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Novel cell penetrating peptides with multiple motifs composed of RGD and its analogs

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ABSTRACT

Cell penetrating peptides (CPPs) have been used to transport macromolecules into cells. Most CPPs have properties such as a strong polycationic charge, amphipathic basic, and hydrophobicity. In this study, we designed the peptides with multiple motifs composed of RGD and its analogs to induce integrin-mediated endocytosis as well as endosomal escape by forming an amphipathic helix in acidic endosomes. These peptides were proved less toxic to animal cells than those without acidic residues. Unexpectedly, peptide conjugated liposomes could penetrate into cells regardless of integrins. The replacement of all aspartic acids by glutamic acids did not prevent the peptide-mediated liposome uptake, and the higher basic and leucine contents enhanced the gene silencing activity of siRNA encapsulated in the liposomes. The peptide is considered to be a new type of CPP which can be used for drug delivery.

1. Introduction

The plasma membrane prohibits the cellular uptake of exogenous hydrophilic therapeutic molecules, and this makes the cell membrane the first barrier for efficient drug delivery. Short peptide sequences, known as cell penetrating peptides (CPPs), are able to cross the membrane efficiently. CPPs were initially discovered from the short sequences of membrane-interacting proteins called protein transduction domains (PTDs) [1–5]. These peptide sequences are cationic, hydrophobic, and/or amphipathic in nature [6]. Many CPPs have been designed and predicted in accordance with these properties [7,8]. Although most amphipathic CPPs are cationic, those with high content of negatively charged residues have also been proved to have cell penetrating activity [9–11]. However, the cellular uptake of CPPs differs depending on the peptide type. MAP12 and VT5 with the α-helical [9] and the β-sheet structures [10], respectively, penetrated into cells in a non-endocytotic manner, whereas p18 and p28 derived from azurin preferentially entered by endocytotic pathways [11].

RGD peptides containing the arginine–glycine–aspartate motif are able to translocate their cargos into target cells through cell membrane integrin receptors [12]. We initially designed peptides with multiple RGD motifs to have the arginine residues face to one side of the helical-wheel diagram, and then changed some glycine residues to leucine residues in order to increase hydrophobicity (Fig. 1A). Our goal was RGD motif-dependent internalization by cell surface integrins and amphipathic α-helix-dependent endosomal escape in acidic endosomes. Unexpectedly, the peptide internalization was independent of the RGD motif. The peptides could induce liposome internalization, and are considered to be a new type of CPP.

2. Material and methods

2.1. Materials

The peptides were synthesized by the solid-phase method using Fmoc-chemistry. The synthesized peptides were purified by HPLC using a C18 column and analyzed by MALDI-TOF mass spectroscopy (Axima Plus, Shimazu Scientific Instruments, Japan). The Fmoc-amino acids and H-Cys(Trt)-trityl resin were obtained from Novabiochem (Merck KGaA, Germany), and 5-carboxy-X-rhodamine, succinimidyl ester (ROX-SE) for the N-terminal fluorescence labeling of peptide was purchased from Molecular Probes, Inc. (Invitrogen, USA). All lipids for the preparation of asymmetric liposomes (ALPs) in this study were purchased from Avanti Polar Lipids, Inc. (USA), including 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy (polyethylene glycol)-2000] (mPEG-PE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[maleimide(polyethylene glycol)-2000] (miPEG-PE), 1,2-dioleoyl-3-dimethylammonium-propane (DODAP), and cholesterol (CH). Double-strand TMPRSS4-siRNA (siTMPR4) was purchased from Samchully Pharm Co., Ltd.

