ORIGINAL ARTICLE

Enhanced specificity of HPV16 E6E7 siRNA by RNA–DNA chimera modification

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Although efforts have been made to develop new drugs for infectious and neoplastic diseases utilizing synthetic small interfering RNA(siRNAs), those intrinsically have undesirable effects, including silencing of unintended genes (off-target effect) and nonspecific cytotoxicity. Off-target effects can be avoided by DNA substitution in the guide strand (GS) seed region of nucleotide positions 1–8 and its complementary part of the passenger strand plus the 3′ overhang, which is designated as a double-strand RNA–DNA chimera (dsRDC). In this study, we found that the specificity of potent siRNAs targeting human papillomavirus 16 (HPV16) E6 and E7 oncogenes, which we previously reported, could be enhanced by short dsRDC modification (first six nucleotides from the 5′ end of the GS and its complementary nucleotides of the passenger strand). Such dsRDC modification reduced nonspecific cytotoxicity in two of three siRNAs (497 and 752), although not in the other (573), which correlated with their off-target effects. In addition, silencing activity was marginally impaired in two dsRDCs (497 and 573) and moderately in one (752). Finally, dsRDC-497 induced E6E7-specific growth suppression of cervical cancer cells as well as E6E7-immortalized human keratinocytes. Our results show that dsRDC modification enhances the specificity of E6E7 siRNA, which is required for use in *in vivo* settings.
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Introduction

A decade has passed since the discovery of RNA interference (RNAi) induction by synthetic 21–23-nucleotide double-stranded small interfering RNA (siRNA) in mammalian cells.[1,2] Since then, siRNA has become a powerful tool for studying gene functions, as well as development of potential drugs for viral infections and cancer treatments. However, the effects of siRNA are not as specific as initially proposed. siRNA silences the expression of unintended targets with partial sequence complementarities (off-target effects),[3,4] although minimal matching of mRNA and the seed region of the siRNA guide strand (GS) (nucleotide positions 2–8 from the 5′ end) may be sufficient for off-target silencing.[3,5,6] Nucleotide modifications at specific positions have been

found to overcome the off-target problem.[7–9] In addition, DNA replacement in the seed region of the GS (positions 1–8 from the 5′ end) and its complementary sequence of the passenger strand (PS), designated as double-stranded RNA–DNA chimera (dsRDC), abolishes the off-target effects with some decrease in silencing activity.[8] Most active siRNAs have been shown to induce a cytotoxic effect by exerting off-target silencing and interfering with the endogenous RNAi pathway,[7,9–12] whereas cytotoxicity might be reduced by use of chemically modified siRNA with low off-target activity[7] or siRNA with high silencing activity at a minimal concentration.

Cervical cancer is the second most common female malignancy throughout the world, with high-risk types of human papillomavirus, such as types 16, 18, 33, 45 and 31, known to be causative agents.[13] Among them, HPV16 is the most prevalent and accounts for about half of reported cases. High-risk types of human papillomaviruses are small DNA viruses with a circular double-stranded DNA genome of about 8000 base pairs (bp) containing an upstream regulatory region, along with six early (E6, E7, E1, E2, E4 and E5) and two late (L1 and L2) genes. High-risk types of human papillomaviruses exert carcinogenic effects through constitutive expressions

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of the E6 and E7 oncogenes.[14–17] Uncontrolled expressions of E6 and E7, which are functionally equivalent to *P53* and *RB* mutations in other cancer types, also contribute to cellular immortalization, genetic instability, accumulation of additional genetic changes and resultant malignant transformation. In addition, their expressions are essential for maintaining a malignant phenotype, whereas their suppression results in apoptosis and cellular senescence by restoring and activating the functions of *P53* and *RB*.[18–24] Thus, E6 and E7 are considered to be ideal therapeutic targets. Prophylactic vaccines covering HPV16 and 18 are now clinically available, and have been shown effective for preventing infections as well as cervical cancer.[25] However, these vaccines do not benefit individuals who are already infected and their long-term effects remain to be confirmed. Thus, it is necessary to develop new therapies for patients who have persistent infections, precancerous lesions or overt cancer.

A large number of siRNA sequences targeting HPV16 E6E7 oncogenes have been found to exert negative growth effects on HPV16-positive cancer cells.[21–23,26] Among them, we previously reported that three siRNAs (497, 573 and 752) were highly potent.[27] In this study, we investigated the effects of short dsRDC modification (positions 1–6 from the 5′ end of the GS and its complementary parts of the PS) on cytotoxicity and off-target effects exerted by some of those potent E6E7 siRNAs. We found that most of the nonspecific cytotoxicity was attenuated by dsRDC modification with a concomitant marginal or moderate reduction in silencing activity. Among those modified dsRDCs, one with high target specificity and preserved silencing activity suppressed the growth of E6E7-immortalized human keratinocytes, an early cancer model, as well as cervical cancer cells. These findings indicate the potential of E6E7 dsRDC in therapeutic applications for HPV16-related diseases.

Materials and methods

Cell lines and plasmid transfection
SiHa (HPV16-positive cervical cancer), HeLa (HPV16-negative cervical cancer) and SNU-1 (gastric cancer) cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD). SiHa and HeLa cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum. SNU-1 cell lines were cultured in RPMI1640 medium containing 10% fetal calf serum. Primary human keratinocytes (HDK) were purchased from Cell Applications (San Diego, CA) and immortalized by the catalytic subunit of *human TERT* basically, as described previously,[28] except that the present HDK were transduced with *human TERT* alone, and not a combination of *human TERT* and *Bmi-1* (HDK1-T). Generation of HPV16 E6E7-immortalized HDK (HDK1-E6E7) was performed as reported previously.[28] HDK, HDK1-T and HDK1-E6E7 were cultured in serum-free keratinocyte media with recombinant epidermal growth factor (5 ng ml^{-1}) and bovine pituitary

extract (5 μg ml^{-1}) (Invitrogen, Carlsbad, CA) at 37 °C in an atmosphere of 5% CO_2. Generation of stable clones expressing firefly luciferase (FLuc) (FL-SiHa-2, FL-HeLa-1) was as reported previously.[27] Plasmid transfection was performed using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions.

siRNAs and transfection
The sequences of the siRNAs used in this study[27] are summarized in Supplementary Table 1. All siRNAs were designed using the siDirect software (http://sidirect2.rnai.jp/), as reported previously.[29] Control siRNA was an artificial sequence designed to have the least homology to human and mouse genes. Control siRNA and its dsRDC-modified form were included in all experiments. For preparation of dsRDCs, six nucleotides from the 5′ end of GS and eight from the 3′ end of PS were substituted with corresponding DNAs. Twenty-one-base nucleotides were synthesized, 2′-deprotected, desalted and annealed in buffer (100 mm potassium acetate, 30 mm HEPES-KOH at pH 7.4, 2 mm magnesium acetate).

