Cancer-Specific RNAi System by Using PNA-Peptide Conjugates

李, 恩卿

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Doctoral Thesis

Cancer-Specific RNAi System by Using PNA-Peptide Conjugates

Graduate School of System Life Sciences

Kyushu University

2016

Eun Kyung Lee

Abstract

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Eun Kyung Lee

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Small interfering RNA (siRNA) received much attention for treatment of intractable disease including cancer. The advantage of siRNA-based cancer therapy is its versatility to target a large number of different genes that are related to distinct cell signaling pathway of cancer cell. However, there are remaining several hurdles to overcome, such as unstable nature, off-target effect, and non-specific delivery. Among these issues, non-specific delivery of siRNA is one of the major causes of adverse effects.

To access the issue, here I designed a new siRNA system which turns on RNAi responding to a cancer cell-specific protease, cathepsin B. The system uses a peptide nucleic acid (PNA)-peptide conjugates to provide a protease-responsive activation. The PNA-peptides were found to form hybrids with double-stranded RNAs with complementary protruding regions, which then affected the susceptibility of dsRNA to Dicer. The dsRNA/PNA-peptide hybrids were activated in cancer cells with a high cathepsin B activity to show RNAi.

Then, I proposed the apoptosis-inducing siRNA system which inhibits the expression of bcl-2 protein for cancer therapy. The dsRNA/PNA-peptide hybrid showed apoptosis induction of cancer cell in response to cathepsin B activity.

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General Introduction

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1-1. RNAi and siRNA

Gene therapy is the gene to replace the abnormal gene that causes the disease or refer to a treatment technique for inserting a gene for treatment of disease such as cancer. Initial gene therapy was to insert the target-specific DNA into the cells or tissues, however, recently, antisense therapy also includes for inhibiting the expression of disease-related gene using small interfering RNA (siRNA) and antisense oligodeoxy nucleotide.

RNA interference (RNAi) is a natural mechanism of gene silencing conserved in plant and mammalian cells.[1,2] This process represents a promising new strategy for gene target identification, and has also rapidly emerged as having promising therapeutic potential for human diseases including cancer.[3,4] In 1990, this mechanism was first reported post-transcriptional gene silencing or co-suppression in plant cells.[1] And, RNAi was discovered in fungi, C. elegans, Drosophila and zebra fish from 1998 to 2001 and received the Nobel Prize in physiology or medicine in 2006.[5]

The RNAi pathway occurs by effector molecules, siRNA in cytoplasm and can be divided into 3 steps which are initiation, effector, and mRNA cleavage steps. In the initiation step, long dsRNA cleaves by Dicer (RNase Ⅲ type protein) and produces siRNA. siRNA is composed of 19-21 nucleotides (nt) of double stranded RNA (dsRNA) fragments with a 2 nt overhang at the 3′-end. Dicer enzyme is necessary for production of siRNA and it consists of 4-types of domains which are RNA helicase, PAZ, tandem RNase Ⅲ, dsRNA binding

domain. [6] In the effector step, siRNA bind to RNA-induced silencing complex (RISC). This RISC complex discovered Argonaute protein that contains PAZ/PIWI domain.[7] In mRNA cleavage step, activated RISC which contains antisense strand RNA binds to target messenger RNA (mRNA) by complementary base pairing. Then, mRNA is cleaved and protein cannot be synthesized (Figure 1.1).

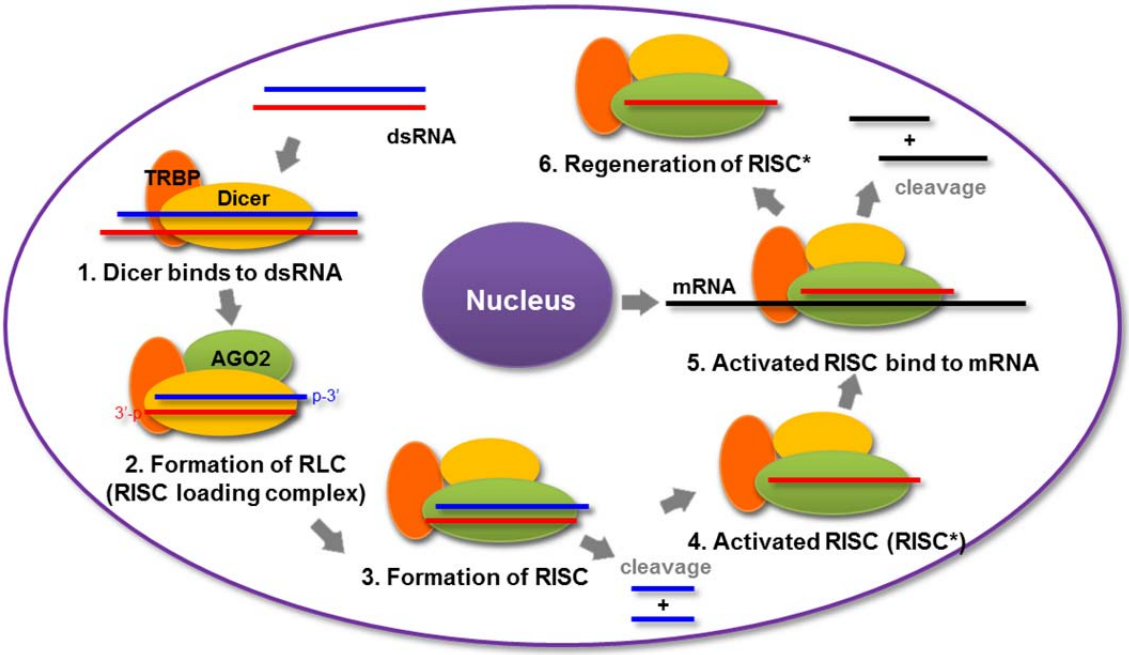

Figure 1.1 Mechanism of RNA interference. siRNA are produced from dsRNA by cleavage of Dicer and then combined to RISC. The antisense strand RNA binds to the target mRNA where mRNA degradation is initiated to inhibit protein synthesis.

1-2. Clinical Trials of siRNA Therapeutics

Gene therapy using siRNA holds the promise to inhibit target gene expression. Both pharmacetutical companies and academic laboratories have committed financial resources to develop siRNA-based therapeutic. Clinical studies of siRNA therapeutics have processed

inhibition of growth and survival in various diseases by target gene such as B-cell CLL/lymphoma 2 (Bcl-2), epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), and ribonucleotide reductase subunit M2 (RRM2) and so on (Table 1.1). Also, most of the target genes are blocked by major cell signaling pathway such as protein tyrosine kinase (PTK), hypoxia-inducible transcription factor (HIF), apoptosis, p53 pathway.[8] For example, Sirna-027 and Bevasiranib were developed for treatment of age-related macular degeneration (AMD) by Allergan and OPKO Health, Inc., respectively. These drugs proposed synthesis of targeting the VEGF inhibits by direct injection in eye. [9,10] In case of Bevasiranib, it is the first drug which entered the phase III clinical trials, however clinical trial was discontinued because the therapeutic mechanism was not clearly determined.[11] In addition, Quark Pharmaceuticals is developing anticancer drug (I5NP) using p53 target gene for decreasing injury of kidney. Also, CALAA-01 which delivers siRNA with cyclodextrin polymer enhances the efficient delivery in target cells and is protected from nuclease.[12]

Although many clinical trials using the siRNA is still progressing, most of studies stays in Phase I because initial research status has not yet developed efficient siRNA delivery system. Thus, development of effective delivery system is required for therapy of disease including cancer.