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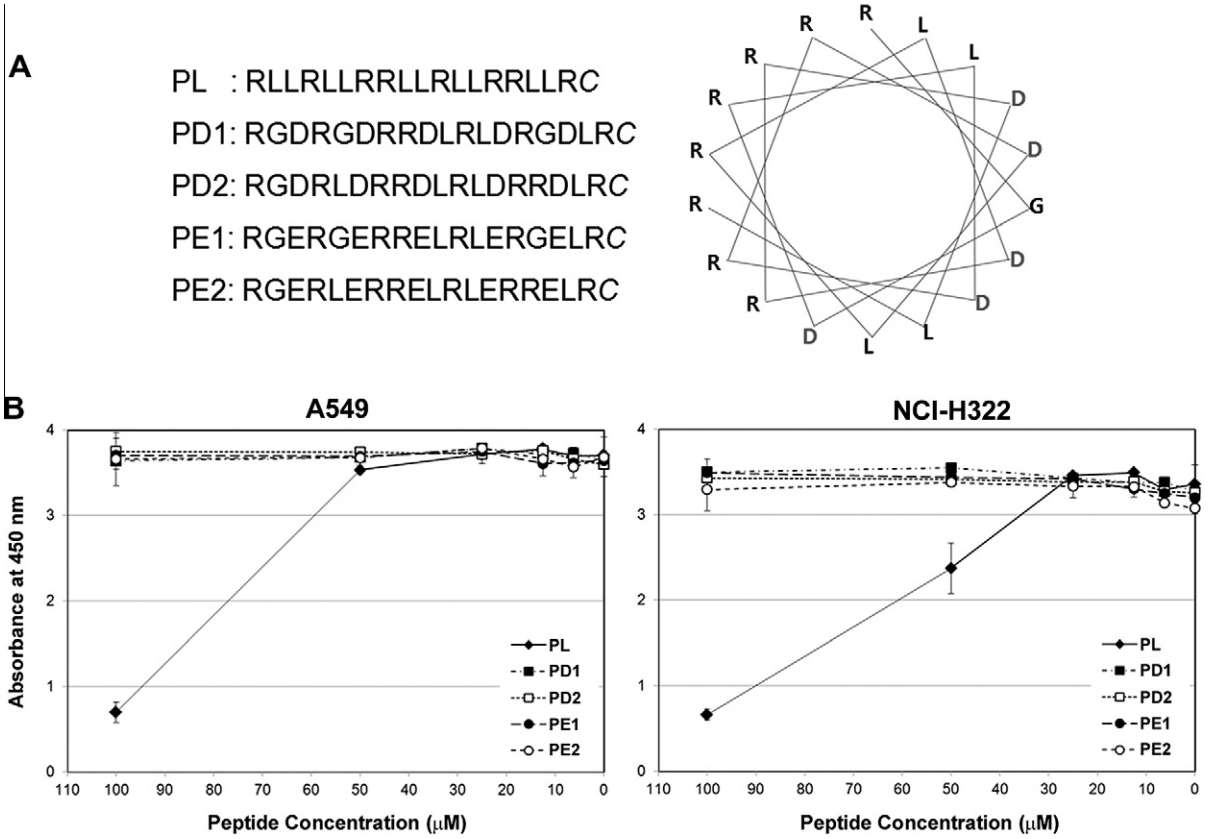

Fig. 1. The sequences of the PD and PE peptides and the helical wheel diagram of PD2 (A) and cytotoxicity of the peptides (B). (A) PL was designed to adopt a well-defined to amphipathic a-helical structure by substitution of leucine residues for all aspartic acid and glycine residues of PD2. C-terminal cysteine residues are introduced for nanoparticle conjugation. (B) A549 and NCI-H322 cells were treated with free peptides for 1 day, and the viable cells were monitored using a premix WST-1 cell viability & proliferation assay system (Takara, Japan). The error bar indicates the standard deviation.

(Korea), and green fluorescence protein (GFP)-siRNA (siGFP) and fluorescein isothiocyanate (FITC)-labeled siRNA (FITC-siRNA) was obtained from the Bioneer Corporation (Korea). Dialysis membranes with molecular weight cut-off values of 10 K and 50 K were purchased from Spectrum Laboratories (USA). The Premix WST-1 Cell Proliferation Assay System was purchased from TAKARA (Japan). All solutions were made up in the diethylpyrocarbonate (DEPC) treated water.

2.2. Cellular toxicity of peptide

The serially diluted peptides (final 0–100 μM) were added to A549 and NCI-H322 cells cultured in a 96-well plate in DMEM supplemented with 10% FBS (DMEM-FBS) overnight in a CO_2 incubator. The viable cells were analyzed using the Premix WST-1 Cell Proliferation Assay System (Takara, Japan) in accordance to the manufacture's protocol. Briefly, 10 μl of the WST-1 reagent was added for 4 h and the absorbance at 450 nm was measured using an ELISA plate reader (Multiskan FC, Thermo Sci., USA).

