1 Systemic delivery of siRNA by actively targeted polyion complex micelles for
2 silencing the E6 and E7 human papillomavirus oncogenes

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14 **Running title:** Delivery of nucleic acid drugs targeting HPVE6/E7

1   **Abstract**

2   Human papillomavirus (HPV) E6 and E7 oncogenes are essential for the immortalization
3   and maintenance of HPV-associated cancer and are ubiquitously expressed in cervical cancer
4   lesions. Small interfering RNA (siRNA) coding for E6 and E7 oncogenes is a promising
5   approach for precise treatment of cervical cancer, yet a delivery system is required for
6   systemic delivery to solid tumors. Here, an actively targeted polyion complex (PIC) micelle
7   was applied to deliver siRNAs coding for HPV E6/E7 to HPV cervical cancer cell tumors in
8   immune-incompetent tumor-bearing mice. A cell viability assay revealed that both HPV type
9   16 and 18 E6/E7 siRNAs (si16E6/E7 and si18E6/E7, respectively) interfered with
10   proliferation of cervical cancer cell lines in an HPV type-specific manner. A fluorescence
11   imaging biodistribution analysis further revealed that fluorescence dye-labeled siRNA-loaded
12   PIC micelles efficiently accumulated within the tumor mass after systemic administration.
13   Ultimately, intravenous injection of si16E6/E7 and si18E6/E7-loaded PIC micelles was found
14   to significantly suppress the growth of subcutaneous SiHa and HeLa tumors, respectively. The
15   specific activity of siRNA treatment was confirmed by the observation that p53 protein
16   expression was restored in the tumors excised from the mice treated with si16E6/E7- and
17   si18E6/E7-loaded PIC micelles for SiHa and HeLa tumors, respectively. Therefore, the
18   actively targeted PIC micelle incorporating HPV E6/E7-coding siRNAs demonstrated its
19   therapeutic potential against HPV-associated cancer.

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21   **Keywords:**

22   Human papillomavirus (HPV) E6/E7
23   Small interfering RNA (siRNA)
24   Polyion complex (PIC) micelle
25   Cervical cancer
26   Drug delivery system

1. Introduction

Cervical cancer is the leading cause of cancer mortality among women in developing countries. Chronic viral proliferation of HPV induces the active proliferation of infected epithelial cells, and some infected cells are incidentally immortalized. The immortalization of HPV-infected cells is accompanied by upregulation of HPV viral oncogenes E6 and E7. HPV types 16 and 18 together account for more than 70% of cervical cancer cases and also develop cancers in other organs, such as vaginal wall, vulva, anus, penis and oropharynx [1]. The combined actions of HPV oncoproteins E6 and E7 are essential for the maintenance of the neoplastic phenotype and the evasion of apoptosis in the HPV-associated cancer cells. E6 binds to cellular ubiquitin ligase E6AP and, synergistic with E6AP, leading to p53 degradation. E6 also activates hTert expression, followed by promotion of anti-apoptotic signaling. E7 binds to and inactivates pRb, resulting in promotion of cell cycles. E7 also binds to several other cellular factors to promote cell cycle. Indeed, E6 and E7 oncogenes are ubiquitously expressed in cervical cancer and the pre-cancer lesions but not in normal cells [2]. Therefore, these viral oncogenes are one of the most reliable candidates for gene silencing therapy for HPV-associated cancers.

Oligonucleotide drugs, including small interfering RNA (siRNA) [3], are one of the most promising formulations to specifically suppress the viral oncogenes. Indeed, several previous studies have reported that the gene silencing of E6 and E7 can induce the apoptosis of cervical cancer cell lines [4–9], suggesting that E6 and E7 oncogenes are attractive targets for selective HPV-associated cancer therapy. In this regard, it is demonstrated that the intra-tumoral injection of siRNA coding for HPV16 and HPV18 E6/E7 (si16E6/E7 and si18E6/E7, respectively) elicited the significant growth inhibition for tumor-bearing SiHa, CaSki and SKG-II [7–9]. Nevertheless, cervical cancer treatment via systemic administration of siE6/E7 still remains to be a major challenge because the negatively charged macromolecular structure of siRNA prevents passive diffusion into the cytoplasm across the cell membranes and also is rapidly eliminated from the bloodstream through renal filtration and enzymatic degradation [10, 11]. To overcome this drawback of siRNA, delivery vehicles have been extensively developed to protect siRNA from enzymatic degradation during the circulation and accelerate the tumor tissue accumulation as well as the uptake of siRNA by cancer cells, by engineering functional materials from cationic lipids, peptides, and polymers [10, 11]. Among them, core-shell type polyion complex (PIC) micelles, which can be constructed through the self-assembly of block cationomers with oppositely charged oligonucleotides, have been engineered as a promising platform for systemic siRNA delivery [12, 13]. The PIC micelles have a biocompatible outer shell of poly(ethylene glycol) (PEG) for reduced nonspecific protein adsorption, allowing the stealthiness in the bloodstream. However, this stealthiness of PIC micelles often compromises their cellular internalization by