Table 1.1 Clinical trials of siRNA therapeutics

Drug name	Target	Disease	Phase	Status	Company
SPC2996	Bcl-2	Chronic Lymphocytic Leukemia	I / II	Completed	Santaris Pharma A/S
CALAA-01	RRM2	Solid Tumor	I	Terminated	Calando Pharmaceuticals
TKM-080301	PLK1	Cancer	I	Completed	National Cancer Institute
Atu027	PKN3	Solid Tumor	I	Completed	Silence Therapeutics
siG12D LODER	KRAS	Pancreatic Cancer	II	Not yet recruiting	Silenseed Ltd.
EZN-2968	HIF-1α	Solid Tumor	I	Completed	Santaris/Enzon
PF-655	VEGF	Diabetic Macular Edema	II	Completed	Quark Pharmaceuticals
Sirna-027	VEGFR1	Wet Age-Related Macular Degeneration	I / II	Completed	Allergan
Bevasiranib	VEGF	Age Related Macular Degeneration	III	Withdrawn	OPKO Health, Inc.
siRNA-EphA2-DOPC	EphA2	Advanced Cancer	I	Recruiting	M.D. Anderson Cancer Center
SYL040012	ADRB2	Glaucoma Ocular Hypertension	I / II	Completed	Sylentis, S.A.
ALN-VSP02	VEGF, KSP	Solid Tumor	I	Completed	Alnylam Pharmaceuticals
PRO-040201	ApoB	Hypercholesterolaemia	I	Terminated	Tekmira Pharmaceuticals
I5NP	p53	Injury of Kidney, Acute Renal Failure	I	Completed	Quark Pharmaceuticals

Data are obtained from ClinicalTrials.gov

1-3. Drawbacks of siRNA-based Therapy

Despite the clinical studies of siRNA-based cancer therapy, it has several issues to overcome, such as chemical instability of siRNA, off-target effects, immune stimulation and non-specific delivery.[13,14]

Instability of siRNA result from rapid degradation by nuclease leading to a short half-life in serum and tissue.[15-17] To prevent degradation of siRNA, a variety of chemical modifications of siRNA have been developed such as phosphorothioate (PS) linkage, 2′-O-methyl (2′-OMe), 2′-fluorine (2′-F), locked nucleic acids (LNA) and unlocked nucleic acids (UNA).[18] These chemical modification of siRNA were found high resistance to nuclease.

siRNA which has large size and negative charge by phosphate group is difficult to cross the cell membranes.[19] For this reason, many researchers are developing siRNA delivery system for improving a transfection efficiency of siRNA. For example, cationic lipid delivery system, as well as siRNA conjugated hydrophilic polymer showed high transfection efficiency than naked siRNA.[20]

Off-target effect includes the non-specific knockdown of non-target genes and inflammatory response. RNAi occurs based on Watson-Crick base pairing between antisense strand RNA (asRNA) and target mRNA, even if mismatch of one or several base pair exist. Due to the mismatch between asRNA and target mRNA, there are possibilities that have effect on not considered target. Birmingham. et al. showed that off-target effect is caused the matching between 3′ untranslated region (UTR) of mRNA and seed region (6 - 8 nt) of siRNA antisense strand.[21] Despite the partial complementarity, siRNA can binds several target mRNAs with seed region. Therefore, to inhibit target gene expression, providing target gene specificity is important.

The off-target effect of siRNA is through immune response is triggered by recognition of long dsRNA by receptors. It has been reported that long dsRNA (more than 29-30 bp) can lead to interferon (IFN) response.[22] Toll-like receptors (TLR) recognize dsRNA to induce immune response.[23] Several TLRs are involved in the recognition of siRNA.[24] TLR3, TLR7 and TLR8 are the RNA sensing receptors among TLR family. siRNA shown to activate TLR3 signaling without sequence specificity. TLR7 and TLR8 are activated by synthetic siRNA in a sequence-dependent manner. Thus, immune response is the problem to be solved in siRNA-based therapy.

1-4. siRNA Delivery System

For effective siRNA delivery, siRNA should be delivered with high efficiency into a cell and it maintains stable expression for a long time. Generally, after injection of siRNA delivery system, siRNA is mostly introduced into the target cell through endocytosis by binding receptors of surface.[25] Then, it is moved in the lysosomes filled with enzyme, and it escapes from endosome to the cytoplasm.[26,27] At the point, siRNA reach the nucleus through the cytoplasm (Figure 1.2).

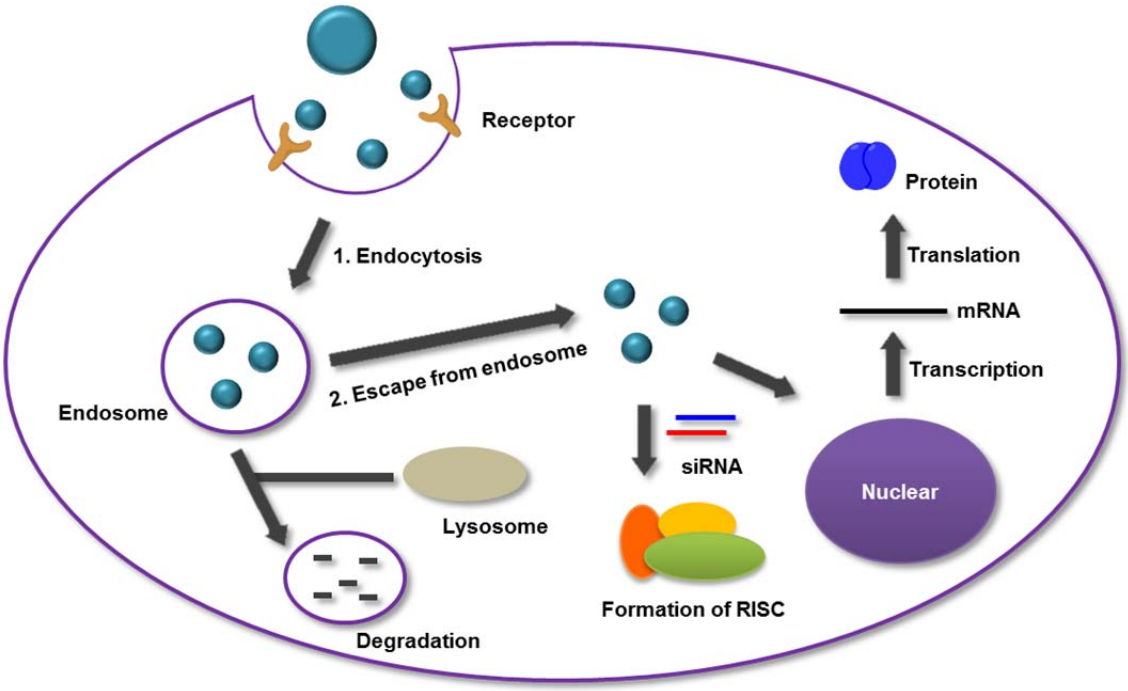

Figure 1.2 Process of siRNA delivery into the cells. First, siRNA with carrier is introduced into cell by endocytosis. The siRNA is released into the cytoplasm.

The methods of siRNA delivery are divided into viral- and non-viral vector.[28,29] Using viral vector such as retrovirus or adenovirus has very high transfection efficiency, but is difficult to long-term administration due to the immune response.[30,31] Therefore, safety of viral vector has been becoming a major problem in the clinical application due to the immune response.

To compensate the drawback of viral vector, cationic lipids and polymer have being developed as non-viral vector such as cationic polymer, lipid, and inorganic material (Figure 1.3). These have attracted attention as a siRNA carrier because they are formed with anionic siRNA by electrostatic interaction. They are introduced into the cell by endocytosis, and it will go through the endosome to the lysosome. At this time, the complexes are degraded in lysosome by nuclease. Polyethylenimine (PEI) has high transfection efficiency and an

incomparable proton-sponge effect is widely used as siRNA carrier.[32] Cyclodextrin had increased the potential for clinical application because it is able to determine the effective siRNA delivery in monkey as well as mouse models.[33-35] The cholesterol-conjugated siRNA has high binding with serum albumin has been reported an excellent delivery and inhibition of target gene. [36]

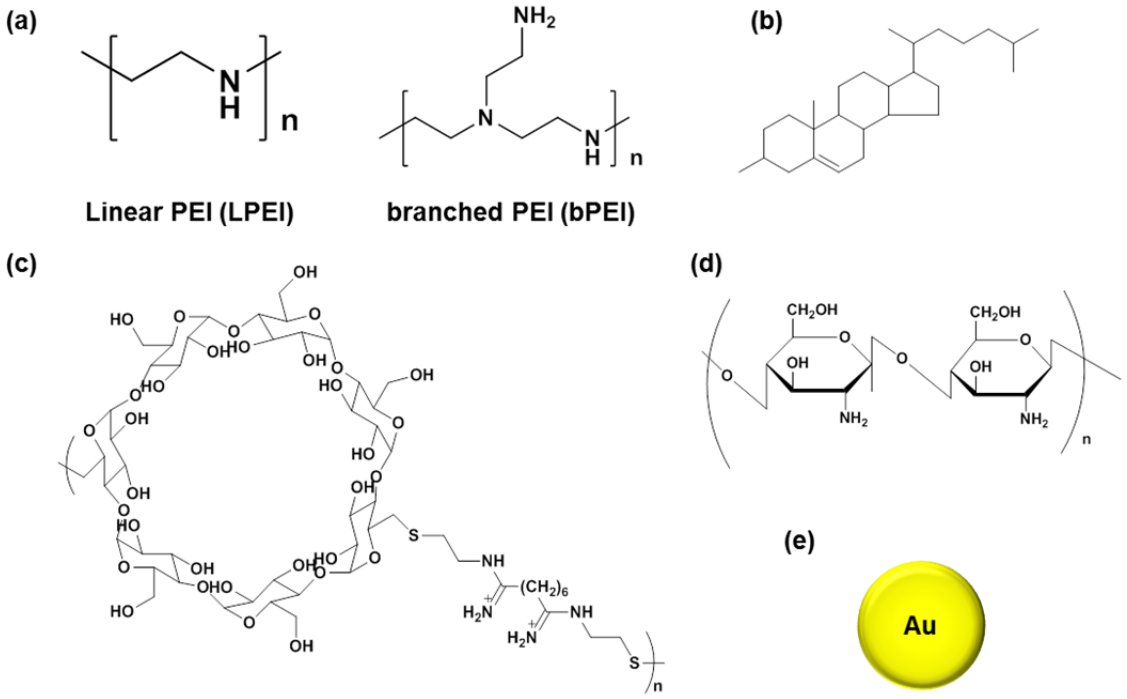

Figure 1.3 Non-viral vectors for siRNA delivery. (a) Polyethlenimine (PEI), (b) Cholesterol, (c) Cyclodextrin, (d) Chitosan [37] and (e) Gold nanoparticles [38]

1-5. Overview of This Thesis

The objective of this thesis is to develop cancer specific RNAi system for application in cancer therapy. Until now, much research is preceded characteristic in respect of siRNA-based cancer therapy. Although significant progress has been made in the design of siRNA, there are remaining many hurdles to overcome including unstable nature, efficient delivery, and avoiding side effect. Especially, it is important to discriminate between normal cell and cancer cell in siRNA-based cancer therapy.