2.3. Asymmetric liposome preparation and peptide conjugation

ALPs encapsulating siRNA were prepared as we reported previously [13]. Briefly, the outer and inner lipid films composed of DSPC:DOPE:mPEG-PE:miPEG-PE:Cholestrol,4:3:0.8:0.2:4 (1.7 μmol total), and DODAP:DOPE, 9:1 (1.5 μmol total), were separately hydrated in a mixture of [200 μl HBS (20 mM HEPES and 150 mM sodium chloride; pH 7.5) and 120 μl ethanol] and 150 μl of 150 mM sodium citrate (pH 4) containing 100 μg of siRNA, respectively. After the outer and inner inverted micelles were prepared by the addition of 600 μl and 400 μl diethylether, respectively, ALPs were made by the serial processes of mixing of the inverted micelles, evaporation, and dialysis in HBS. To conjugate the peptide to ALPs, a 5-times molar excess peptide than miPEG-PE on ALPs was incubated with the ALPs for 3 h at room temperature, and then the unbound peptide was removed by dialysis with a 50 K dialysis membrane in HBS.

2.4. Cellular uptake of the peptide-modified ALPs

The NSCLC cells (A549, NCI-H322 and NCI-H460) and the NIH-3T3 cells were cultured in micro-slide 8-well microscopy chambers (ibidi, Germany) in DMEM-FBS. The cells were treated with FITC-siRNA-ALPs (1 μg FITC-siRNA per well) modified by the peptides in DMEM-FBS. After 1 day of incubation, the cellular uptake of the FITC-siRNA/ALPs was monitored using a confocal microscope (Olympus FV1000, Japan). The total green fluorescence intensity of a confocal image (Fig. 2A) was measured by densitometric analysis using the Bio2D program (Vilber Lourmat, Germany), and the average fluorescence intensity of an individual cell was calculated by dividing the total intensity to the total cell numbers.

2.5. Gene silencing activity

To test the gene-silencing efficiency of the peptide-conjugated ALPs entrapping siGFP (antisense: 5′-AACUUCAGGGUCAG-CUUGCdTdT-3′), we cultured the MDA-MB-435 cells expressing GFP in micro-slide 8-well microscopy chambers (ibidi, Germany) in DMEM-FBS. The cells were treated with the peptide modified siGFP/ALPs (0.6 μg siGFP per well). After 3 days of incubation, the