targeted cells (termed PEG dilemma) [13]. To overcome the PEG dilemma, we have recently developed an actively targeted and disulfide cross-linked PIC micelle, which was prepared from a block cationomer of cyclo(arginine-glycine-aspartic acid) peptide (cRGD)-installed PEG and poly(L-lysine) (PLL) modified to contain thiol-containing functional groups [14–16]. This “targeted” PIC micelle had a well-defined size of 35 nm in diameter and increased stability through disulfide cross-links in biological milieu. Notably, the cRGD ligands conjugated on the micellar surface dramatically facilitated the uptake of siRNA payloads by cultured HeLa cell, a cervical cancer cell line, which overexpresses $\alpha v \beta 3$ and $\alpha v \beta 5$ integrin receptors on the cell-surface. Ultimately, the significantly enhanced accumulation of siRNA in the subcutaneous tumors was achieved by systemic administration of the targeted micelles without changes in siRNA accumulation in healthy organs under the comparison with non-targeted control micelles, leading to the significant gene silencing in the tumor tissues [15, 16].

Considering the advantages of tumor-targeted (or cRGD-conjugated) PIC micelles and the cancer-cell specific molecular target of E6/E7 viral oncogenes, the micellar delivery of siE6/E7 is a promising approach for precise treatment of HPV-associated cancers. As far as we know, there is no study to address systemic delivery of siE6/E7 using a polymeric nanocarriers to cervical cancer models. Herein, we demonstrate that siE6/E7 encapsulated in the targeted PIC micelle can be efficiently delivered into the subcutaneous tumor tissues established from cervical cancer cells by intravenous injection and significantly suppress the tumor growth in the mouse model.

2. Materials and Methods

2.1. Materials

α -3,3-Diethoxy-1-propyl- ω -propylamine poly(ethylene glycol) (acetal-PEG-NH₂, M_w = 12,000) and α -methoxy- ω -propylamine poly(ethylene glycol) (MeO-PEG-NH₂, M_w = 12,000) were purchased from NOF corporation (Tokyo, Japan). ϵ -Trifluoroacetyl-L-lysine *N*-carboxy anhydride (Lys(TFA)-NCA) was prepared by the Fuchs-Farthing method using triphosgene [17]. Dimethyl-3,3'-dithiobispropionimidate 2HCl (DTBP) and 2-iminothiolane HCl (IT) were purchased from Thermo Scientific (Rockford, IL). Cyclo-[RGDfK(C- ϵ -Acp) peptide (cRGD peptide) was purchased from Peptide Institute Inc. (Osaka, Japan). Dithiothreitol (DTT) and 5,5-dithio-bis-(2-nitrobenzoic acid) (Ellman's reagent) were purchased from Wako Pure Chemical Industries (Osaka, Japan). All siRNAs used in this study were synthesized by Hokkaido System Science (Hokkaido, Japan) or Gene Design (Osaka, Japan) and their sequences are as follows. 18E6/E7 siRNA (si18E6/E7) sense: 5'-CAUUUACCAGCCCGACGAGTT-3' and antisense: 5'-CUCGUCGGGCUGGUAAAUGTT-3' [2], 16E6/E7 siRNA (si16E6/E7) sense: 5'-GAC

CGG UCG AUG UAU GUC UUG-3' and antisense: 5'-AGA CAU ACA UCG ACC GGU CCA-3' [5], scramble siRNA (siScr) sense: 5'-UUC UCC GAA CGU GUC ACG UdTdT-3' and antisense: 5'-ACG UGA CAC GUU CGG AGA AdTdT-3' and GL3 luciferase siRNA (siGL3) sense: 5'-CUU ACG CUG AGU ACU UCG AdTdT-3' and antisense: 5'-UCG AAG UAC UCA GCG UAA GdTdT-3'. Cy5-labeled siRNA (Cy5-siRNA) and Alexa647-labeled siRNA (Alexa647-siRNA) were obtained from Hokkaido System Science and Gene Design, respectively. Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (NY, USA). Fetal bovine serum (FBS) was purchased from Gibco (NY, USA). Lipofectamine 2000 Transfection Reagent and OptiMEM-I Reduced Serum Media were purchased from Thermo Fisher Scientific (Waltham, MA). Cell Counting Kit 8 (CCK8) was purchased from Dojindo Laboratories (Kumamoto, Japan). CellLytic™-MT Mammalian Tissue Lysis/Extraction Reagent was purchased from Sigma-Aldrich (St. Louis, MO). HeLa, SiHa, and C33A cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). BALB/c nude mice were purchased from Charles River Laboratories (Tokyo, Japan). All animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals as stated by The University of Tokyo.