In this thesis, novel RNAi system for siRNA delivery is proposed, and the RNAi effect is evaluated for cancer therapy. To provide efficient siRNA delivery, cancer protease-responsive siRNA carrier is designed and synthesized. Chapter 2 and 4 consist of studies on RNAi system for cancer therapy.

Chapter 2 reports design of cancer protease-responsive RNAi system based on PNA-peptide conjugates. The PNA-peptide was designed and synthesized. Formation of hybrid between dsRNA and PNA-peptide was examined and the physicochemical characteristics and response against enzymes (dicer and cathepsin B) of the hybrid was examined.

Chapter 3 introduces the in vitro evaluation of suppression of gene expression of the cancer protease-responsive RNAi system in response to cancer-specific cathepsin B.

Chapter 4 suggests a dual-targeting RNAi system using bcl-2 siRNA and PNA-peptide conjugate for cancer therapy. This RNAi system was sought to evaluate whether induces apoptosis while responses protease.

Finally, chapter 5 summarized the conclusions that can be drawn from this work and proposes future studies for siRNA-based cancer therapy.

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CHAPTER 2

Cancer Protease-Responsive RNAi system; Design and Characterizations

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2-1. Introduction

A key challenge of siRNA-based cancer therapy is development of effective delivery system. And, the non-specific delivery of siRNA to normal tissue can cause serious adverse effects because the same signaling pathways may also be essential for normal cells. To overcome non-specific delivery, researchers have used siRNAs modified with ligand molecules such as peptides,[1-4] or antibody,[5,6] or aptamers,[7-11] which recognize cancer cell surface antigens.

An alternative way to target cancer cells with siRNA is to activate siRNA upon recognition of the specific intracellular environment of the cancer cell. Several promising strategies have been reported using this concept. Sando et al. reported an siRNA which is activated upon hybridization with a specific mRNA existing specifically in the target cell.[12] Koehn et al. developed a protease-responsive siRNA by covalent modification of siRNA with a protease-specific peptide sequence.[13] With the cleavage of the peptide region by caspase 4, which exists in apoptotic cells, the siRNA can be recognized by Dicer to induce RNAi. This strategy is potentially applicable to any kind of protease, although covalent modification of a peptide with siRNA is troublesome because of the chemically unstable nature of siRNA.

Here, I developed a simple protease-responsive RNAi system without covalent modification of siRNA. I designed a peptide nucleic acid (PNA)-peptide conjugate which

renders protease responsive characteristics to siRNA. The PNA-peptide conjugate is readily prepared by Fmoc-solid phase peptide synthesis.[14,15] The mechanism of action of the hybrid between PNA-peptide and dsRNA is shown in Figure. 2.1. To provide the protease responsive action, dsRNA that composes of normal antisense RNA (asRNA) and sense strand RNA (ssRNA) with a protrusion at 3′-terminus, was also designed and synthesized. And, asRNA of 3′-terminus contained dTdT which prevent degradation of siRNA by RNase in lysosome and improve binding efficiency with target mRNA. The PNA region of the PNA-peptide (gray sequence) hybridizes with ssRNAs protruded region and the peptide tail (green sequence) hinders the dsRNA from the recognition and editing by Dicer. However, this peptide tail can be removed with cathepsin B which is known to be highly expressed in lysosomes of many types of cancer cells.[16-19] This peptide cleavage is expected to start the editing by Dicer to activate RNAi.

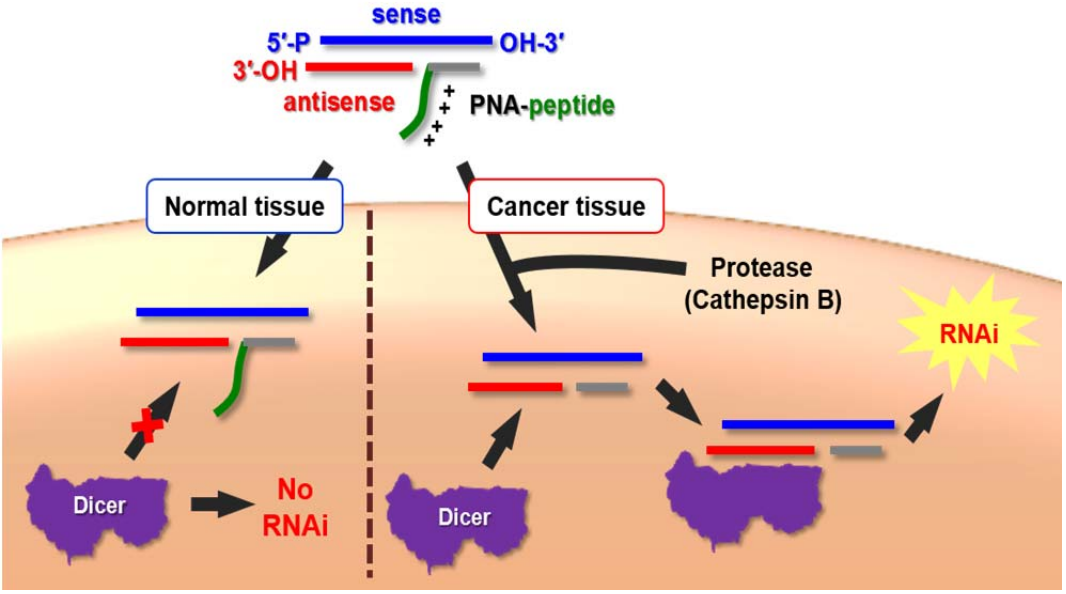

Figure 2.1 Mechanism of action of cancer protease-responsive siRNA. The PNA-peptide conjugate forms hybrid with complementary dsRNA by electrostatic interaction.

PNA was first reported by Niselsen et al. in 1991 is an artificially synthesized polymer

similar to DNA or RNA.[20] The backbone of PNA has replaced with 2-aminoethyl glycine instead phosphodiester of DNA (Figure 2.2).

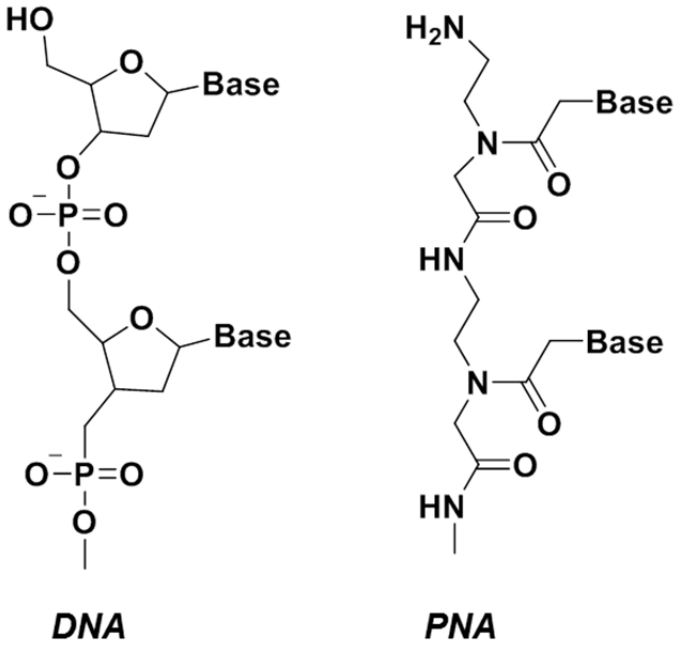

Figure 2.2 Structural comparisons between DNA and PNA.