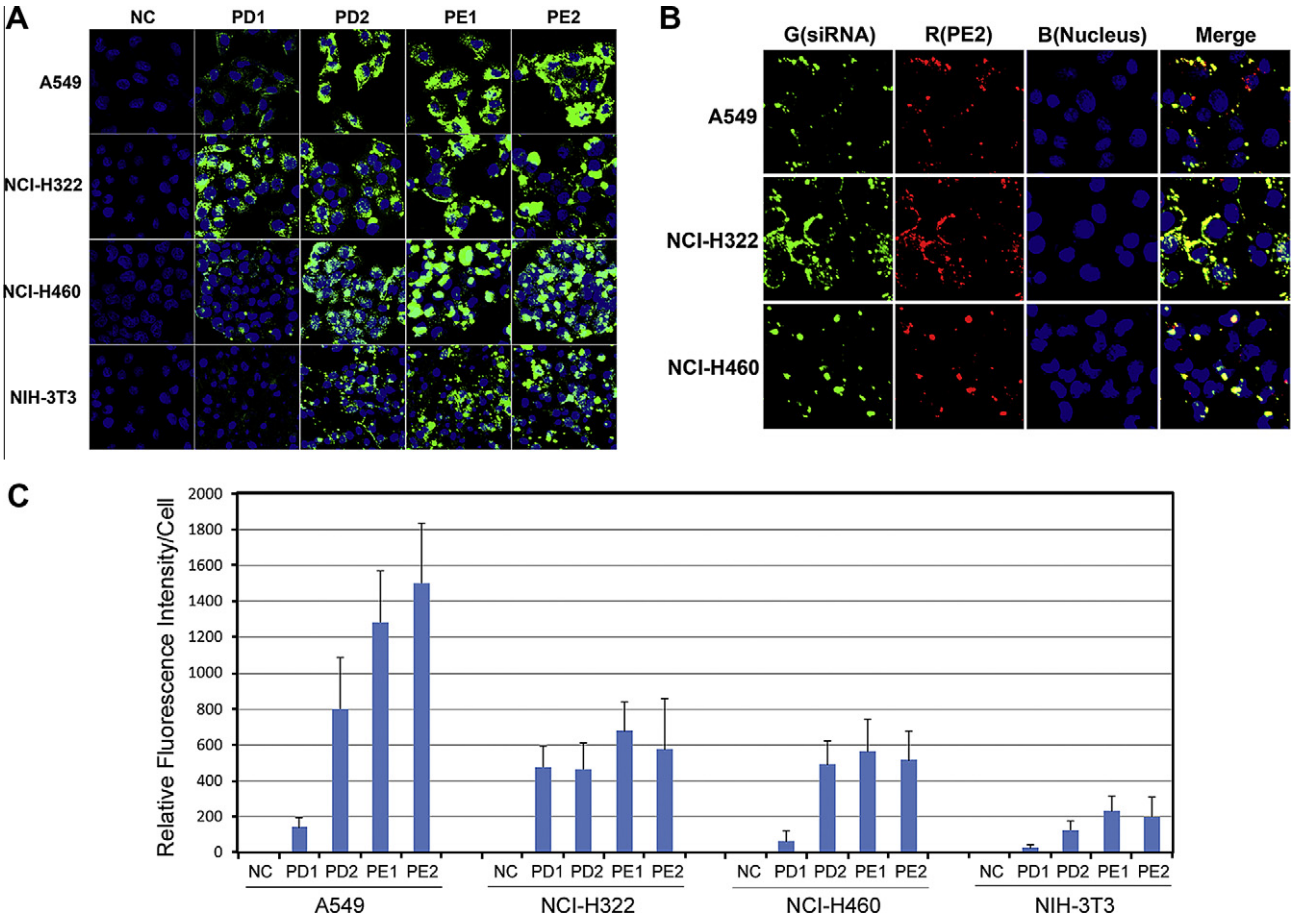

Fig. 2. Cellular uptake of ALPs with and without modification of one of the PD and PE peptides (A) and ROX-PE2 (B). (A) Four cell lines, namely, A549, NCI-H322, NCI-H460, and NIH-3T3 were transfected with FITC-siRNA/APLs with and without modification of either the PD or PE peptides on their surfaces. The non-conjugated ALPs (NC) showed no penetration into any of the cell lines, whereas the peptide-modified ALPs internalized in all of the cell lines, except PD1, which showed NCI-H322 preference. (B) Three NSCLC cell lines, namely, A549, NCI-H322 and NCI-H460 were treated with the ROX-PE2-modified FITC-siRNA/ALPs for 1 day, and the localizations of the peptide (red) and siRNA (green) were observed. Red and green colors were colocalized in the vesicle-like compartments in the cells. The nuclei were stained using DAPI (blue). (C) The average fluorescence intensity of an individual cell was calculated by dividing the total intensity of green fluorescence (siRNA) to the total cell numbers. The error bar indicates the standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

GFP fluorescence intensity in MDA-MB-435 cells was monitored using a confocal microscope (Olympus FV1000, Japan). The mean intensity of GFP fluorescence per cell was analyzed using the ImageJ 3D program (IJ 1.46r version, NIH, USA).

To inhibit the TMPRSS4 expression, we applied the peptide-modified ALPs encapsulating siTMPR4 (antisense: 5′-AAGUUGUC-GAAACAGGCAGAGAACC-3′) to NCI-H322 cells cultured in DMEM-FBS in a 6-well plate. After 48 h incubation, the expression of TMPRSS4 mRNA and the inhibition of cell migration and invasion were analyzed as described previously [14].