2.2. Polymer synthesis and characterization

A ligand-installed block copolymer, cRGD-PEG-PLL (molecular weight of PEG: 12,000 Da, degree of polymerization of PLL: 46), was synthesized according to our previous reports [14–16]. The detailed synthetic procedure was described in Supporting Information. The PLL side chains in cRGD-PEG-PLL were functionalized with 1-(3-mercaptopropyl)amidine (MPA) and IT as follows. cRGD-PEG-PLL was dissolved in 100 mM sodium borate buffer (pH 9.0) at 10 mg/mL polymer concentration. DTBP (0.1 molar equivalent to the amine in PLL) was dissolved in 0.5 mL of cool water and added to the polymer solution dropwisely. The reaction mixture was stirred for 45 min at room temperature. Subsequently, IT (2.4 molar equivalent to the amine in PLL) was added to the reaction mixture directly and stirred for 30 min at room temperature. The reaction mixture was warmed up to 35 °C and stirred over 6 h. The solution was put into a dialysis tube (molecular weight cutoff: 6,000–8,000) and dialyzed against 10 mM phosphate buffer (pH 7.4) for 30 min. DTT (2 molar equivalent to the amine in PLL) was added to the solution and reacted for 30 min. The reaction solution was dialyzed against 150 mM NaCl solution for 2 h and then de-ionized water for 2 h. The dialyzed solution was frozen with liquid nitrogen and lyophilized to obtain the final product. The composition of the obtained polymer was determined from the ¹H-NMR spectrum (Figure S2). The introduction rates of MPA and IM moieties were calculated to be 7 and 36 from the peak intensity ratios of ethylene protons in PEG (-CH₂CH₂O-, δ = 3.7) to mercaptoethyl protons in MPA (HS-(CH₂)₂-, δ = 2.7–2.9) and PEG protons to ethylene group in IT (-CH₂CH₂CH₂S-, δ

=) 2.4), respectively.

2.3. Polyion complex (PIC) micelle preparation and characterization

cRGD-PEG-PLL(MPA/IM) was dissolved at a concentration of 5 mg/mL in 10 mM HEPES buffer (pH 7.4). The polymer solution was mixed with 100 mM DTT for 15 min at room temperature to reduce disulfide bonds contained in cRGD-PEG-PLL(MPA/IM). siRNA solution (15 μ M in 10 mM HEPES buffer (pH 7.4)) was mixed with the polymer solution at a mixing ratio of 2:1 (v/v) for preparation of PIC micelles. To remove DTT and facilitate disulfide cross-linking in the micellar core, the mixed solution was dialyzed against 5 mM HEPES buffer (pH 7.4) with 0.5% DMSO (v/v) for 1 day and 5 mM HEPES buffer (pH 7.4) for another 1 day. The 90% conversion of thiol groups to disulfide bonds was confirmed by Ellman's method, as previously described [15,16]. The hydrodynamic diameter and polydispersity indices (PDIs) of PIC micelles were determined at 25 °C by dynamic light scattering (DLS) using ZetaSizer Nano ZS instrument (Malvern Instruments Ltd., Worcestershire, UK). The ζ -potential of PIC micelles was also determined at 25 °C using the same apparatus. The obtained cumulant diameter, PDI, and ζ -potential of a series of PIC micelles are expressed as mean and standard deviation ($n = 3$) in Table 1. The size distribution histograms are also shown in Figure S3.

2.4. In vitro RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

HPV16-positive SiHa and HPV18-positive HeLa cells, cervical cancer cell lines, (30,000 cells) were seeded onto 24-well plate and allowed to attach for 24 h in 0.5 mL DMEM containing 10% FBS/penicillin/streptomycin under 37 °C and 5% CO₂. Then, the media were replaced with fresh one and treated with siRNA samples using Lipofectamine 2000, according to the manufacturer's protocol, at 20 nM siRNA under the same incubation condition. After 48 h, the siRNA-containing media were replaced with fresh one. Then, total RNAs were extracted from the cells using an RNeasy Mini Kit (QIAGEN, Hilden, Germany). Extracted RNAs were reverse transcribed using an RT-PCR kit (TOYOBO, Osaka, Japan), according to the manufacturer's instruction. To assess mRNA expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), E6, and E7 (HPV16 and HPV18), qRT-PCR was performed using a Light Cycler 480 (Roche Diagnostics, Mannheim, Germany). Expression of E6 and E7 mRNAs was normalized to that of GAPDH mRNA as an internal standard. Primer pairs for E6, E7 (HPV16 and HPV18), and GAPDH are shown in Table S1. The PCR conditions for GAPDH, E6, and E7 were as follows: for GAPDH, 35 cycles at 95 °C for 10 s, 64 °C for 10 s and 72 °C for 18 s; for HPV16 E6, 35 cycles at 95 °C for 10 s, 61 °C for 10 s and 72 °C for 8 s; for HPV16 E7, 35 cycles at 95 °C for 10 s, 60 °C for 10 s and 72 °C for 12 s; for HPV18 E6, 35 cycles at 95 °C for 10 s, 64 °C for 10 s and 72 °C for 5 s; for HPV18E7, 35 cycles at 95 °C

for 10 s, 66 °C for 10 s and 72 °C for 13 s. All PCR reactions were followed using a melting curve analysis.