PNA has several chemical characteristics such as higher melting temperature and resistance against enzyme. PNA is resistant against enzyme degradation because of amide backbone. For this reason, PNA exists for a long-term in vitro and in vivo.[21] PNA is easily synthesized by Fmoc-solid phase synthesis.[14,15] PNA forms more stable hybrid with complementary RNA and DNA than RNA and DNA hybrid because having neutral electric charge imparts the stronger binding force between nucleic acid and PNA.[22] Due to this advantage of PNA, PNA have been used in biomedical studies such as diagnosis and antisense therapy.[23-25]

Proteolysis is one of the most important biological reactions. The activity has been attributed to a class of enzymes called proteases. There are currently 596 known human protease and related to cancer.[26,27] Recently, many proteases have been determined as makers

or target for diagnosis and treatment of cancer.[28-32] The substrate of protease contains specific sequences for recognition of protease. Among various proteases, we focused on cathepsin B for the cancer-specific protease to utilize activation of our RNAi system. Cathepsin B is reported one of the cysteine protease of papain family and accumulates in the lysosome which is slightly low pH than the cytosol.[16-19] The main function of cathepsin B is the degradation of proteins that have entered the lysosomal system from the cell.[33] Also, the protease acts as an endopeptidase, cleaving internal peptide bonds, and assists a hydrophobic side chain in the substrate which are two residues N-terminal to the scissile peptide bond (Figure 2.3). Cleavage of peptide bond is catalyzed by two residues, cysteine (Cys 29) and histamine (His 199). The reaction site residues, Cys29-His199, are mediated by nucleophilic attack by S- from Cys29 on the carbonyl carbon, followed by proton donation from His199.[34] The substrate sequences of cathepsin B was reported as cleavage site is between Phe and Leu,[35,36] or Lys and Lys.[37] Cathepsin B has been used as a trigger to release drug for drug delivery system [38-40] and it has been also utilized to turn on fluorescent signal of molecular probe of cancer. [41-43]

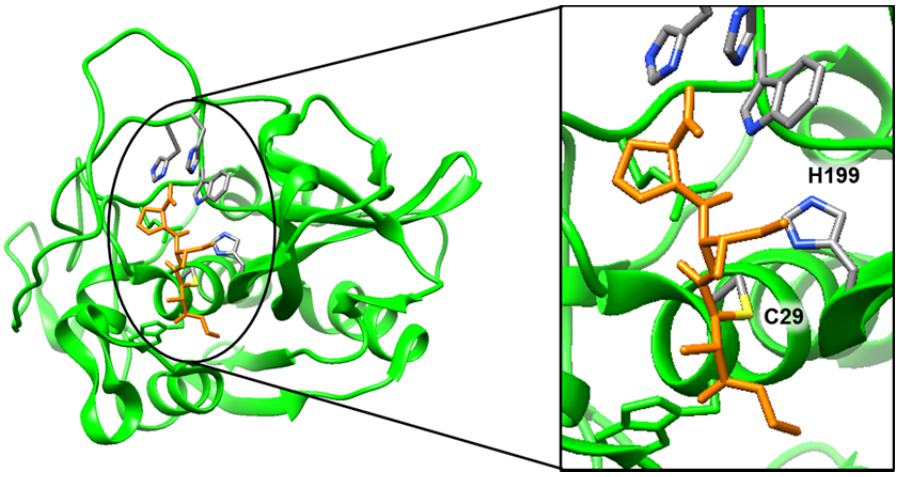

Figure 2.3 The three-dimensional structure and reaction site of cathepsin B (From Protein Data bank, 1CSB). Cathepsin B consists of active site residues which are His199 (H199) and Cys29 (C29).[34]

2-2. Results and Discussion

2-2-1. Design and Synthesis of PNA-peptide

All of PNA-peptides summarized in Table 2.1 were synthesized by Fmoc-solid phase peptide synthesis. PNA-peptides were purified by reverse phase HPLC and were identified by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. D-amino acid was used so that the sterically-hindering region was resistant to non-specific proteolytic degradation (shown as lowercase letters in Table 2.1). The PNA regions of PNA-peptide are designed for preventing complementary binding of itself and have the GC contents for obtaining high melting temperature (T_m). Also, the PNA-peptide includes a substrate sequence for cathepsin B (GFLG) in r8S, r4S and r4L, and this sequence was replaced with protease-resistant linker X in the negative control PNA-peptides (r8S-N, r4S-N, r4L-N) (Figure 2.4). The substrate seqeunce for cathepsin B was used for cancer specific delivery of doxorubicin.[44]

I checked whether the PNA-peptides can be cleaved by cathepsin B. The PNA-peptides were dissolved in reaction buffer and cathepsin B was added to the solution.[45] After incubation of PNA-peptide with cathepsin B, the reaction mixture was analyzed by MALDI-TOF mass spectrometry. As shown in Figure 2.5a, r8S was cleaved selectively at the various positions of the GFLG sequence. In contrast, a negative control r8S-N, which does not include this sequence, was not cleaved by cathepsin B (Figure 2.5b). Also, r4S and r4L are cleaved by cathepsin B and, negative control PNA-peptide were not cleavage by cathepsin B (Figure 2.5c-f). The peaks observed in negative control PNA-peptide are assignable to impurities which were also detected in a mass spectrum of original PNA-peptide.

Table 2.1 Sequences of siRNA, sticky dsRNA, dsDNA and PNA-peptide (X: 8-amino-3,6-dioxaoctanoic acid, FAM: 6-carboxyfluorescein). Sequences shown in gray and green in PNA-peptide are sterically-hindering region and complementary region, respectively. The lowercase letters indicate D-amino acids.

Sample	Sequences
ssRNA	5′-CUUACGCUGAGUACUUCGAdTdT-3′
sssRNA-S	5′-CUUACGCUGAGUACUUCGAGAUGAUGdAdU-3′
sssRNA-L	5′-CUUACGCUGAGUACUUCGAGAUGAUGAUGAUdGdA-3′
asRNA	3′-dTdTGAAUGCGACUCAUGAAGCU-5′
sssDNA-L	5′-CTTACGCTGAGTACTTCGAAATGTCGATGATGATGA-3′
asDNA	3′-TTGAATGCGACTCATGAAGCT-5′
asDNA-FAM	3′-FAM- TTGAATGCGACTCATGAAGCT -5′
r8S	C-NH_2-rrrrrrrr-X-GLFG-X-CTACTACTA-H-N
r8S-N	C-NH_2-rrrrrrrr-X- X -X-CTACTACTA-H-N
r4S	C-NH_2-rararara-X-GLFG-X-CTACTACTA-H-N
r4S-N	C-NH_2-rararara-X- X -X-CTACTACTA-H-N
r4L	C-NH_2-rararara-X-GLFG-X-CTACTACTACT-H-N
r4L-N	C-NH_2-rararara-X- X -X-CTACTACTACT-H-N