3. Results

3.1. Peptide design and cytotoxicity assay

We designed a peptide (PD1) with multiple RGD motifs to induce RGD/integrin-dependent internalization and to promote endosomal escape by the formation of an amphipathic α-helical structure in acidic endosomes (Fig. 1A). We also designed PD2 with alteration of two glycines to an arginine and a leucine to increase the basic and hydrophobic properties, respectively. We also changed all aspartic acids to glutamic acids of both PD1 and PD2, and named PE1 and PE2, respectively, to remove the RGD motif. PL was designed to adopt well an amphipathic α-helical structure. We initially tested the cytotoxic activities of the peptides (Fig. 1B). The peptide cytotoxicity was investigated using A549 and NCI-H322 cells cultured in 96-well plates using the WST-1 assay system. Antimicrobial peptides with basic and amphipathic natures usually have cytotoxic activities for bacteria, fungi, and animal cells (especially cancer cells) by formation of pores in the plasma membranes [15,16]. PL with the property elicited a potent cytotoxicity on A549 and NCI-H322 cells, while the other peptides with acidic residues displayed no cytotoxic activity for the cells at 100 μM (Fig. 1B). The lower toxicity of the peptides may provide a beneficial effect in the development of a CPP-mediated delivery system for drugs including nanoparticles.

3.2. Peptide-mediated liposome delivery

The fluorescence-labeled PD1 and PD2 showed cell-penetrating activity for the NSCLC cells and NIH3T3 cells (data not shown). To test the peptide-mediated cellular uptake of ALPs, we prepared the ALPs encapsulating FITC-siRNA with miPEG-PE, and found no non-specific delivery into cells tested as we reported previously [13]. PD1 or PD2 with the C-terminal cysteine residue was conjugated to the maleimide group of miPEG-PE on the ALP surface. The unmodified ALPs could not penetrate into cells as we reported [13], while the peptide-conjugation to ALPs successfully induced

their cell penetration (Fig. 2A and C). Interestingly, PD2 showed higher cell penetrating activity than PD1, even though it had less RGD motif. The RGE peptide is currently used as a negative control for the RGD peptide [14,17]. We tested the effect of the RGD motif in the peptide-mediated ALP uptake. Unexpectedly, the substitutions of glutamic acids for aspartic acids did not prevent the cellular uptake of the PE peptide-modified ALPs (Fig. 2A and C). Moreover, PE1 and PE2 displayed higher delivery efficiency than PD1 with three RGD motifs. These results demonstrate that the peptide-mediated uptake of ALPs may not be dependent on the RGD motif. To confirm whether PE2 induces the ALP uptake, the ROX-labeled PE2 (ROX-PE2) was conjugated to the ALPs and treated to the NSCLC cells for overnight. Fig. 2B shows that the red signals (ROX-PE2) colocalize with the green signals (FITC-siRNA), indicating that the ROX-PE2 is responsible for the ALP uptake. Although the uptake mechanism cannot be explained using the present data, the ability of the peptide-mediated liposome transduction suggests that the peptide with multiple RGD/RLD or RGE/RLE motifs will be developed as an effective CPP with less toxicity.

3.3. Peptide-mediated gene silencing of siRNA/ALPs

To analyze whether the peptide-mediated cellular uptake of the siRNA encapsulated in ALPs induces the target gene-silencing, we initially tested the gene-silencing activity of siGFP in MDA-MB-435 cells overexpressing GFP [18]. We prepared ALPs encapsulating siGFP (siGFP/ALPs) and then conjugated the peptides to the ALPs. Fig. 3 shows the peptide-dependent GFP gene-silencing activity of siGFP/ALPs. The siGFP transfection using lipofectamine 2000 (LF2K) effectively reduced the GFP expression (32.4 ± 6.3%) as compared with the non-treated group (NT), whereas the ALPs without conjugation (NC) displayed a similar GFP intensity with NT. In contrast, the Tat conjugation significantly reduced the GFP expression (44.2 ± 8.6%), and the PD and PE peptides also elicited the reduction of GFP expression ranging from 51.8 ± 11.7% to 37.3 ± 7.6%. These results indicate that the siGFP/ALPs uptake is responsible for the gene silencing. We further confirmed the peptide-mediated gene silencing by siTMPR4/ALPs. Fig. 4 shows inhibition of TMPRSS4 expression as well as TMPRSS4-mediated NCI-H322 cell invasion and migration. Previously, we found that