At 1 day before transfection, cells were plated at a density of $2–4 \times 10^4$ cells per well in 24-well plates containing 0.5 ml of Dulbecco's modified Eagle's medium with 10% fetal calf serum. For analysis of the effects of siRNA on cell growth and viability, cells were plated at a low density ($1–2 \times 10^3$ cells per well) in 96-well plates containing 0.1 ml of Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Various amounts of siRNA and Lipofectamine RNAiMAX (0.6 μl) (Invitrogen), both of which were added along with Opti-MEM I reduced serum medium (Opti-MEM, Invitrogen) up to 50 μl, were combined, incubated at room temperature for 20 min and then added to 0.5-ml cultures. For transfection of SiHa cells and their derivatives, 1 μl of Lipofectamine RNAiMAX was used. For transfection of immortalized keratinocytes, the culture medium was replaced at 5 h after transfection. For co-transfection of HeLa cells with the plasmid and siRNA or dsRDC, Lipofectamine 2000 was used.

Plasmid construction
Renilla luciferase (RLuc) cDNA fused with HPV16 E6 (231–559) (RLuc-ΔNE6) and RLuc-E7 (562–858) were isolated from 16ΔNE6/psiCheck-2 and 16E7/psiCheck-2,[27] respectively, and then cloned into pZeoSV2 (Invitrogen) at the *Nhe*I and *Not*I sites. The resultant plasmids were designated as 16ΔNE6/pZeoSV2 and 16E7/pZeoSV2, respectively. Construction of RLuc-ΔNE6E7/psiCheck-2 was reported previously.[27] Next, 19-nucleotide human gene sequences carrying 3-nucleotide mismatches to GS of each E6E7 siRNA were selected using the siDirect software[29] (Table 1). The sense and antisense oligoDNAs of these sequences are summarized in Supplementary Table 2. Sense and antisense oligoDNAs were phosphorylated, annealed and subcloned at the *Xho*I and *Not*I sites of psiCheck-2 (Promega, Madison, WI).

Table 1 Off-target candidate human genes carrying 3-nucleotide mismatches to GS of siRNAs targeting HPV16 E6E7

Sequence name	OT gene accession number	Gene definition and gene symbol	GS sequence (3′ ← 5′) / OT mRNA (5′ → 3′)
497OT-1	BX647877	cDNA clone DKFZp313E2325	GAUCUGUCGAUGUAUGUUU ||X|X|||||||||||X| CUGGCCAGCUACAUACAGA
497OT-2	NM_006237.2	*POU4F1*	UACCUGUCCAUGUAUGUCU X|||X|||X||||||||| CUGGCCAGCUACAUACAGA
573OT-1	NM_000922.2	*PDE3B*	CACAUACAUUGAAUAAAUA |||X|||||||X||X||| GUGGAUGUAACGUACUUAU
573OT-2[a]	AL832149	cDNA clone DKFZp686P15111	CACCUACACAGCAUGAAUU ||||||||XX|||||||X GUGGAUGUAACGUACUUAU
573OT-3	NM_001517.4	*GTF2H4*	CACCUACAAUGCAGGAAUC ||||||||X||||X||||X GUGGAUGUAACGUACUUAU
573OT-4[a]	BF693154	cDNA clone IMAGE:4244422 5′	CACCUACACAGCAUGAAUU ||||||||XX|||||||X GUGGAUGUAACGUACUUAU
573OT-5[b]	AF138859	cDNA clone FLB2932	CAACUACAUUACAUUAAUA ||X|||||||X|||X|||| GUGGAUGUAACGUACUUAU
573OT-6[b]	BC036590	cDNA clone IMAGE:4801089	CAACUACAUUACAUUAAUA ||X|||||||X|||X|||| GUGGAUGUAACGUACUUAU
573OT-7	NM_004582.2	*RABGGTB*	CAACUUCAUCGCAUGAAUA ||X||X|||X||||||||| GUGGAUGUAACGUACUUAU
573OT-8	AA861522	cDNA clone IMAGE:1407653 3′	CUCCUACAUUGUAGGAAUA |X||||||||X|X||||| GUGGAUGUAACGUACUUAU
573OT-9	F03276	cDNA clone c-1sa08 3′	CACCUCCAUUUAAUGAAUA |||||X|||||XX|||||| GUGGAUGUAACGUACUUAU
752OT-1	BQ054887	cDNA clone IMAGE:5806941 5′	CUUCGGCUGCGCGUAAAAA ||||||X||X|||||X||| GAAGCCAACACGCAUGUUU

Abbreviations: GS, guide strand; HPV, human papillomavirus; OT, off-target candidate; PS, passenger strand; siRNA, small interfering RNA.

All human genes with 3-nucleotide mismatches to the GS of each E6E7 siRNA were selected using the siDirect software.

Matched and mismatched base pairs between off-target candidate sequences and GSs are visualized by vertical lines and cross marks, respectively.

[a]The sequence of 573OT-4 is identical to that of 573OT-2.

[b]The sequence of 573OT-6 is identical to that of 573OT-5.

Immortalized human keratinocytes stably expressing FLuc

FLuc cDNA was amplified using a polymerase chain reaction (PCR) method with Platinum PCR Supermix High Fidelity (Invitrogen) and sense and antisense primers, with psiCheck2 utilized as a template. The PCR product was cloned into pCR8/GW/TOPO (Invitrogen) using Topoisomerase I (Invitrogen) and sequenced (FLuc/pCR8/GW). FLuc cDNA was then subcloned in a lentivirus expression plasmid (pLenti6.3/V5-DEST; Invitrogen) with LR Clonase II (Invitrogen) and the resultant lentivirus plasmids were designated as FLuc/pLenti6.3. An infectious recombinant virus was produced by co-transfection of 293FT cells (Invitrogen) with FLuc/pLenti6.3 and packaging plasmids (pLP1,

pLP2 and pLP/VSVG) (Invitrogen) using Lipofectamine 2000, according to the manufacturer's instructions. The culture medium was changed at 24 h and collected at 96 h after transfection. HDK1-T and HDK1-E6E7 were infected with a recombinant lentivirus for 6 h in the presence of polybren ($6 \mu g \, ml^{-1}$), and then selected using $2.5 \mu g \, ml^{-1}$ of blasticidin (Invitrogen), and FLuc-expressing cells were designated as HDK1-T-luc and HDK1-E6E7-luc, respectively.