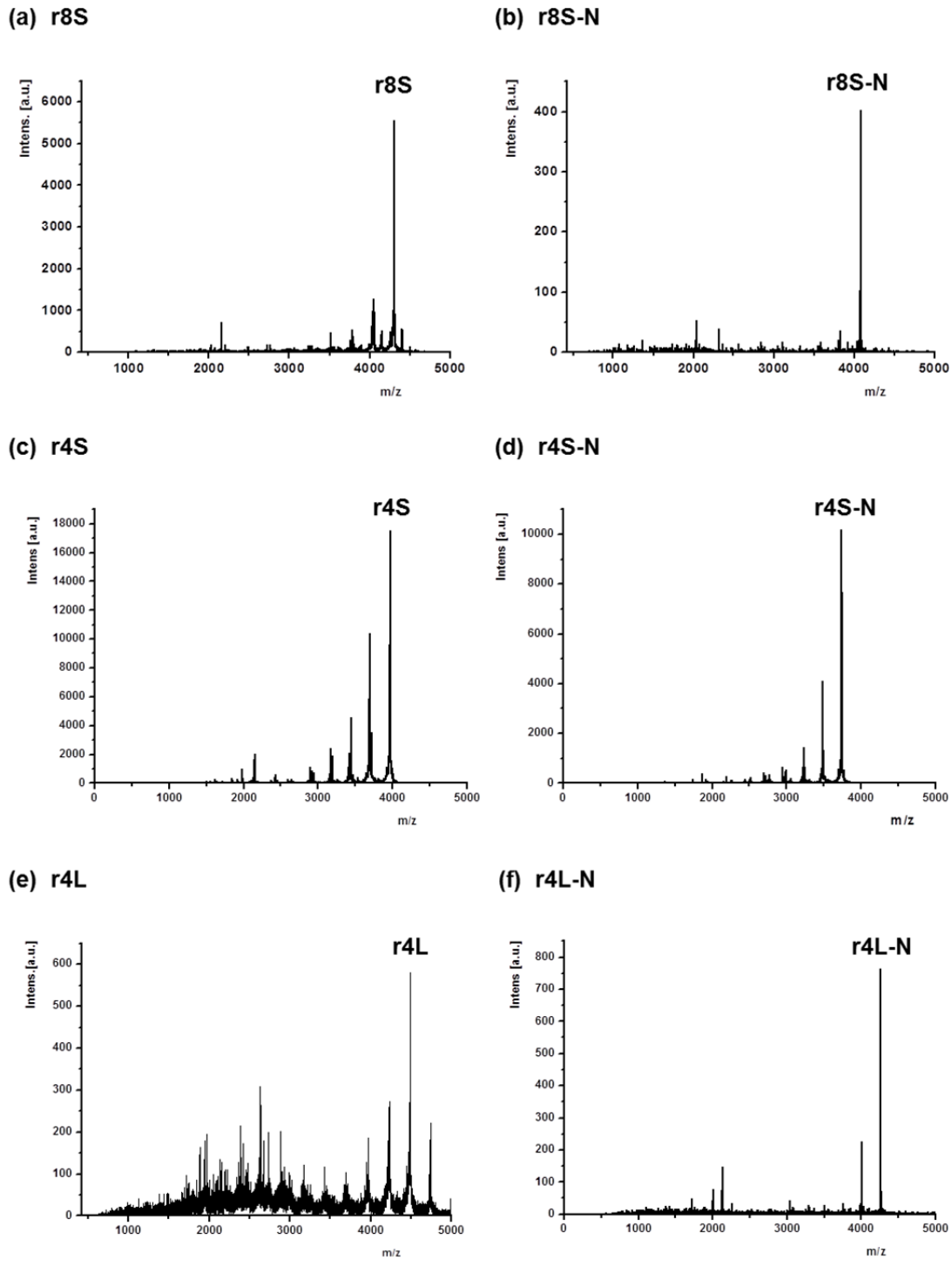

Figure 2.4 Synthesized PNA-peptide were eluted with a gradient of 0-30% eluent A and B within 30 min at a flow rate of 1.0 ml/min, where A was 0.1% (v/v) TFA in water and B 0.1% (v/v) TFA in acetonitrile; detection was at 260 nm. The molar masses of all peptides were determined by MALDI-TOF mass spectrometry.

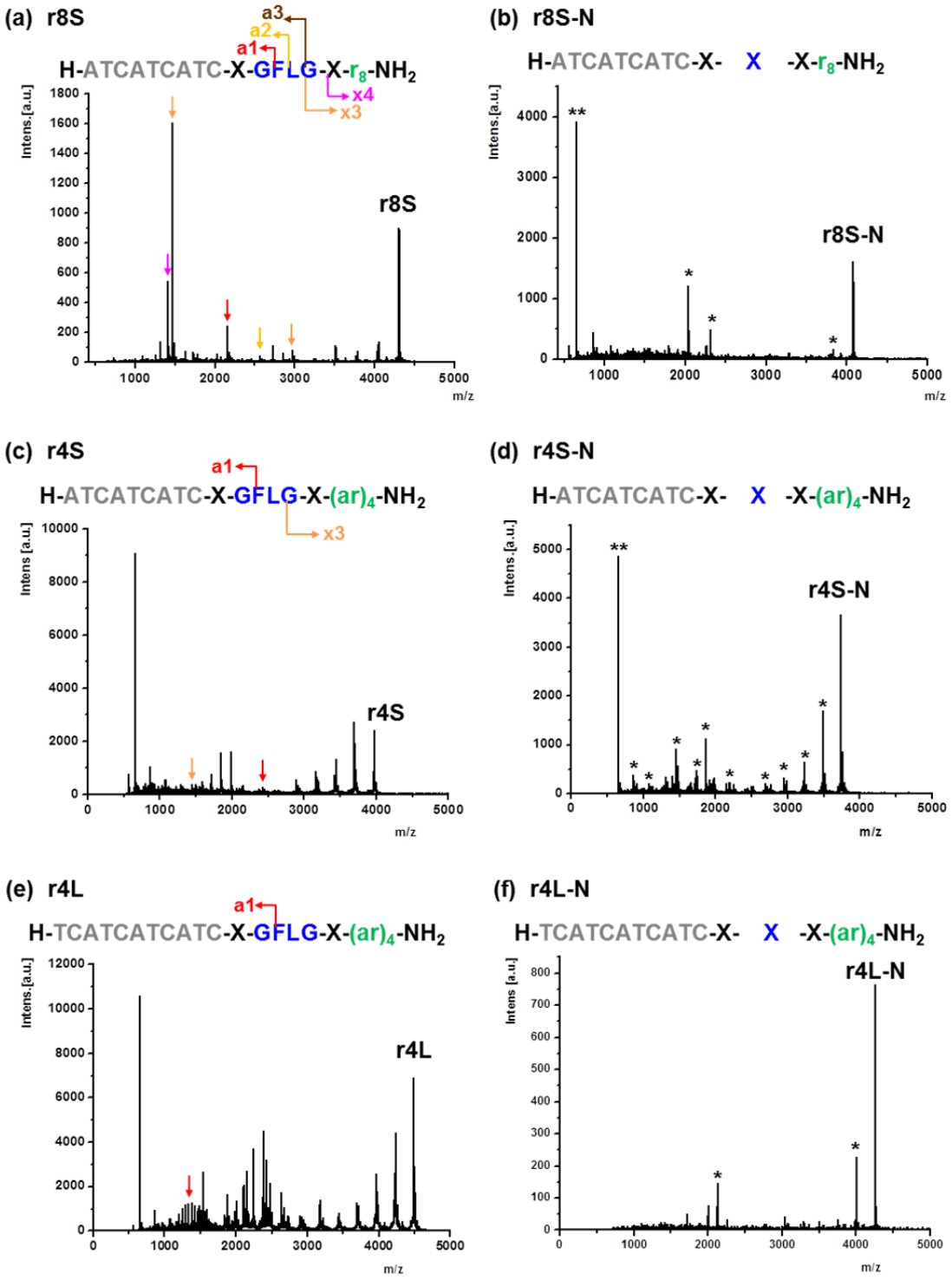

Figure 2.5 All of PNA-peptides were incubated with cathepsin B in reaction buffer at 37 ^{o}C for 30 min. Cleavage fragment were analyzed by MALDI-TOF mass spectra. *: impurities, **: matrix

2-2-2. Stability and Formation of dsRNA/PNA-peptide hybrid

The solubility of PNA-peptides and their hybrids with complementary RNA in physiological saline was examined by turbidimetry (Figure 2.6). When r8S was mixed with dsRNA-S (ssRNA-S/asRNA duplex), the solution became slightly turbid. This insolubility of the hybrid could result from the strong electrostatic interaction between anionic dsRNA-S and cationic r8S. Then I examined r4S, which contained half the number of cationic D-arginine (Table 2.1). Although r4S was not completely soluble in PBS due to the less cationic charge and hydrophobic nature of PNA, it became soluble upon hybrid formation with dsRNA-S (Figure 2.6b). Then I measured the melting curve of this hybrid. The hybrid of dsRNA/PNA-peptide should be stable at physiological condition. Thus, the thermal stability of dsRNA/PNA-peptide was evaluated by the melting temperature. Solutions of PNA-peptide and dsRNA were all brought to 3 μM. The duplexes were annealed at 90 ^{o}C for 1 min and then cooled down slowly to room temperature in the spectrometer. Thermal program: starting and returning temperature was 25 ^{o}C. Heating to 90 ^{o}C, cooling to 5 ^{o}C, each at 1 ^{o}C /min and holding for 3 min at the end-temperatures. The procedure was repeated three times. As shown in Figure 2.7, dsRNA-S/r4S hybrid (blue line) showed two transitions at 40°C and 70°C. The higher *T*m was found to be the melting temperature of dsRNA-S (black line). Thus, the lower *T*m was attributed to the melting point of dsRNA-S and r4S. The *T*m between dsRNA-S and r4S was not suited to further experiments because the hybrid would be instable at physiological temperature, 37°C. Thus, I designed another PNA-peptide, r4L (Table 2.1), that was extended by two bases in the PNA region of r4S to stabilize the hybrid with dsRNA-L (ssRNA-L/asRNA duplex). The dsRNA-L/r4L hybrid was fully soluble in PBS (Figure 2.6c) and like the dsRNA-S/r4S hybrid showed two transitions (Figure 2.7, red line). From the

melting curve, the *T*m value between dsRNA-L and r4L was determined to be 52°C, which is much higher than physiological temperature. Thus, I selected r4L for RNAi applications as shown below.