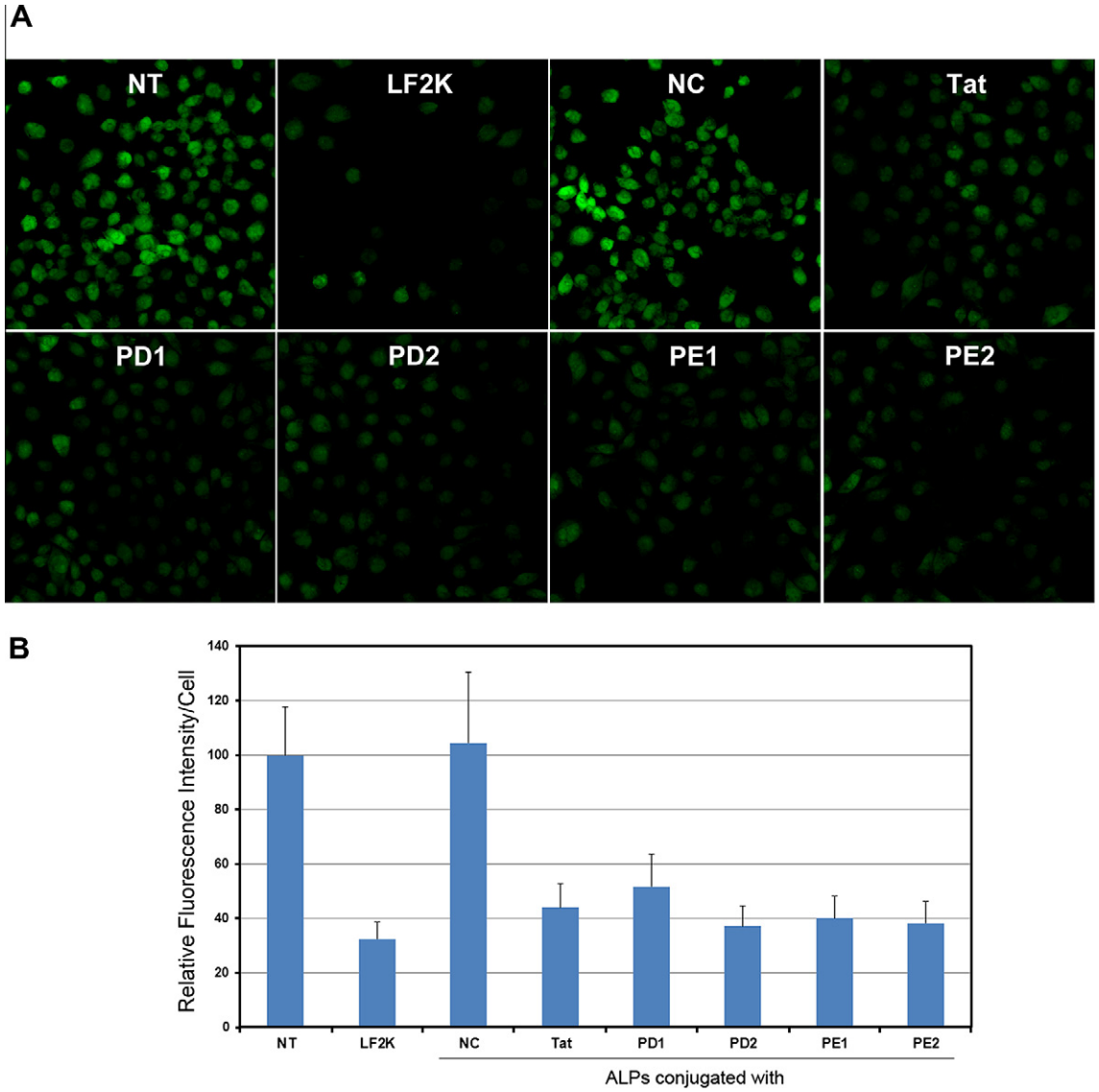

Fig. 3. In vitro GFP gene-silencing by siGFP. MDA-MB-435 cells were treated with siGFP/ALPs with or without conjugation of one of the peptides as indicated. After 48 h of incubation, the cells were monitored by a confocal microscope (A), and the average fluorescence intensity of an individual cell was analyzed by using the ImageJ 3D program (B). The non-modified siGFP/ALPs (NC) had no effect on the inhibition of GFP expression in MDA-MB-435 cells, similar to non-treatment (NT). The error bar indicates the standard deviation.