SiHa cells stably expressing FLuc and RLuc-E6, or RLuc-E7, and luciferase assay

SiHa cells stably expressing both FLuc and RLuc-E6 (RL-E6-FL-SiHa-10), and those expressing both FLuc and RLuc-E7 (RL-E7-FL-SiHa-2) were obtained by

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transfection of FL-SiHa-2 cells[27] with 16ΔNE6/pZeoSV2 and 16E7/pZeoSV2, respectively, and antibiotic selection using both zeocin (1 mg ml^{-1}; Invitrogen) and G418 (1 mg ml^{-1}; Invitrogen). For luciferase assays, cells were harvested at 48 h after transfection with siRNA or dsRDC, and then luciferase activity was determined using a Dual Luciferase Reporter Assay System (Promega).

Real-time quantitative reverse transcription-PCR

Total RNA was extracted from cells using a High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. cDNA was synthesized using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics). Real-time quantitative reverse transcription-PCR assays were performed using a LightCycler 480 System (Roche Diagnostics) in 96-well plates. Each reaction was carried out in 20 μl containing 10 μl of LightCycler 480 Probes Master (Roche Diagnostics), 5 μl of cDNA from a 10 × dilution of the reverse transcriptase reaction (equivalent to 25 ng of reverse-transcribed RNA), 900 nM of a forward and reverse prime pair and 250 nM of 6-carboxyfluorescein-labeled probe. Serial dilutions of plasmid DNA (10–0.01 ng) were used to generate a standard curve. The LightCycler protocol was used as follows: initial cycle of 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Water was used as the template for negative control amplifications included with each PCR run. All reactions were performed in triplicate in each 96-well plate. Data were analyzed using the Roche LightCycler 480 software and the crossing point (Cp) value was calculated with the Second Derivative Maximum Method. The amount of target mRNA was examined and normalized to that of 18S ribosomal RNA.

The primers and probes for E6-E7 and E6*I-E7 have been described by others.[30] Those for 18S ribosomal RNA and AL832149 were designed by Sigma Aldrich Japan (Tokyo, Japan) and Applied Biosystems (Foster City, CA), respectively. Primers and probes for POU4F1 (NM_006237.3) and GTF2H4 (NM_001517.4) were obtained from Applied Biosystems (POU4F1, Assay ID Hs00366711_m1; GTF2H4, Assay ID Hs00231008_m1). All probes were labeled at the 5′ end with the reporter dye 6-carboxyfluorescein and at the 3′ end with a non-fluorescent quencher. The PCR primer and probe sequences are listed in Supplementary Table 3.

Analysis of cell growth and senescence-associated β-galactosidase staining

Cell viability was examined with a colorimetric assay using WST-8 (Cell Counting Kit 8; Dojin Laboratories, Kumamoto, Japan), according to the manufacturer's instructions. Senescence-associated β-galactosidase (SAβ-gal) staining was performed using a Senescent Cell Staining Kit (Sigma-Aldrich). The viability of HDK1-T-luc and HDK1-E6E7-luc cells was determined using a Luciferase Assay System (Promega).

Statistical analysis

The statistical significance of differences was determined using an unpaired two-tailed Student's t-test. Differences were considered statistically significant at $P < 0.05$.

Results

Silencing activities of E6E7 siRNAs and their dsRDC counterparts

In this study, we examined whether dsRDC modification of HPV16 E6E7 siRNAs with high silencing activity (497, 573 and 752; Figure 1a) would increase their target specificity and reduce nonspecific cytotoxicity. dsRDCs with an 8-nucleotide DNA substitution often show lower silencing activity than those with a 6-nucleotide DNA substitution (data not shown). We used the shorter DNA substitution in this study (Figure 1b).

HPV16 E6E7 siRNAs (497, 573 and 752) and their dsRDC forms were compared with regard to their silencing activity using HeLa cells transiently expressing RLuc-E6E7. HeLa cells were co-transfected with RLuc-E6E7-expressing plasmids along with siRNAs (control, 497, 573 and 752) and their dsRDC counterparts. At 48 h after transfection, a dual luciferase assay was performed. As shown in Figure 1c, siRNAs 497, 573 and 752 exhibited potent silencing activity (8.4, 11.6 and 5.6%, respectively), whereas control siRNA showed very mild suppression (72.1%). dsRDC modification of these siRNAs slightly reduced the silencing activities of siRNAs 497 and 573 (16.2 and 13.6%), and moderately that of 752 (22.0%). SiHa cells stably expressing both RLuc-E6 and FLuc (RL-E6-FL-SiHa-10) were used for determining the half-maximal inhibitory concentration (IC$_{50}$) values of siRNA-497 and its dsRDC. Various concentrations of siRNA and dsRDC were transfected into RL-E6-FL-SiHa-10 cells, and then a dual luciferase assay was performed 48 h after transfection. siRNA-497 showed maximal suppression of RLuc activity to 4.6% at 5 nM with an IC$_{50}$ of 0.015 nM, whereas dsRDC modification slightly decreased the silencing activity by 6.8-fold (IC$_{50}$, 0.105 nM) (Figure 1d). Similarly, siRNAs targeting E7 (573 and 752) were analyzed with RL-E7-FL-SiHa-2 cells expressing RLuc-E7 and FLuc. siRNA-573 had a high silencing activity with an IC$_{50}$ of 0.019 nM and dsRDC modification increased the IC$_{50}$ by 5.2-fold (0.098 nM; Figure 1e). In contrast to the other two dsRDCs, dsRDC-752 had a low level of silencing activity and was 32-fold less active than its siRNA form (IC$_{50}$ 1.774 vs 0.055 nM) (Figure 1f). Both control siRNA and dsRDC had negligible effects at 1 nM on RLuc expression in RL-E6-FL-SiHa-10 and RL-E7-FL-SiHa-2 cells (data not shown).