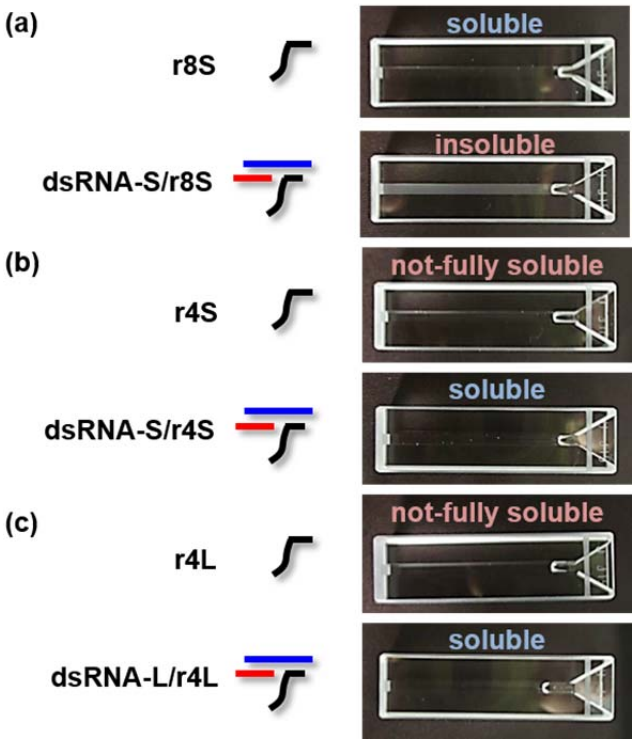

Figure 2.6 Solubility of three kinds of PNA-peptide and their hybrid with dsRNA in PBS. Concentration of PNA-peptide and dsRNA is 3 μM, respectively.

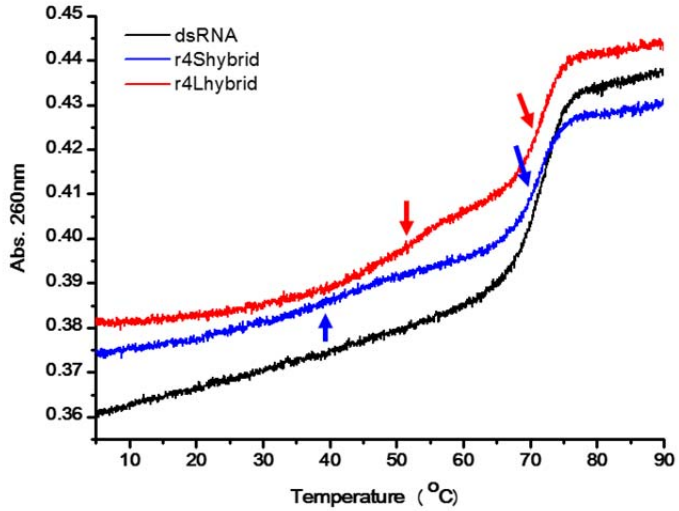

Figure 2.7 Melting behaviour of dsRNA (black line), dsRNA-S/r4S hybrid (blue line) and dsRNA-L/r4L hybrid (red line) in PBS. Arrows indicate melting temperatures. Concentration of each hybrid is 3 μM. Heating rate is 1.0 ^{o}C min^{-1}.

The formation of dsRNA-L/r4L hybrid was confirmed by polyacrylamide gel electrophoresis (PAGE). After annealing r4L and RNA, the mixture was applied to PAGE. As shown in Figure 2.8, dsRNA-L/r4L hybrid showed a single band with no remaining fluorescence in the well, indicating the quantitative formation of the hybrid and complete dissolution of the hybrid in aqueous medium. The lower mobility of the hybrid compared with dsRNA-L could be explained by an increase in molecular weight and addition of D-arginine's cationic charge following hybridization with r4L.

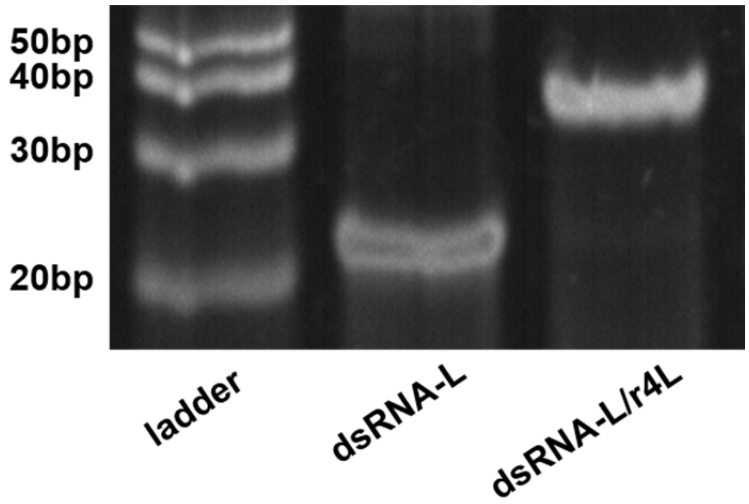

Figure 2.8 Formation of dsRNA/r4L hybrid confirmed by PAGE.

2-2-3. Dicer Processing

Then I examined resistance of dsRNA-L/r4L hybrid to Dicer as conceptually depicted in Figure 2.1. After treating dsRNA-L/r4L hybrid with recombinant human Dicer, the resulting mixture was analyzed by PAGE. As shown in Figure 2.9, in the case of dsRNA, a new band resulting from the blunt-ended dsRNA created by Dicer appeared (lane 2). However, the dsRNA-L/r4L hybrid did not provide the blunt-ended dsRNA and seemed to be completely intact (lane 4). This indicated that the peptide region of PNA-peptide hinders Dicer's

nuclease activity.

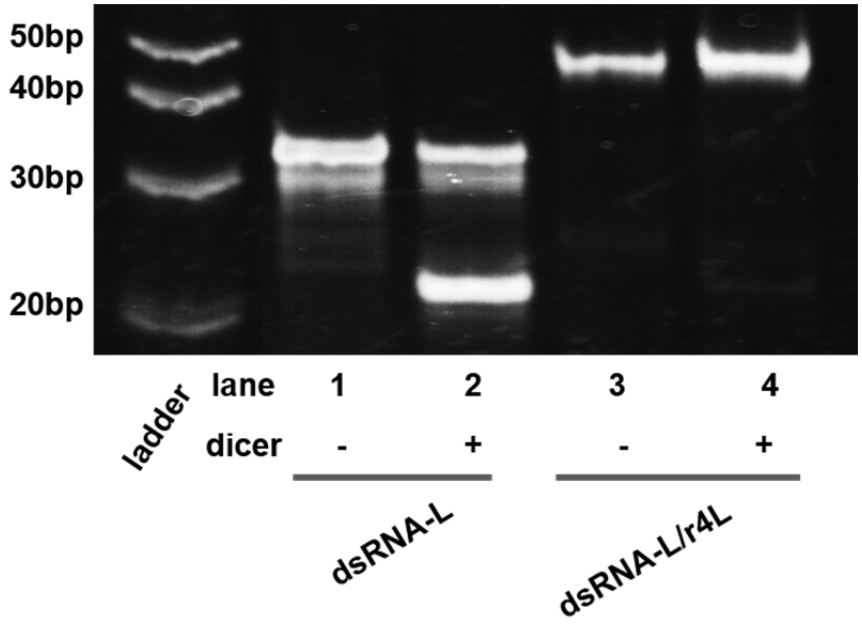

Figure 2.9 Resistance of dsRNA/r4L hybrid was analysed by gel electrophoresis. dsRNA/PNA-peptide hybrid was incubated with Recombinant Dicer Enzyme Kit in reaction buffer at 37 ^{o}C for 20 h.

2-3. Summary

In this chapter, PNA-peptides were designed to form hybrid with dsRNA for cancer-specific RNAi effect. I found that PNA-peptide was cleaved by cathepsin B at a sequence composed of natural amino acids. In the view of the thermal stability, dsRNA/r4L hybrid was expected to be suitable for RNAi evaluation because its melting temperature was more than 10 ^{o}C higher than the physiological temperature. And, the peptide region of PNA-peptide hinders recognition by dicer.