2.5. *In vitro* cytotoxicity assay

SiHa (HPV16-positive), HeLa (HPV18-positive) or C33A (an HPV-negative cervical cancer cell line) cells were seeded onto 24-well plates (10,000-20,000 cells/well). They were allowed to attach for 24 h in 0.5 mL DMEM containing 10% FBS/penicillin/streptomycin under 37 °C and 5% CO₂. Then, the media were replaced with fresh one and treated with siRNA samples using Lipofectamine 2000, according to the manufacturer's protocol, at 20 nM siRNA under the same incubation condition. After 8 h, the siRNA-containing media were replaced with fresh one. After 48 h, the cell metabolism was assessed by CCK8 solution (1 µL/10 µL media), according to the manufacturer's instruction. The absorbance at 480 nm was recorded using Epoch (BioTek Instruments, Winooski, VT) and normalized to that obtained from untreated control cells (n = 6).

2.6. *Tumor accumulation*

HeLa tumors were prepared by *in vivo* passage of solid tumor fragments. Donor tumors were prepared by subcutaneous injection of HeLa cells (2.0×10^6 cells) to 6 weeks old female BALB/c nude mice and allowed to mature for 2 weeks. The donor tumors were excised and cut into 3 × 3 mm pieces. The tumor fragments were transplanted subcutaneously to 6 weeks old female BALB/c nude mice. On day 9 after tumor transplantation, micelle samples prepared with Alexa647-siRNA (20 µg) were intravenously administered from the tail vein. At 24 h after systemic injection, tumors were excised and their fluorescence intensity was measured using an IVIS instrument (PerkinElmer, Waltham, MA).

2.7. *Intravital confocal microscopy*

The tumor distribution of the micelles was assessed using intravital confocal microscopy [18–19]. Two subcutaneous tumor models were prepared. BALB/c nude mice (4 weeks old) were inoculated subcutaneously with HeLa cells (2.0×10^6 cells per mouse). SHO mice (6 weeks old) were inoculated subcutaneously with SiHa cells (2.0×10^7 cells per mouse). Both tumor models were allowed to mature for 2-3 weeks. Cy5-siRNA-loaded cRGD-conjugated micelles were injected intravenously (20 µg Cy5-siRNA/mouse) into mice bearing HeLa or SiHa tumors. At 24 h after injection, mice were anesthetized and intravenously injected with DyLight488-labeled lectin derived from *lycopersicon esculentum* (Vector Laboratories, Burlingame, CA) to stain the endothelial cells. Then, the tumor was exposed by a series of dorsal cuts in the skin surrounding the tumor to create a hinged skin flap with tumor attached to the skin, while leaving blood vessels feeding the tumor intact [15]. The exposed tumor was

1 mounted under a cover slip and imaged using intravital confocal laser scanning microscopy
2 (IVCLSM, Nikon A1R, Nikon Corp., Tokyo, Japan) equipped with a Nikon CFI Apo Lambda
3 S LWD 40x WI NA 1.15 objective. Images were analyzed using the Nikon NIS-Elements C
4 software provided by the manufacturer.

5 6 2.8. Antitumor activity assay

7 In order to evaluate the *in vivo* antitumor activity of siE6/E7-loaded PIC micelles,
8 subcutaneous xenograft models were established by transplanting HeLa into BALB/c nude
9 mice or SiHa into SHO (hairless SCID) mice. Donor tumors were prepared by subcutaneously
10 injecting cells (HeLa: 2.0×10^6 cells, SiHa: 2.0×10^7 cells) into 6 weeks old female mice and
11 allowed to mature for suitable weeks (HeLa: 2 weeks, SiHa: 6 weeks). After that, donor
12 tumors were excised and cut into 3×3 mm pieces. Tumor fragments were subcutaneously
13 transplanted to 6 weeks old female mice. At 5 days after implantation, the mice were treated
14 with siRNA samples (20 μ g/body/shot) (n = 5–6), siE6/E7-loaded micelles, siScr-loaded
15 micelles, and 10 mM HEPES buffer as controls. The intravenous administration was
16 performed for HeLa tumors on day 0, 1, 3, 4, 6, and 7, and for SiHa tumors on day 0, 1, 2, 4,
17 5, 6, 8, 9, and 10. Tumor volumes were measured on day 0, 3 (or 4), 6, 8, 10, and 12. Tumor
18 volume was calculated as $1/2a \times b^2$ (a: major axis, b: minor axis).

19 20 2.9. TUNEL assay

21 After the antitumor activity assay of HeLa tumors described in 2.8, the kidney was excised
22 on day 12 for evaluation of the renal toxicity after treatment. Paraffin embedding was made
23 from the excised kidney. The cell apoptosis was evaluated by TUNEL staining, where
24 ApopTag Peroxidase *In Situ* Apoptosis Detection Kit (Millipore, Billerica, MA) was used for
25 brown coloring of apoptotic cells.