Effects of E6E7 dsRDC on endogenous E6E7 mRNAs

The effects of dsRDCs targeting E6E7 on endogenous E6-E7 and E6*I-E7 mRNA expressions were compared with their siRNA counterparts using HPV16-positve cervical cancer cells. FL-SiHa-2 cells were transfected with siRNAs (control, 497, 573 and 752) or their dsRDC
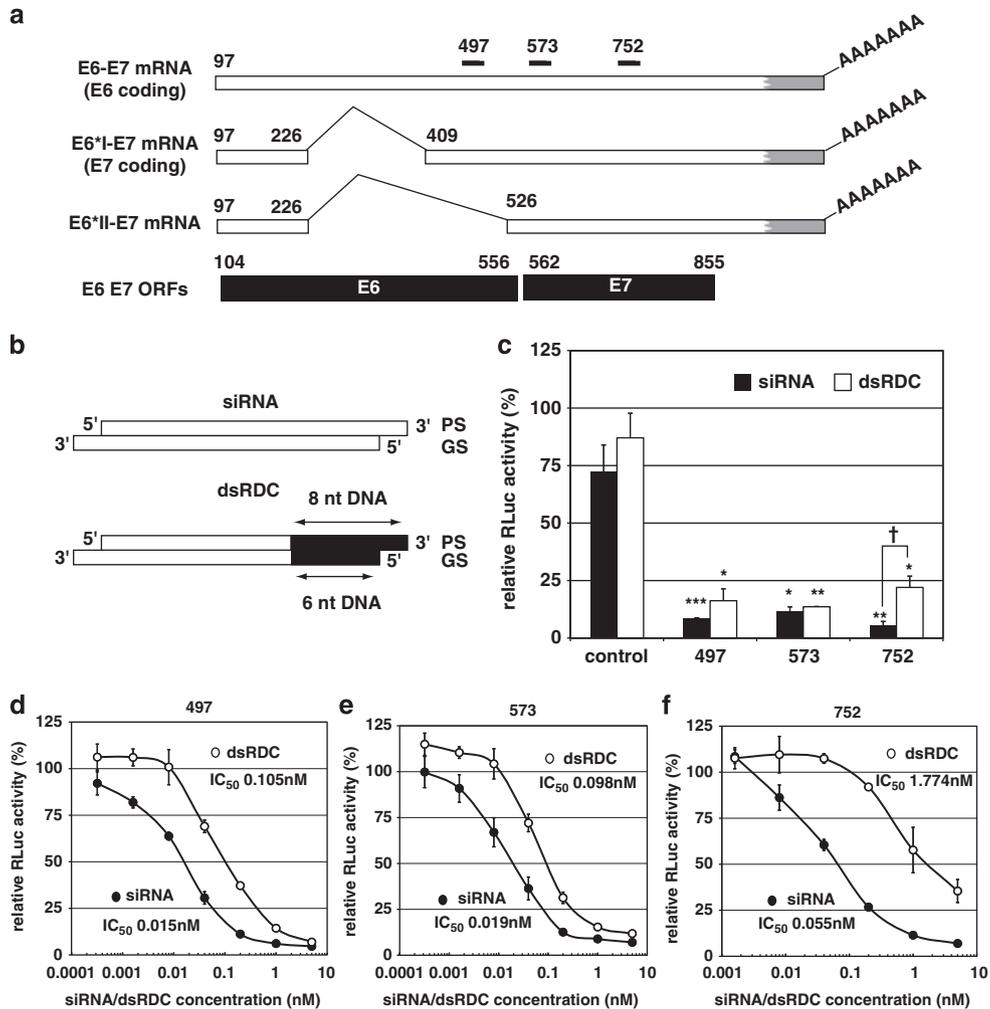
Figure 1 RNA–DNA chimera modification of small interfering RNA (siRNA) targeting human papillomavirus 16 (HPV16) E6E7. (**a**) E6E7 mRNA and splicing variants, and E6E7 siRNAs. siRNAs targeting E6 and E7 are depicted as short lines (top). Open bars represent mRNAs coding E6 and E7 with introns within E6 (middle). Gray area indicates cellular sequence derived from the chromosomal integration site. Closed boxes show locations of the E6 and E7 coding regions of the HPV16 prototype (accession number K02718) (bottom). Numbers refer to the locations of the transcription initiating site and splicing junctions. ORF, open reading frame. (**b**) Structure of double-strand RNA–DNA chimera (dsRDC). Conventional siRNA is a 19-bp long double-stranded RNA with 2-nucleotide 3′ overhangs. The dsRDCs used in this study contained 6-nucleotide DNA in the 5′ region of the guide strand and 8-nucleotide DNA in the 3′ region of the PS. Open and closed bars represent ribonucleic acid and deoxyribonucleic acid, respectively. PS, passenger strand; GS, guide strand. Silencing activities of control and HPV16 E6E7 targeting siRNAs (497, 573 and 752), and their dsRDC forms were determined by transient transfection of HeLa cells with a *Renilla* luciferase (RLuc)-E6E7/psiCheck-2 plasmid, as well as siRNAs and dsRDCs. At 48 h after transfection, a dual luciferase assay was performed. RLuc activities are shown as relative to mock-transfected cells (**c**). The half-maximal inhibitory concentration (IC_{50}) values of siRNA and dsRDC targeting E6 (497) were determined using SiHa cells stably expressing firefly luciferase (FLuc) and artificial fusion mRNA of RLuc-E6 (RL-E6-FL-SiHa-10) (**d**), whereas those targeting E7 (573 and 752) were analyzed using SiHa cells stably expressing FLuc and RLuc-E7 (RL-E7-FL-SiHa-2) (**e**, **f**). Cells were transfected with various concentrations of siRNAs or dsRDCs, and then analyzed using a dual luciferase assay 48 h after transfection. RLuc activities were normalized to those of FLuc. RLuc activities are shown as relative to mock-transfected cells. Experiments were performed in triplicate and error bars represent the standard deviation from the mean. Data points marked with asterisks are statistically significant as compared with control transfection (*$P < 0.001$; **$P < 0.01$; ***$P < 0.05$). Data points marked with crosses are statistically significant ($^{\dagger}P < 0.01$).

counterparts at 5 nM for 72 h, and then analyzed for levels of mRNAs coding E6 and E7 by quantitative reverse transcription-PCR. As shown in Table 2, transfection with siRNAs 497, 573 and 752 decreased the levels of both E6-E7 and E6*I-E7 mRNA to 13.5–27.6 and 3.2–6.4%, respectively. Their dsRDC counterparts 573 and 752 were less active, although they still suppressed those levels to 30.9–32.7 and 17.2–31.2%, respectively. dsRDC-497 also

suppressed E6*I-E7 mRNA expression to 24.9%, however, its effect on E6-E7 mRNA expression was indistinguishable from that of control dsRDC (64.7 vs 67.2%). Thus, dsRDC modification of most E6E7 siRNAs slightly decreased the silencing activity towards E6-E7 and E6*I-E7 mRNA expressions, except for dsRDC-497, which exerted a silencing effect only on E6*I-E7 mRNA and not on E6-E7 mRNA.