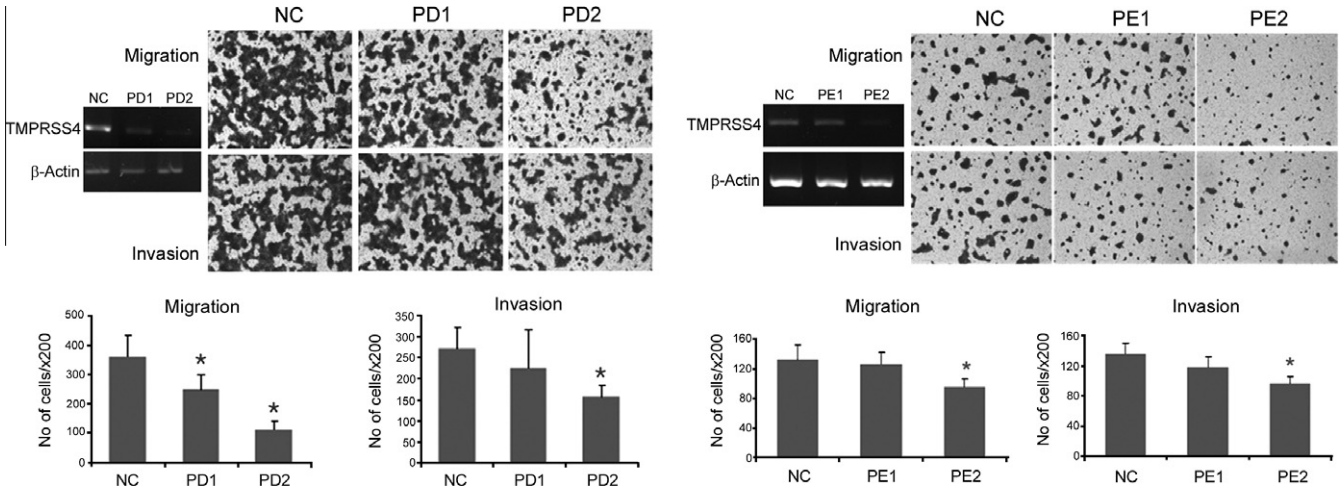

Fig. 4. TMPRSS4 knockdown and inhibition of cell migration and invasion by peptide-modified siTMPR4/ALPs. NCI-H322 cells were treated with siTMPR4/ALPs with or without conjugation of one of the PD and PE peptides. After 48 h of incubation, the cells were harvested and subjected to total RNA extraction as well as cell invasion and migration assays. TMPRSS4 mRNA levels were determined by RT-PCR, and β-actin was used as the internal control. For the cell invasion and migration assays, 4×10^4 cells were allowed to migrate or invade Matrigel for 48 h. Representative high-power (×200) fields (HPFs) were photographed, and the number of cells that had migrated or invaded was counted in six to eight representative HPFs per a Transwell insert. The error bar indicates the standard deviation ($^*P < 0.05$). NC means the non-conjugated siTMPR/ALPs.

the cells treated with the unmodified siTMPR4/ALPs (NC) showed the similar gene-silencing activity with the untreated cells [13], and a similar result was also found in this study (data not shown). As compared to NC without cell penetrating ability, the siTMPR4/ALPs conjugated with PD2 or PE2 displayed strong inhibition of TMPRSS4 transcription as well as the NCI-H322 cell migration and invasion. Although PD1 and PE1 induced good delivery efficiency of ALPs in NCI-H322 cells (Fig. 2A and C), PD1 and PE1 displayed less efficacy than PD2 and PE2 with more basic and hydrophobic natures, respectively (Fig. 4). These results were correlated with the TMPRSS4 mRNA expression levels (Fig. 4).