26 27 2.10. Western blot analysis

28 On day 12 in *in vivo* experiment, tumors were excised from mice. Proteins were extracted
29 from the excised tumors using CelLytic^{TM}-MT Mammalian Tissue Lysis/Extraction Reagent
30 with a protease inhibitor (Roche, Basel, Switzerland). Protein concentrations were determined
31 using a Protein Assay Kit (BioRAD, Hercules, CA). Antibodies to p53 (DO1, Santa Cruz
32 Biotechnology, Santa Cruz, CA) were used for immunoblotting, as recommended by the
33 manufacturer. For detection of p53 protein, primary antibodies were labeled with HRP using
34 the appropriate Zenon HRP Mouse IgG_{2a} labeling kit (Thermo Fisher Scientific). Signals were
35 detected using BioRAD western blotting systems (BioRAD) with the chemiluminescent HRP
36 Substrate (Immobilon^{TM} Western; Millipore corporation, Billerica, MA).

2.11. Apoptosis detection assay in cultured cervical cancer cells

HeLa and SiHa cells (2×10^3 cells/well) were seeded in an 8-well Lab-Tek chambered borosilicate cover glass (Nalge Nunc International, Rochester, NY) in 200 μ L DMEM containing 10% FBS/penicillin/streptomycin and incubated for 1 day. siE6/7- or siGL3-loaded PIC micelles were added to the cells in an siRNA concentration of 200 nM. The cells were cultured for 48 h at 37 °C. Then, the medium was removed and washed gently with PBS twice, followed by adding 200 μ L of fresh medium containing 3 μ L of FAM-FLICA® Caspase 3 and 7 Assay Kit (ImmunoChemistry Technologies, Bloomington, MN). The cells were further incubated for 1 h at 37 °C. After the cells were washed with fresh DMEM containing 10% FBS/penicillin/streptomycin 3 times, Hoechst 33342 solution was added to the cells and incubated for 5 min at 37 °C. The medium was then removed and the cells were washed with fresh DMEM containing 10% FBS/penicillin/streptomycin twice. The cells were observed using a confocal laser scanning microscope, LSM510 (Carl Zeiss) with excitation of Hoechst 33342 and FLICA at 710 nm (MaiTai laser, two photon excitation) and 488 nm (Ar laser), respectively. Data were analyzed by the LSM imaging software, Zen (Carl Zeiss).

2.12. Statistical analysis

All data are expressed as mean $\pm$ standard deviation (SD). The *p* values were determined by the Student's *t* test using a two-tailed distribution and two-sample un-equal variance with the *t*-test function of Microsoft Excel. The *p* values of less than 0.05 were considered as statistically significant.

3. Results and Discussion

3.1. In vitro E6/E7 gene silencing and its suppressive effect on cervical cancer cell proliferation

E6 and E7 transcripts are generated by RNA splicing from a single E6/E7 transcript (pre-mRNA), which is transcribed using a common promoter [20]. In this study, two E6/E7-targeting siRNAs, HPV16 E6/E7-targeting (si16E6/E7) and HPV18 E6/E7-targeting (si18E6/E7) were tested, referring the siRNA sequences by which previous studies demonstrated the depletion of both E6 and E7 transcripts [2, 5]. Although both sequences of the siRNAs map the E7 open reading frame, the siRNAs have been reported to silence either E6 or E7 gene by depletion of E6/E7 pre-mRNA and/or E6 and E7 transcripts [21–23]. The si16E6/E7, si18E6/E7 or control siRNA (siScr or siGL3) were firstly transfected into SiHa and HeLa cells, respectively, by Lipofectamine 2000, according to the manufacturer's instruction. The depletion of E6 and E7 transcripts was compared between siE6/E7 and irrelevant siRNA (siScr) by measuring E6 and E7 mRNA levels in the cells by qRT-PCR (Figure 1). For si16E6/E7, mRNA levels of both E6 and E7 in siE6/E7-treated SiHa cells

were decreased significantly compared with that in siScr-treated controls. For si18E6/E7, both mRNA levels in siE6/E7-treated HeLa cells were also decreased significantly compared with that in siScr-treated controls. Therefore, each siE6/E7 was confirmed to work for sequence-specific RNA interference in the cervical cancer cells in which the corresponding type of HPV maintains cell proliferation.

Figure 1. E6 and E7 mRNA expression levels in cultured SiHa cells (A) and HeLa cells (B) determined by qRT-PCR. The cells were treated with siE6/E7RNA or controls using Lipofectamine 2000 at an siRNA concentration of 20 nM for 6 h and further incubated for 48 h without siRNA. The expression levels of E6 and E7 mRNA were normalized to that of GAPDH. Results are expressed as mean $\pm$ SD (n = 6, *: $p < 0.05$).