Effects of HPV16 E6E7 siRNAs and dsRDCs on growth of HPV16-positive and -negative cancer cells

To examine if dsRDCs targeting E6E7 are capable of inducing specific growth inhibition of HPV16-related cancer cells, the effects of dsRDC on cell growth were examined using HPV16-positive (FL-SiHa-2) and -negative cells (FL-HeLa-1). Control and E6E7 dsRDCs (497,

573 and 752) as well as their siRNA counterparts were transfected into FL-SiHa-2 cells at 2 nM. All E6E7 dsRDCs tested in this study suppressed the growth of FL-SiHa-2 cells, although the effects of two of the dsRDCs (497 and 752) were less potent as compared with those of their siRNA counterparts (Figure 2a).

HeLa and FL-HeLa-1 cells are highly vulnerable to siRNA-mediated nonspecific cytotoxicity.[9,27] By taking advantage of this characteristic, E6E7 dsRDC and siRNA (497, 573 and 752) were evaluated for their nonspecific cytotoxicity by determining cell viability 7 days after transfection. As shown in Figure 2b, all siRNAs including the control showed moderate to strong growth suppression toward FL-HeLa-1 cells. dsRDC modification of these siRNAs (control, 497 and 752), except for 573, also decreased cytotoxicity as compared with their siRNA counterparts. These results suggest that most siRNAs induce nonspecific cytotoxicity[9] and that dsRDC modification reduces the level of cytotoxicity.

The negative growth effect of E6E7 siRNA on E6E7-positive cancer cells is mediated in part by inducing cellular senescence.[24,26,27] We examined if E6E7 dsRDCs capable of specific growth suppression (497 and 752) also induced senescence in FL-SiHa-2 cells. siRNA-573 and its dsRDC were omitted, because of their strong nonspecific toxicity. Cells were transfected with either control siRNA, E6E7 siRNA or their dsRDC counterparts for 7 days, and then tested for the expression of SAβ-gal, a hallmark of cellular senescence. The proportions of SAβ-gal-positive cells transfected with control siRNA and dsRDC was 8.6 and 3.5%, respectively, whereas those of siRNA-497- and dsRDC-497-induced cells were 64.1 and 70.3%, respectively. dsRDC-752 induced a slightly lower percentage of SAβ-gal-positive cells (46.6%) than its siRNA counterpart (78.8%) (Figure 2c). Thus, it was shown that E6E7 dsRDC exerts growth suppression in HPV16-positive cancer cells through a mechanism similar to that of siRNA.

Table 2 qRT-PCR analysis of E6-E7 and E6*I-E7 mRNA levels in FL-SiHa-2 cells transfected with siRNAs and dsRDCs targeting HPV16 E6E7

siRNA/dsRDC	E6-E7 mRNA (%)	E6*I-E7 mRNA (%)
Mock	100.0 ± 5.0	100.0 ± 10.8
Control		
siRNA	53.8 ± 4.1	55.3 ± 8.1
dsRDC	67.2 ± 1.3	72.2 ± 3.6
497		
siRNA	27.6 ± 0.7	5.9 ± 0.6
dsRDC	64.9 ± 4.2	24.9 ± 1.9
573		
siRNA	21.4 ± 1.1	6.4 ± 0.7
dsRDC	30.9 ± 0.9	17.2 ± 0.9
752		
siRNA	13.5 ± 1.1	3.2 ± 0.4
dsRDC	32.7 ± 0.9	31.2 ± 2.9

Abbreviations: dsRDC, double-strand RNA–DNA chimera; FL, firefly luciferase; HPV, human papillomavirus; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; s.d., standard deviations; siRNA, small interfering RNA. E6-E7 and E6*I-E7 mRNA levels were analyzed by qRT-PCR and were normalized to that of 18S rRNA. Mean values and s.d. of relative mRNA levels (%) to mock-transfected cells are shown. Experiments were carried out in triplicate.