2-4. Experimental Section

Materials

NovaSyn TGR resin and all 9-fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids were purchased from Novabiochem, Merck (Tokyo, Japan). Peptide nucleic acids (PNA) were purchased from PANAGENE (Daejeon, Korea). 1-hydroxybenzoriazole monohydrate (HOBt·H_2O), *O*-benzotriazol-1-*N,N,N′,N′*-tetramethlyuronium hexafluorophosphate (HBTU), *N,N′*-diisopropylethlamine (DIEA), pyperidine (PPD), and trifluoro acetic acid (TFA) were purchased form Watanabe Chemical Industries (Hiroshima, Japan). *N,N*-dimethylformamide (DMF) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Wako Pure Chemical Industries (Tokyo, Japan). Triisopropylsilane (TIS) was purchased from Tokyo Chemical Industry (Tokyo, Japan). 10% fetal bovine serum (FBS), 100U/mL penicillin, 100μg/mL streptomycin, 0.25μg/mL amphotericin B, and Opti-MEM were purchased from Giboco Life Technologies (Grand island, NY, USA). Tris-Borate-EDTA (TBE) buffer and TritonX-100 were purchased from Sigma Aldrich (St. Louis, MO, USA). Human liver cathepsin B was purchased from MBL (Nagoya, Japan). siRNA ladder was purchased from Takara biotechnology (Tokyo, Japan). Recombinant Dicer Enzyme Kit was purchased from Genlantis, Inc. (San Diego, CA, USA). Lipofectamine^{TM}2000 reagent was purchased from Invitrogen (Carlsbad, California, USA). Luciferase assay substrate was purchased from Promega (Madison, WI, USA). Luciferase (Luc) siRNA and double strand (ds) RNA were purchased from BONAC Corporation (Kurume, Japan) with the following sequence; Luc sense strand: 5′-CUUACGCUGAGUACUUCGAdTdT-3′, Luc antisense strand: 5′-UCGAAG UACUCAGCGUAAGdTdT-3′, ds sense strand: 5′-CUUACGCUGAGUACUUCGAAAUGU

CGAUGAUGAUdGdA-3′, and ds antisense strand: 5′-GACAUUUCGAAGUACUCAGCGU AAGdTdT-3′.

Synthesis of PNA-peptides

All of PNA-peptides were synthesized according to standard Fmoc-chemistry using a NovaSyn TGR resin (Novabiochem, Tokyo, Japan). Coupling reactions were performed using the amino acids and PNAs activated with HOBt/HBTU and DIPEA in DMF. After cleavage for the protection group using TFA/water/triisopropylsilane (95/2.5/2.5), the obtained PNA-peptide was reprecipitated against cold diethyl ether. The crude PNA-peptides were purified by a LaChrome Elite reverse-phase liquid chromatography (Hitachi High-Technologies Corporation, Tokyo, Japan). The molecular weight of PNA-peptide was identified by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry on a Bruker autoflex-III spectrometer using cyano-4-hydroxycinnamic acid (CHCA) as matrix. m/z : r8S calcd for $C_{175}H_{267}N_{87}O_{45}$ 4307.43; found 4306.52, r8S-N calcd for $C_{162}H_{252}N_{84}O_{44}$ 4078.27; found 4079.20, r4S calcd for $C_{163}H_{239}N_{75}O_{45}$ 3967.22; found 3967.96, r4S-N calcd for $C_{150}H_{224}N_{72}O_{44}$ 3738.06; found 3740.66, r4L calcd for $C_{184}H_{266}N_{84}O_{52}$ 4484.50; found 4485.34, r4L-N calcd for $C_{171}H_{251}N_{81}O_{51}$ 4255.34; found 4258.20.

Concentration determination of PNA-peptide

Concentration of PNA-peptide are calculated by each base extinction coefficient at 260nm ($\varepsilon_{PNA,A}$ = 13700 M^{-1} cm^{-1}, $\varepsilon_{PNA,G}$ = 11700 M^{-1} cm^{-1}, $\varepsilon_{PNA,C}$ = 6600 M^{-1} cm^{-1}, $\varepsilon_{PNA,T}$ = 8600 M^{-1} cm^{-1}).[46]

Cleavage assay of PNA-peptide by Cathepsin B

The PNA-peptides were dissolved in reaction buffer (50 mM NaOAc, 1 mM EDTA, 1 M DTT, pH 5.4) and then 5.9 U/ml human liver Cathepsin B (MBL, Nagoya, Japan) was added to the solution and incubated a 37 ^{o}C for 30 min. After 30 min, cleavage site of PNA-peptides were diluted with 10 mg/mL CHCA solution and was analyzed by MALDI-TOF mass spectrometry.

Melting temperature of PNA-peptide with dsRNA

Solution of PNA-peptide and dsRNA were all brought to 3 μM. The duplex were formed by annealing at 90 ^{o}C for 1 min and then gradually cooled to 5 ^{o}C. Heating rate of the melting measurement was 1 ^{o}C/min.

Gel retardation assay

dsRNA and PNA-peptides (3 μM) in PBS were heated to 90 ^{o}C for 1 min for annealing, then gradually cooled to room. The resulting dsRNA/PNA-peptide hybrid was electrophoresed in a 40% polyacrylamide gel in TBE buffer (Sigma Aldrich, St. Louis, MO, USA). During the electrophoresis, the gel temperature was ice-cooled to avoid the dissociation of the hybrid. RNAs and hybrids were visualized by soaking the gel in distilled water containing SYBR Gold nucleic acid gel stain (Molecular Probe, Inc., Eugene, Oreg.).

Dicer processing

dsRNA/PNA-peptide (8 μM) hybrid was diced using a 1 U/mL of Recombinant Dicer Enzyme Kit (Gene Therapy Systems, Inc., San Diego, CA, USA) in reaction buffer at 37 ^{o}C for 20 h (total 10 μL). After the reaction, reaction was quenched by adding 2 μL of stop solution to the reaction mixture. The resulting solution was analyzed by 40% polyacrylamide gel electrophoresis in TBE buffer under ice-cooling. RNAs and hybrids were visualized by soaking the gel in distilled water containing SYBR Gold nucleic acid gel stain (Molecular Probe, Inc., Eugene, Oreg.).

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CHAPTER 3

Cancer Protease-Responsive RNAi System; in vitro Evaluation

CHAPTER 3
Cancer Protease-Responsive RNAi System; in vitro Evaluation

3-1. Introduction

Cathepsin B is abundantly expressed peptidase of the papain family in lysosome.[1-4] Many studies have been reported that cathepsin B played a role as growth, migration, invasion, and metastasis of various cancers.[5-7] Moreover, Cathepsin B is secreted in the particular cancer cells such as human breast, colon and prostate carcinomas.[8,9] And cathepsin B expression level of cancer tissues is reported to be two-fold higher than normal tissues.[10-13]

Cathepsin B can serve as a cancer target for prodrug to induced anticancer drug release to cancer cell.[14-17] For example, Soepenberg et al. focused on providing accumulation and investigation of clinical study using DE-310 prodrug which occurs a cleavage of the peptidyl spacer by cathepsin B in cancer cells.[17] Actually, cathepsin B-activated prodrugs have been started for clinical therapy.[18,19] In addition, cathepsin B imaging probes have been applied as useful markers for diagnosis of cancer under the cathepsin B expression.[20-22] Ryu et al. developed a cathepsin B-sensitive nanoprobe based on cathepsin B cleavage peptide linker in SCC7 cancer cells. The probe showed high specificity of activation in in vitro and in vivo under overexpression of cathepsin B.[20] Thus, these properties of cathepsin B can be used as attractive target in many cancers.

In this chapter, I examined the in vitro evaluation of cellular uptake and gene expression of the cancer protease-responsive RNAi system in response to cancer-specific cathepsin B. And the RNAi system was evaluated inhibitory effect in presence of inhibitor.

3-2. Results and Discussion

3-2-1. Cytotoxicity of PNA-peptide

Before examining whether RNAi occurred, I checked the cytotoxicity of the PNA-peptide, r4L, on CT-26 cells (Figure 3.1). The cytotoxicity was almost negligible when the r4L concentration was less than 20 nM. Therefore, I used the PNA-peptide at 20 nM in subsequent RNAi experiments.

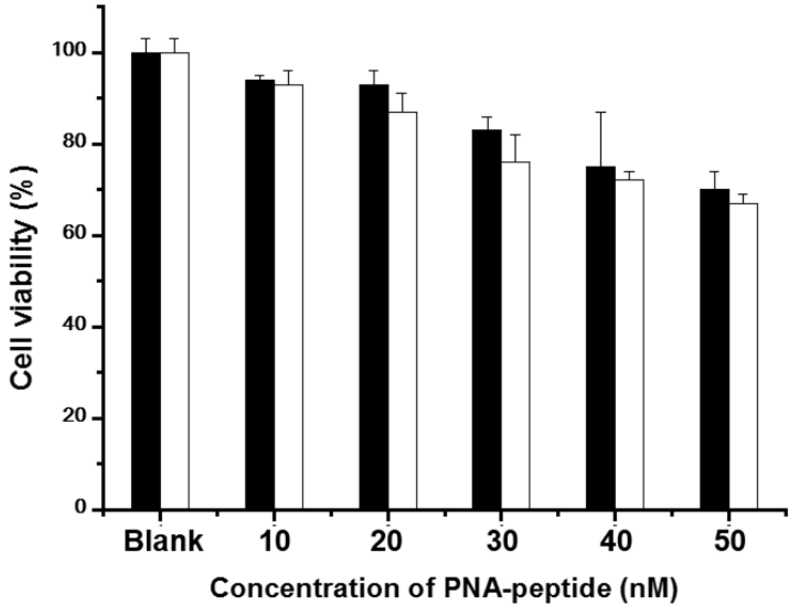

Figure 3.1 Cytotoxicity of PNA-peptide against CT-26 in the absence (filled bar) or the presence of Lipofectamine 2000 (open bar).