Next, HPV type-specific pharmacological action of siE6/E7 was assessed in several cervical cancer cell lines, SiHa (HPV16-positive), HeLa (HPV18-positive), and C33A (HPV-negative) cells. si16E6/E7, si18E6/E7, and control siRNA (siScr or siGL3) were transfected into these three cell lines. The cell viability of each cell line was quantitatively evaluated by a colorimetric cell viability assay 48 h after transfection. si16E6/E7 treatment suppressed significantly the viability of SiHa cells, but neither HeLa cells nor C33A cells. By contrast, si18E6/E7 treatment significantly suppressed the viability of HeLa cells, but neither SiHa cells nor C33A cells. The suppressive effect of siE6/E7 on cell viability was obtained in an HPV type-specific manner, although comparison in suppressive efficiency between si16E6/E7 and si18E6/E7 might be difficult due to different cell lines. Together with the data shown in Figure 2, the siE6/E7s used here were demonstrated to have the ability to suppress the proliferation of HPV-associated cervical cancer cells through depletion of E6 and E7 transcripts.

Figure 2. Viability of cultured cervical cancer cells after treatment with si16E6/E7 (A–C) and si18E6/E7 (D–F) or controls using Lipofectamine 2000 at an siRNA concentration of 20 nM. The cells were incubated for 6 h in the transfection medium containing siRNA and additionally incubated for 48 h in the medium without siRNA. The metabolic activity of cells was determined by CCK8 and normalized to that of non-treated cells. Results are expressed as mean $\pm$ SD (n = 6, *: $p < 0.05$).

3.2. Preparation and characterization of siRNA-loaded PIC micelles

For construction of the targeted siE6/E7-loaded PIC micelles, a cRGD-installed block cationomer was synthesized from PEG-PLL bearing acetal group at the α -end of PEG segment, according to the previously reported protocol [14–16]. The quantitative introduction of cRGD moiety was confirmed from the peak intensity ratio between ethylene groups in PEG and benzyl group in cRGD ($C_6H_5CH_2-$) at 7.2–7.5 ppm in the ^1H NMR spectrum (data not shown). The PLL side chains in cRGD-PEG-PLL were further functionalized with MPA and IM moieties to generate disulfide cross-linking as well as hydrophobic (or dipole) interaction in the micellar core for micelle stabilization [15, 24]. The modification rates of MPA and IM were determined to be 7 and 36 from the peak intensity ratios of ethylene groups in PEG to ethylene groups in MPA, and ethylene groups in PEG to ethylene group in IT, respectively, in the ^1H NMR spectrum (Figure S2). The obtained polymer, cRGD-PEG-PLL(MPA/IM), was mixed with siRNA in 10 mM HEPES buffer (pH 7.4) at a residual molar ratio (*i.e.* primary amines and amidines in block cationomer to phosphates in siRNA) of 1.8 to form siRNA-loaded

PIC micelles. Of note, this residual molar ratio was selected for preparation of monodispersed PIC micelles, according to our previous study [15, 24]. Indeed, the micelle formation with narrow size distributions (PDI = ~0.1) was confirmed by DLS for a series of micelle samples used in this study (Table 1 and Figure S3).

Also, the PIC micelles were characterized to have a cumulant diameter of ~40 nm and almost neutral ζ -potential (Table 1), consistent with the formation of micellar architecture featuring the nonionic and hydrophilic PEG palisade [24]. This PEG palisade is expected to compromise the adsorptive endocytosis of micelles by nontarget cells as well as the capture by reticuloendothelial system during systemic circulation [12, 13]. On the other hand, cRGD peptide ligands were conjugated on the micellar surface (or PEG termini) to accelerate the preferential cellular uptake of micelles by the target cancer and cancer-related endothelial cells that overexpress the $\alpha_v\beta_3/\alpha_v\beta_5$ integrins for overcoming the so-called PEG dilemma [25, 26]. It should be noted that the similar physicochemical properties were observed among siScr-, si16E6/E7-, and si18E6/E7-loaded micelles, suggesting that siRNA payload sequence did not elicit substantial changes in the micelle structure.

Table 1. Cumulant diameter, PDI, and ζ -potential of siRNA micelles. Results are expressed as mean $\pm$ SD (n = 3).

siRNA	cRGD	Cumulant diameter (nm)	PDI	ζ -potential (mV)
siScr	+	35 $\pm$ 0.6	0.10 $\pm$ 0.01	-1.0 $\pm$ 0.2
si16E6/E7	+	35 $\pm$ 0.3	0.11 $\pm$ 0.00	-0.6 $\pm$ 0.1
si18E6/E7	+	36 $\pm$ 0.1	0.10 $\pm$ 0.01	-1.1 $\pm$ 0.2

3.3. Accumulation of targeted PIC micelles in subcutaneous tumors

With regard to tumor-bearing animal models for HPV-associated cancers, most of previous studies have utilized subcutaneous tumor derived from cervical cancer cell lines, including SiHa and HeLa, for *in vivo* evaluation of therapeutic effects of anticancer agents [5, 21, 27]. In the treatment for cervical cancer, systemic chemotherapy using anti-cancer drug is usually performed as first line therapy for systemically-advanced stage patients with distant metastases. The subcutaneous tumor is thought to be one of the metastatic lesions from human cervical cancer and clinically treated by systemic chemotherapy. Considering the clinical application, subcutaneous tumor-bearing mouse model was appropriate for evaluation of systemic therapy against cervical cancer, although the mouse model had limitation that the tumor was not metastatic but artificially formed.