Figure 2 Effects of E6E7 double-strand RNA–DNA chimera (dsRDCs) on growth of human papillomavirus 16 (HPV16)-positive and -negative cervical cancer cells. HPV16-positive FL-SiHa-2 (**a**) and HPV16-negative (FL-HeLa-1) cells (**b**) were transfected with either mock, small interfering RNA (siRNA) (control, 497, 573 and 752) or their dsRDC counterparts at 2 nM. Cell viability was determined using a WST-8 assay 5–7 days after transfection. Cell viability relative to the mock-transfected cells is shown. (**c**) Induction of senescence-associated β-galactosidase (SAβ-gal) activity in FL-SiHa-2 cells by dsRDCs targeting E6E7. FL-SiHa-2 cells were transfected with mock, siRNA (control, 497 and 752) (1 nM) or dsRDC (control, 497 and 752) (2 nM) for 7 days, and then stained for SAβ-gal activity. Values for SAβ-gal-positive cells are shown as percentages. All assays were performed in triplicate and error bars represent the standard deviation from the mean. Data points marked with asterisks are statistically significant as compared with control transfection (*$P < 0.001$; **$P < 0.01$; ***$P < 0.05$). Data points marked with crosses are statistically significant (†$P < 0.01$).
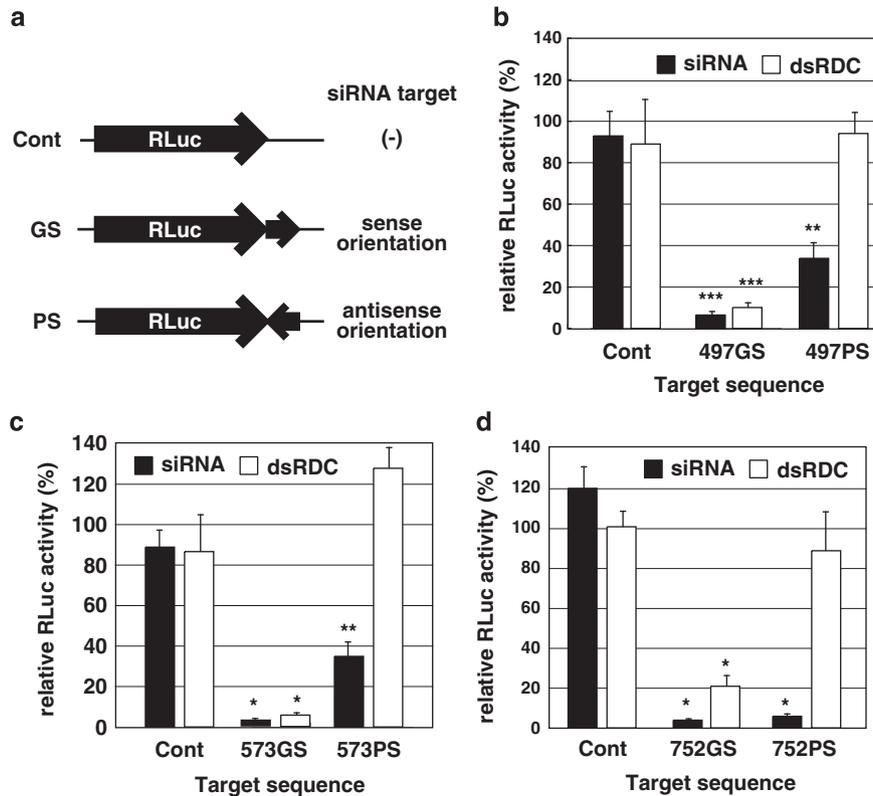
Figure 3 Lack of passenger-strand mediated RNA interference (RNAi) in E6E7 double-strand RNA–DNA chimera (dsRDC). Plasmids ligated with a small interfering RNA (siRNA) target sequence in a sense orientation (497GS, 573GS and 752GS) expressed targets of the guide strand (GS)-loaded RNA-induced silencing complex (RISC), whereas those in an antisense orientation (497PS, 573PS and 752PS) expressed targets of the passenger strand (PS)-loaded RISC (**a**). HeLa cells were co-transfected with *Renilla* luciferase (RLuc)-GS (497GS, 573GS and 752GS) or RLuc-PS expression plasmids (497PS, 573PS and 752PS), along with respective siRNA and dsRDC (497 (**b**), 573 (**c**) and 752 (**d**)). Data points marked with asterisks are statistically significant as compared with control transfection (*$P<0.001$; **$P<0.01$; ***$P<0.05$). All siRNAs examined here exhibited PS- and GS-mediated RNAi, whereas all examined dsRDCs showed only GS-mediated RNAi.

Lack of PS-mediated RNAi in E6E7 dsRDCs

To examine the differences between E6E7 siRNAs and their dsRDC forms with regard to PS-mediated silencing activity, we used an expression plasmid comprised of RLuc fused with 23-bp siRNA target sequences in sense and antisense directions as GS and PS targets, respectively (Figure 3a). As shown in Figure 3b–d, all siRNAs tested (497, 573 and 752) suppressed their respective RLuc-GS as well as RLuc-PS expression, whereas their dsRDC counterparts suppressed only RLuc-GS.

Effects of HPV16 E6E7 siRNAs and dsRDCs on off-target candidate genes

We also examined whether 6-nucleotide DNA substitution in the seed region (nucleotides 1–6 from the 5′ end of GS) could alter the off-target property of siRNA towards human gene sequences with 3-nucleotide mismatches to each E6E7 siRNA GS (Table 1), using plasmids of RLuc fused with 19-bp off-target candidate gene sequences (OT). HeLa cells were transfected with siRNA or dsRCD (2 nM) along with a corresponding RLuc-OT plasmid, and then analyzed using a dual luciferase assay. E6E7 siRNAs and dsRDCS showed no or very mild inhibitory effects on 7 of 10 sequences with less than 35%

suppression (data not shown). Furthermore, 497OT-2 was inhibited by siRNA-497 to 23.6%, whereas its suppression by dsRDC-497 was significantly less (65.6%, $P<0.001$; Figure 4a), indicating that dsRDC modification of 497 reduced off-target activity. In addition, 573OT-2 and 573OT-3 expressions were suppressed by siRNA-573 to 20.3 and 49.5%, respectively. Surprisingly, dsRDC modification of siRNA-573 did not reduce the inhibitory effect on 573OT-2 or OT-3 (Figure 4b and c).