4. Discussion

Target-specific delivery and endosomal escape of siRNA play important roles in enhancing the therapeutic efficacy of siRNA. We focused on the nature of the RGD motif that is currently used for specific delivery to cancer cells overexpressing integrins, especially αvβ3. Since the amino acid with a carboxylic group has some hydrophobic property in an acidic endosome environment, we initially designed the peptides to localize at different sides of Arg and Asp/Leu on the α-helix wheel diagram (Fig. 1A). The hydrophobic moment of an amphipathic CPP is considered to play a critical role in cell penetration [6,9]. The PD peptides have lower hydrophobic moments at pH 7.0–7.4 because of the negatively charged acidic residues, and therefore we initially thought that the peptide-mediated internalization may be caused by the RGD motif(s). The RGE motif is currently being used as a negative control with no cellular uptake or less cellular uptake than that of the RGD motif [19,20]. Surprisingly, PD1 with three RGD motifs was less effective for cellular uptake of ALPs than PD2, PE1, and PE2 with one or no RGD motif (Fig. 2A), indicating that the increase of the basic and hydrophobic properties of the peptides seems to be more important for their CPP activity rather than the RGD motif. The result suggests that the RGD-independent mechanism may play an essential role for the peptide-mediated uptake of ALPs. However, the PD1-modified ALPs could be effectively internalized into NCI-H322 cells known to express TMPRSS4 on the cell surface unlike the other NSCLC cell lines (A549 and NCI-H460) showing almost no the protein [14,21]. TMPRSS4 expression has been reported to be responsible for the binding to the RGD motif [14]; therefore, we assume that the RGD-dependent interaction may be a possible mechanism for the PD1-mediated uptake of ALPs in TMPRSS4 expressing cells.

The direct penetration of the peptide may be difficult because of the existence of five acidic residues and less hydrophobic moment. Moreover, the peptide conjugated ALPs with an average size 250 nm may be much harder to directly penetrate the cell membrane. The Tat and polyarginine peptides are known to be internalized into cells by polyanionic heparan sulfate proteoglycan receptor-mediated endocytosis [22,23]. In addition, azurin-derived acidic peptides have been recently reported to preferentially penetrate into cancer cells via endocytotic, caveosome-directed and caveosome-independent pathways [11]. Localization of internalized FITC-siRNA/ALPs and colocalization of ROX-PE2 and FITC-siRNA (Fig. 2B) at the vesicle-like compartments also suggest that the endocytotic pathway may be essential for the peptide-mediated uptake of ALPs. The PD and PE peptides are hard to group with other CPPs [6,7], and therefore they are considered to be a new type of CPP.

The ideal CPPs should be non-toxic and could transport any target molecule and/or particle into every cell. Basic amphipathic CPPs with higher amphipathic moments induce significant leakage of cancer cells and hemolysis [24], and PL with basic amphipathic property also shows cytotoxicity (Fig. 1B). The basic rich CPPs such as Tat and antennapedia display much less toxicity [24], while it also has been reported that the basic rich CPPs reduce the viable cells [25]. In contrast, the PD and PE peptides used in this study do not have any toxicity for A549 and NCI-H322 cells at 100 μM (Fig. 1B). Based on cell line-independent internalization and almost no cytotoxicity, the peptides seem to satisfy the category of ideal CPPs.

CPP-mediated endocytosis does not usually correlated to the cytoplasmic distribution of the target molecule required for siRNA function. The siRNA/ALPs conjugated to one of the peptides could effectively induce the siRNA-mediated gene-silencing in MDA-MB-435 and NCI-H322 cells with slightly different patterns (Figs. 3 and 4, respectively). Although we found no significant difference in the gene silencing activity by siGFP between the peptides, PD2 and PE2 with more basic and hydrophobic residues induced much higher siTMPR4-mediated gene silencing than, PD1 and PE1 in

NCI-H322 cells, respectively (Fig. 4), whereas no significant differences among the PD and PE peptides were observed in MDA-MB-435 cells (Fig. 3). Although we cannot explain the different results using the present data, at least PD2 and PE2 may have the ability to increase siRNA release to cytoplasm.

In conclusion, peptides with multiple R-G/L-D/E motifs have nonspecific cell penetrating activity and induce ALP uptake in many cells. These peptides have almost no toxicity at 100 μM for A549 and NCI-H322 cells. The peptide sequences are different from other reported CCP ones; therefore, they seem to be a new type of CPP. Although we obtained some ambiguous gene-silencing results induced by the peptides, at least PD2 and PE2 may be considered to be effective carriers for siRNA/ALPs. The peptides with CPP activity and almost no toxicity to cells will be useful in the development of carriers for drug and nanoparticles.

Acknowledgments

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