For tumor-targeted systemic siRNA delivery, siRNA-loaded PIC micelles were conjugated with cRGD peptide ligands for specific binding to the cancer and cancer-related endothelial cells that overexpress $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins on their surface [25, 26]. To confirm the micelle accumulation in a cervical cancer model, Alexa647-siRNA was encapsulated into PIC micelles and administered intravenously into the mice bearing a subcutaneous HeLa tumor. At 24 h after administration, the tumors were resected and assessed for their fluorescence intensity by an imaging analyzer. The obtained images show that the fluorescence intensity of Alexa647 dyes was high (from green to red) in all tumors from the mice treated with the targeted micelles (upper panel), whereas barely detectable (from blue to green) in tumors treated with naked siRNA (middle panel) or without treatment (lower panel) (Figure 3A). The quantitative analysis displays that the fluorescence intensity of Alexa647 dyes in tumors from the mice treated with the targeted micelle was significantly higher than those treated with naked siRNA or without treatment (Figure 3B). These result demonstrates that the targeted micelle more efficiently delivered siRNA payloads to the HeLa tumor via intravenous administration, probably due to the targetability derived from cRGD ligands as well as protection of siRNA from the rapid renal clearance [15, 16]. Of note, there is no significant difference in the fluorescence intensity between naked siRNA-treated tumors and non-treated tumors, indicating negligible accumulation of naked siRNA (or Alexa647 dyes) in the tumor because of its rapid renal clearance as well as enzymatic degradation.

Figure 3. Accumulation of Alexa647 dye in subcutaneous HeLa tumors. (A) IVIS images of excised tumors 24 h after injection of Alexa647-siRNA (20 µg/mouse) in naked and micelle formulations or non-treated tumors. (B) Fluorescence intensity of Alexa647 in HeLa tumors, determined from IVIS images. Results are expressed as mean ± SD (n = 3, *: $p < 0.05$).

Next, the micelle localization in tumor tissues was further observed by IVCLSM imaging

in mice at 24 h post-injection of Cy5-siRNA-loaded targeted micelles. In this experiment, DyLight488-labeled lectins were used to stain endothelial cells. Apparently, the fluorescence signals of Cy5 dye (red) were not colocalized with those of lectins (green) and observed outside of the blood vessels in both tumor tissues (Figure 4). These results indicate that the targeted micelles were more effectively internalized by cervical cancer cells, compared with endothelial cells, 24 h after systemic administration.

Figure 4. IVCLSM images of subcutaneous HeLa (A) and SiHa (B) tumors 24 h after systemic injection of Cy5-siRNA-loaded targeted micelles (20 μ g siRNA/mouse). Endothelial cells were stained in green with DyLight488-labeled lectins and Cy5 signals were shown in red.

3.4. Tumor suppressive effect of intravenously administered siE6/E7-loaded micelle

Both the inhibitory effect of siE6/E7 on cervical cancer cell proliferation and the enhanced tumor accumulation of siE6/E7 by targeted PIC micelles encouraged us to further examine the antitumor effect of siE6/E7-loaded PIC micelles for the cervical tumor-bearing mouse models. The si18E6/E7- and si16E6/E7-loaded micelles were intravenously injected into HeLa tumor- and SiHa tumor-bearing mice, respectively. Relative tumor volumes of HeLa and SiHa tumors were measured on the designated days by three-dimension measuring method (Figure 5). In the mice treated with buffer or siScr-loaded micelles, the mean tumor volumes similarly increased from day 3–4 until day 12, and eventually became 12-fold and 8-fold larger than those on day 0 in HeLa and SiHa tumors, respectively. By contrast, in the mice treated with siE6/E7-loaded micelles, the suppression of tumor growth was clearly observed compared with buffer- or siScr-treated controls in both tumor models. Ultimately, the mean tumor volumes after treatment with siE6/E7-loaded micelles were approximately 4-fold and 3-fold larger than those on day 0 in HeLa and SiHa tumors, respectively. The significant difference in tumor volumes between siE6/E7- and siScr-loaded micelles was observed on day 8, 10, and

12 in both tumor models. Therefore, it is demonstrated that intravenous administration of siE6/E7-loaded micelles significantly suppressed the tumor growth in two different cervical cancer models in an siRNA sequence-dependent manner. These results are consistent with the enhanced tumor accumulation of siE6/E7 delivered by targeted PIC micelles (Figures 3 and 4). In our *in vivo* experiment, the treatment regimens were set at six doses (two doses per three days) for HeLa tumors and nine doses (three doses per four days) for SiHa models. The six doses for HeLa tumors were determined according to our previous study, where the cRGD-conjugated micelles incorporating siRNAs for vascular endothelial growth factor and its receptor demonstrated the significant antitumor activity after six doses of 20 μ g siRNA [15]. We initially set six doses of the si16E6/E7-loaded micelles for SiHa models as well, but the anti-tumor growth effect on SiHa model was barely observed but the difference was not statistically significant (Figure S4). Then, we changed the treatment regimen for si16E6/E7-loaded micelles and SiHa model.