Next, we examined whether E6E7 siRNA (497 and 573) could suppress mRNA expression of the off-target candidate genes (497OT-2, *POU4F1*; 573OT-2, AL832149; 573OT-3, *GTF2H4*) using quantitative reverse transcription-PCR. As neither SiHa nor HeLa cells expressed *POU4F1*, we used SNU-1 cells, which expressed all three OT genes. SNU-1 cells were transfected with siRNA-497 or dsRDC-497 for 2 days, and then analyzed for mRNA expression of *POU4F1* (Figure 4d). siRNA-497 suppressed the *POU4F1* mRNA level to 57.7%, whereas dsRDC-497 had no effect. The mRNA levels were parallel to RLuc activities of 497OT-2 (Figure 4a). Similarly, the effects of siRNA-573 and its dsRDC form on AL8321449 mRNA level were examined using SNU-1 cells (Figure 4e). siRNA-573 decreased its level to 65.4% and
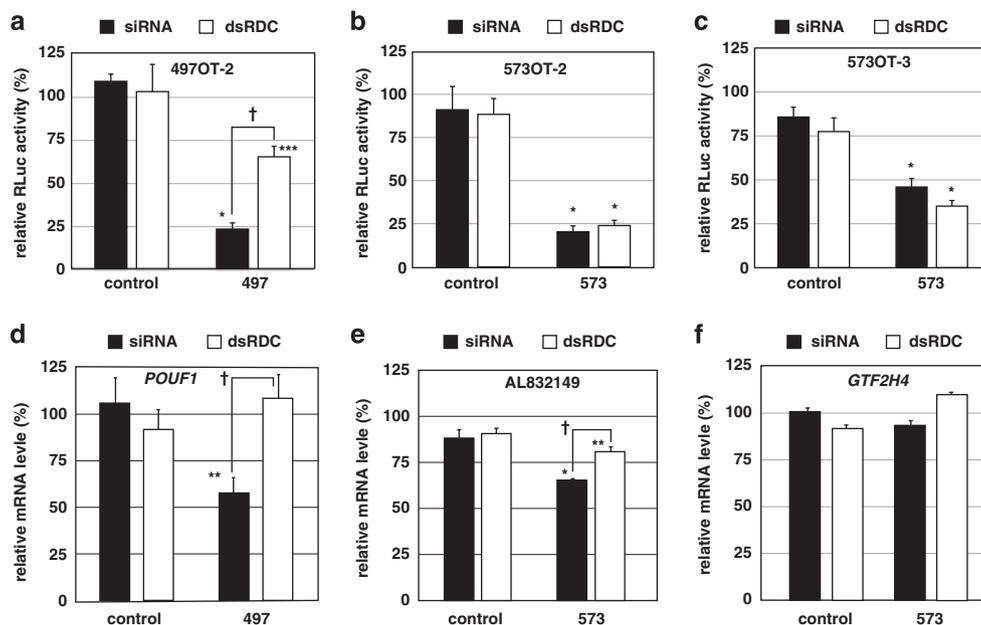
Figure 4 Effects of human papillomavirus 16 (HPV16) E6E7 small interfering RNA (siRNA) and double-strand RNA–DNA chimera (dsRDC) on off-target candidate sequences. All human gene sequences carrying 3-nucleotide mismatches to the guide strand (nucleotides 1–19 from the 5′ end) of E6E7 siRNAs are listed in Table 1. The 23-nucleotides oligoDNAs encompassing these 19-nucleotide sequences were synthesized and ligated downstream to the stop codon of *Renilla* luciferase (RLuc) in a psiCheck2 plasmid. HeLa cells were co-transfected with RLuc-497OT-2 (**a**) or RLuc-573OT-2 (**b**), or an RLuc-573OT-3 expression plasmid (**c**), along with respective siRNAs and their dsRDC counterparts (2 nM). After 48 h of incubation, cells were analyzed using a dual luciferase assay. RLuc activities were normalized to those of firefly luciferase (FLuc). RLuc activities relative to mock-transfected cells are shown. SNU-1 cells transfected with siRNAs (473 and 573) and their dsRDC counterparts at 100 nM were incubated for 48 h, and then analyzed for 497OT-2 (*POU4F1*, **d**), 573OT-2 (AL832149, **e**) and 573OT-3 (*GTF2H4*, **f**) expressions using quantitative reverse transcription-PCR (qRT-PCR). mRNA levels were normalized to that of 18S rRNA and those relative to mock-transfected cells are shown. All experiments were performed in triplicate and error bars represent the standard deviation from the mean. Data points marked with asterisks are statistically significant as compared with control transfection (*$P < 0.001$; **$P < 0.01$; ***$P < 0.05$). Data points marked with crosses are statistically significant (†$P < 0.01$).

dsRDC modification partially relieved the suppression (80.8%). *GTF2H4* mRNA expression was unaffected by siRNA-573 and its dsRDC form (91.9 and 93.4%, respectively; Figure 4f), whereas expression of 573OT-3 carrying the same target sequence was inhibited by more than twofold (Figure 4c), suggesting that *GTF2H4* mRNA might have lower accessibility to RNA-induced silencing complex than artificial 573OT-3 mRNA. These results showed that E6E7 siRNA exerts off-target effects toward some human genes carrying 3-nucleotide mismatches to siRNA and that dsRDC modification can reduce the off-target effects of some, if not all, mRNAs.

Effects of HPV16 E6E7 siRNAs and dsRDC on growth of E6E7-positive and -negative keratinocytes
Finally, we examined whether E6E7 dsRDC (497) suppressed the growth of E6E7-immortalized keratinocytes (HDK1-E6E7) with features of early cervical cancer cells.[31] *TERT*-immortalized keratinocytes (HDK1-T) were used as an E6E7-negative control. The vulnerability of these cells to FLuc siRNA transfection was tested using HDK1-E6E7-luc and HDK1-T-luc. The dose effect of FLuc siRNA on FLuc activity was similar between the cells at concentrations of 0.004–1.0 nM (data not shown),

indicating that these cells could be transfected with siRNA to induce target gene silencing with comparable efficacy.

HDK1-E6E7-luc and HDK1-T-luc cells were transfected with control and E6E7 siRNAs, as well as their dsRDC-modified forms (497 and 752) at 0.5 nM for 6 days. A WST8 assay did not reflect a viable cell number of E6E7 siRNA-treated HDK1-E6E7 cells (data not shown), thus we took advantage of the constitutive FLuc expression of these cells and relative cell viability was determined based on FLuc activity, which paralleled cell viability. As shown in Figure 5a and b, siRNA-497 suppressed the growth of *TERT*-immortalized keratinocytes as well as E6E7-immoratalized keratinocytes, indicating that siRNA-497 had nonspecific growth suppression, as also seen in FL-HeLa-1 cells (Figure 2b). When siRNA-497 was modified to a dsRDC form, nonspecific growth inhibition of HDK1-T-luc was reduced and cell viability recovered to 62.5%, with a marginal loss of growth suppression of E6E7-immortalized cells (19.1%). siRNA-752 also showed specific growth suppression as compared with siRNA control and siRNA-497. However, dsRDC modification compromised the E6E7-specific growth suppression of siRNA-752. Taken together,
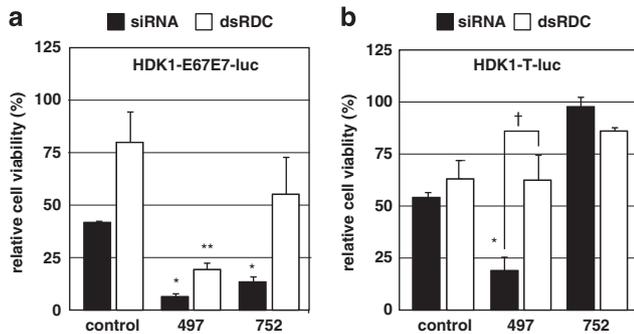
Figure 5 Specific growth suppression of E6E7-immortalized human keratinocytes by E6E7 double-strand RNA–DNA chimeras (dsRDCs). Human papillomavirus 16 (HPV16) E6E7-immortalized keratinocytes (HDK1-E6E7) and *TERT*-immortalized (HDK1-T) stably expressing luciferase were obtained, and designated as HDK1-E6E7-luc and HDK1-T-luc, respectively. HDK1-E6E7-luc (**a**) and HDK1-T-luc (**b**) cells were transfected with mock, small interfering RNA (siRNA) (control, 497 and 752) or their dsRDC counterparts at 0.5 nM. At 5 days after transfection, relative cell viability was determined using a luciferase assay. Data points marked with asterisks are statistically significant as compared with control transfection (*$P < 0.001$; **$P < 0.01$). Data points marked with crosses are statistically significant ($^\dagger P < 0.01$).

