our data proves that the dual shRNA constructs can effectively silence their target genes. 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).

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Conflict of interest:

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

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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 this lasted for 9 weeks (A). No changes in the E7 level were observed in the negative control, HeLa cells transduced with ineffective lentiviral shRNA 16E7-2. In both cases, human β-tubulin (Tub) was 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, FACS result of eGFP expression of 18E6-1 and PLL transduced HeLa cells over a 5-week period. MFI: mean fluorescent intensity. The 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 two-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, with single 18E6-1 shRNA insert; 4, with twin 18E6-1 shRNA insert; 5, two-target shRNA insert.

Figure 3. Gene silencing efficacy of dual shRNA constructs. The real-time RT-PCR results of HPV 18E7 (A) and human VEGF-A (B) gene expression in HeLa cells after transduction of lentiviral shRNAs. Each column represents at least 3 repeats. C shows the Western blotting analysis for 18E7 protein levels in HeLa cells after transduced with LV-shRNAs. E6-1: 18E6-1shRNA; Tw: twin 18E6-1

shRNA; E6-1VE: two-target shRNA; 16E7: 16E7-2. D is the densitometry result of C. **: 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 result of transduced HeLa cells by using the 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. Dual shRNA treated cells are more sensitive to chemotherapeutic drugs. The cell viability assay (Cell–titre blue assay) result (CTB counts) of HeLa cells transduced with LV-shRNA and treated with Akt pathway inhibitor rapamycin (A) and chemotherapeutic drugs SAHA (B). The bars represent four to six repeats of each group of cells treated (+) or non-treated with the drugs. *: P<0.05; **: P<0.01; ***: P<0.001.

Figure 6. Tumor weight and apoptosis analysis of transduced HeLa cells. A shows the result of tumor growth (weight) after 35 days in xeno-transplant $Rag^{-/-}$ mouse model. Each bar represents 5 mice. B. Western blotting result of Bax protein, one of the downstream components of p53 apoptosis pathway, the cell lysate samples were collected from the same sources that were used to inject the mice for tumor growth.