15
16 **Figure 5.** Antitumor activity of si18E6/E7 and si16E6/E7-loaded PIC micelles for
17 subcutaneous cervical cancer models derived from (A) HeLa cells and (B) SiHa cells,
18 respectively. Arrows indicate the day for systemic administration of samples (20 μ g
19 siRNA/mouse/shot). Results are expressed as mean $\pm$ SD (n = 5–6). *: $p < 0.05$ for
20 buffer-treated control.

21
22 Systemic administration of siE6/E7- and siScr-loaded micelles showed no significant body
23 weight loss in the treated mice, compared with buffer-treated control mice (Figure S5),
24 suggesting that the targeted micelles might have negligible systemic toxicity under the tested
25 condition. In addition, the kidney was excised from HeLa tumor-bearing mice treated with
26 buffer, siScr-loaded micelles, and siE6/E7-loaded micelles on day 12, and embedded in

paraffin for TUNEL staining to estimate the renal toxicity. This is because our previous studies demonstrated that siRNA (or degraded products) mainly accumulated in the kidney after systemic administration in micellar formulations [15, 16, 24]. Apparently, apoptotic cells were not observed in all the kidney tissues (Figure S6), suggesting that the PIC micelles might elicit no significant tissue damage.

3.5. Biological effects of siE6/E7-loaded micelles on cervical cancer cells

To validate whether antitumor effects of siE6/E7-loaded micelles were caused by RNA interference, subcutaneous tumors were excised on day 12 and examined on mRNA levels of E6 and E7 in the excised tumors by qRT-PCR. E6 and E7 transcripts were decreased in both HeLa and SiHa tumors in the mice treated with siE6/E7-loaded micelles compared with siScr-loaded micelles, even though significant difference was observed only for E7 mRNA in SiHa tumors (Figure S7). Thus, we additionally examined the amount of p53 in SiHa and HeLa tumors by western blotting, because p53 degradation by HPV E6 oncoprotein can be suppressed by depletion of E6/E7 transcripts. While p53 was barely detected in the HeLa tumors treated with siScr-loaded micelles or buffer, it was rescued clearly in those treated with si18E6/E7-loaded micelles (Figure 6A). Similarly, p53 was detectable only in the SiHa tumors treated with si16E6/E7-loaded micelles (Figure 6B). These results suggest that the antitumor effect of siE6/E7-loaded micelles was the result from the cell death of cervical cancer cells by p53-induced apoptosis. Thus, the apoptotic change was further validated by immunocytochemistry in cultured HeLa and SiHa cells after incubation with si18E6/E7- and si16E6/E7-loaded micelles, respectively (Figure 7). In the obtained CLSM images, the activated caspase 3/7 and the nuclei are shown in green and blue, respectively. Apparently, more green spots are observed in both HeLa and SiHa cells treated with siE6/E7-loaded micelles when compared with siScr-loaded micelles or buffer control. These indicated that the siE6/E7-loaded micelles shifted two cervical cancer cells to p53-induced apoptosis as well as that our targeted micelle itself did not induce cell death meaning the negligible cytotoxic effects.

1 **Figure 6.** Western blotting of p53 in (A) HeLa and (B) SiHa tumors treated with si18E6/E7
2 and si16E6/E7-loaded micelles, respectively. The tumors were excised on day 12 in Figure 5
3 and subjected to the analysis described in 2.10.

4

5

6 **Figure 7.** Apoptosis detection assay in (A) HeLa and (B) SiHa cells treated with buffer,
7 siScr-loaded micelles, or siE6/E7-loaded micelles at an siRNA concentration of 200 nM. The
8 cancer cells were incubated with the samples for 48 h, followed by the apoptosis (or activated
9 caspase 3 and 7) detection using FAM-FLICA Assay Kit.

10

11 **4. Conclusion**

12 In this study, a cRGD-conjugated PIC micelle was prepared from siRNAs with functional
13 block cationomers to silence the HPV oncogenes for treatment of HPV-associated cervical
14 cancers. This micelle formulation was designed to reduce nonspecific interactions with
15 biological components, target the cancer cell surface, and trigger the intracellular release of
16 siRNA. Systemic administration of siE6/E7-loaded micelles resulted in the significant tumor
17 growth inhibition for two different cervical cancer tumors, associated with the
18 sequence-specificity of siRNA. These antitumor effects of siE6/E7-loaded micelles were in
19 good agreement with the enhanced accumulation of siRNA payloads and the p53 recover in
20 the tumor. The obtained results suggest that the present HPV oncogene-targeted
21 siRNA-loaded micelles may be a promising therapeutic candidate for a wide variety of

1 HPV-associated cancers, including oropharyngeal, penile, vaginal, vulvar, and anal cancers.

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*Graphical Abstract