these results showed that dsRDC-497 and siRNA-752 are able to induce specific growth suppression of E6E7-expressing keratinocytes.

Discussion

In this study, we assessed the off-target activities of E6E7 siRNAs (497, 573 and 752) and their dsRDC modified forms using 10 potential human off-target sequences carrying 3-nucleotide mismatches to the GS of E6E7 siRNA (nucleotides 1–19 from the 5′ end). In most cases, 3-nucleotide mismatches were adequate to avoid suppression of artificial mRNA of RLuc fused with a single copy of the off-target candidate sequence. However, the silencing effect was significant when targeted mRNA contained a single copy of the 19-nucleotide sequence with a completely matched seed region as well as one of three mismatches located at either nucleotide 1 or 19 from the 5′ end of the GS. These sequences (497OT-2, 573OT-2 and 573OT-3) were found to have mismatches to their respective siRNAs at position 10, or at positions 10 and 11 from the 5′ end of the GS. Pairing of these GS nucleotides to target mRNA is essential for mRNA cleavage and mismatch of these positions is a hallmark of miRNA-mediated translational inhibition.[32–34] Thus, the off-target effects observed in our study might have been mediated through miRNA-like activity without mRNA cleavage.

Perfect RNA–RNA duplex formation between the siRNA seed region (GS positions 2–8 from the 5′ end) and unintended target mRNA is essential for off-target silencing.[3,5,6] Using unmodified authentic siRNA, we previously found that the melting temperatures of seed–mRNA duplexes (T_m 2–8) are divergent and positively correlated to the extent of off-target suppression.[35] We also reported that this rule is applicable to the off-target effects mediated by dsRDCs.[36] Substitution of seed RNA by DNA lowers T_m 2–8, which explains why most dsRDCs, if not all, might have reduced off-target effects as compared with siRNA.[8] In this study, dsRDC modification reduced the inhibitory effects of siRNA-497 on RLuc-497OT-2 and *POU4F1* mRNA expressions, which was consistent with our previous observation.[8,36] In contrast, similar DNA substitution of siRNA-573 did not compromise the suppression of RLuc-573OT-2. According to results of our calculation formula,[35] the T_m 2–8 change induced by dsDNA modification was highest in siRNA-497 (11.5 °C), followed by siRNA-752 (7.7 °C) and lowest in siRNA-573 (6.9 °C), which was well correlated with their reduction of off-target effects.

All HPV16 E6E7 siRNAs (497, 573 and 752) showed potent nonspecific cytotoxicity in HeLa-derived cells cultured at a low density. As HeLa cells express HPV18 E6E7 mRNAs with a relatively low homology to HPV16 E6E7 mRNAs, we analyzed the HPV18 E6E7 region and found no sequences perfectly matching the seed regions of these siRNAs, which is the minimal requirement for knockdown of HPV18 E6E7 (data not shown).[3,5,6] Control siRNA also exhibited cytotoxicity to FL-HeLa-1 cells under the same conditions. Furthermore, siRNA (497) was highly toxic to *TERT*-immortalized keratinocytes lacking E6E7 expression. Thus, these results suggested that the nonspecific cytotoxicity of these siRNAs was not mediated through suppression of E6E7 in FL-HeLa-1 cells. Reduction of off-target effects by dsDNA modification was correlated with their reduction of cytotoxicity. These results favor the hypothesis that nonspecific cytotoxicity is induced by off-target suppression of genes that regulate cell growth and viability.[7] Alternatively, such cytotoxicity might be mediated by disturbing the endogenous miRNA pathways through competition of molecules involved in RNAi by exogenous siRNA.[10–12] It remains to be determined how siRNA exerts toxic effects in transduced cells.

We previously reported that most E6E7 siRNAs, with a few exceptions including 573,[8] exerted PS-mediated silencing toward mRNA containing inverted E6E7 (628 bp fragment). However, we detected PS-mediated silencing in all siRNAs (497, 753 and 573) tested in this study when mRNAs containing inverted short target sequences were used as targets. Detection of PS RNAi might be influenced by the secondary structure of the target mRNA. Our results suggest that PS RNAi is unavoidable in conventional siRNAs. Thus, modifications such as dsRDC would be applicable for avoiding unwanted PS-induced silencing.

Deregulation of E6 and E7 expression is an initial event during multistep carcinogenesis in cervical cancer. In addition to expressions of these oncogenes, progression into overt cancer requires additional genetic changes. Human keratinocytes immortalized with HPV16 E6E7 were shown to be less invasive and have a less tumorigenic phenotype.[31] Using E6E7-immortalized keratinocytes as

an early disease model and *TERT*-immortalized keratinocyte s as normal keratinocytes, we examined the effects of E6E7 siRNA and dsRDC on their growth and specificity, and found that dsRDC-497 and siRNA-752 suppressed the growth of E6E7-positive keratinocytes, but not that of E6E7-negative keratinocytes. Thus, those might be useful for the treatment of pre-cancer and early cancer lesions.

In summary, we found that dsRDC modification enhanced the specificity of HPV16 E6E7 siRNA by reducing its nonspecific cytotoxicity and off-target property. E6E7 dsRDC inhibited the growth of E6E7-expressing human keratinocytes as well as HPV16-positive cervical cancer cells; however, not that of non-expressing keratinocytes. Taken together, our results indicate a large potential for HPV16 E6E7 dsRDC in treatments for HPV16-related cancer and early disease stages.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

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