3-2-2. Quantitative cellular uptake of dsRNA/PNA-peptide hybrid

Prior to transfection of dsRNA/PNA-peptide hybrid, intracellular localization of PNA-peptide with 6-FAM labeled dsDNA (dsDNA-F, 100 nM) were measured by fluorescence imaging and flow cytometry. As shown in Figure 3.2, the cellular uptake of dsDNA-L/r4L hybrid was almost same with naked dsDNA, indicating that transfection reagents are needed for the cellular uptake of the hybrid. And, dsDNA/r4L hybrid mixed Lipofectamine (LF) determined higher intracellular localization than dsDNA-L/r4L hybrid. These result showed cationic peptide region of PNA-peptide did not have function to interact with anionic cell membrane for accelerating cellular uptake. Also, cationic peptide region may play a role as formation with complementary dsRNA by electrostatic interaction.

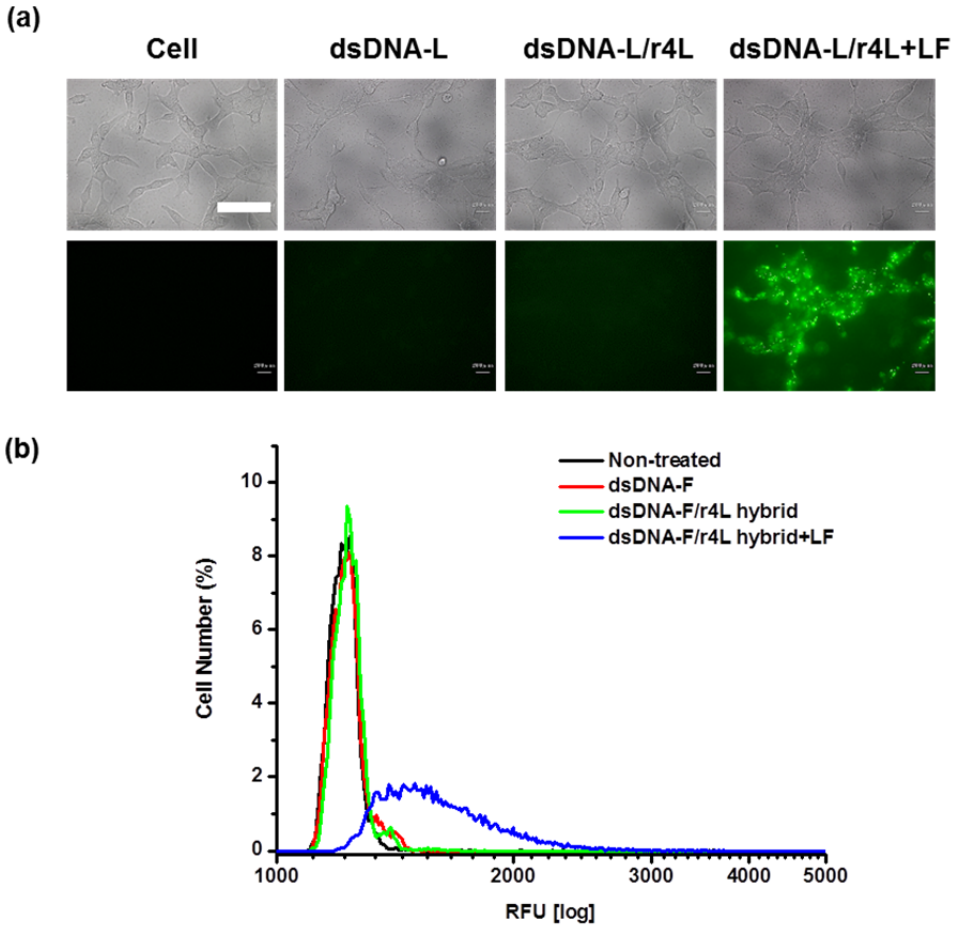

Figure 3.2 Uptake of fluorescein-labeled dsDNA-L/r4L hybrid was analyzed by fluorescence microscope (a) and flow cytometry (b) in CT-26 cells. Fluorescence is 6-carboxyfluorescein (λex/λem: 495/517nm). The scale bar is 20 μm.

3-2-3. RNAi effect of dsRNA/PNA-peptide hybrid in CT-26 cells

I evaluated RNAi of dsRNA-L/r4L hybrid in CT-26 cells that constitutively expressed firefly luciferase. CT-26 cells have been reported to highly express cathepsin B.[23] Lipofectamine 2000 was used to transfect the hybrid into the cells because I found that cellular uptake of the hybrid alone was negligible. RNAi effect was evaluated 48 hours after addition of the hybrid. As shown in Figure 3.3, the dsRNA-L/r4L hybrid reduced the luciferase activity to ca.20%, which is similar to that of siRNA and dsRNA alone. In contrast, the negative control, dsRNA-L/r4L-N hybrid, which does not include a cathepsin B cleavable site shows high level of luciferase activity (> 60%) compared with the dsRNA-L/r4L hybrid. Thus, the significant difference of luciferase activity between these two hybrids should result from the activation of RNAi by the dsRNA-L/r4L hybrid that corresponded to cathepsin B activity as depicted in Figure 2.2. An incomplete suppression of the RNAi effect of the negative control dsRNA-L/r4L-N hybrid may be due to the dissociation of some amount of the hybrid in intracellular conditions to generate free dsRNA.

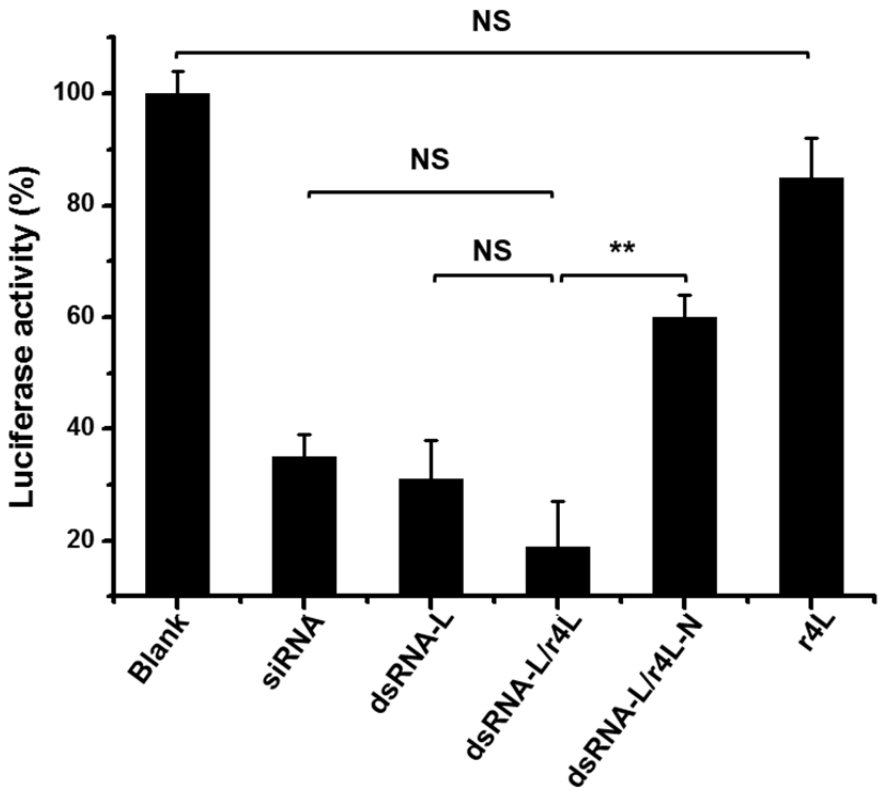

Figure 3.3 RNAi effect of dsRNA/PNA-peptide hybrids in CT-26 cells expressing luciferase. Luciferase activity was measured for 48 hours after treatment with the hybrids. **: $p < 0.01$, NS: Not significant.

I examined the effect of concentration of dsRNA/r4L hybrid on the RNAi effects. The RNAi effects were analyzed by siRNA concentration from 5 to 20 nM. As shown in Figure 3.4, luciferase activity was reduced with increasing concentration of dsRNA-L/r4L hybrid, which is almost similar effect with siRNA and dsRNA-L. The concentration dependent suppression of luciferase expression also indicates that this suppression results from RNAi of the hybrid. GFP siRNA, negative control, was not reduced luciferase activity.

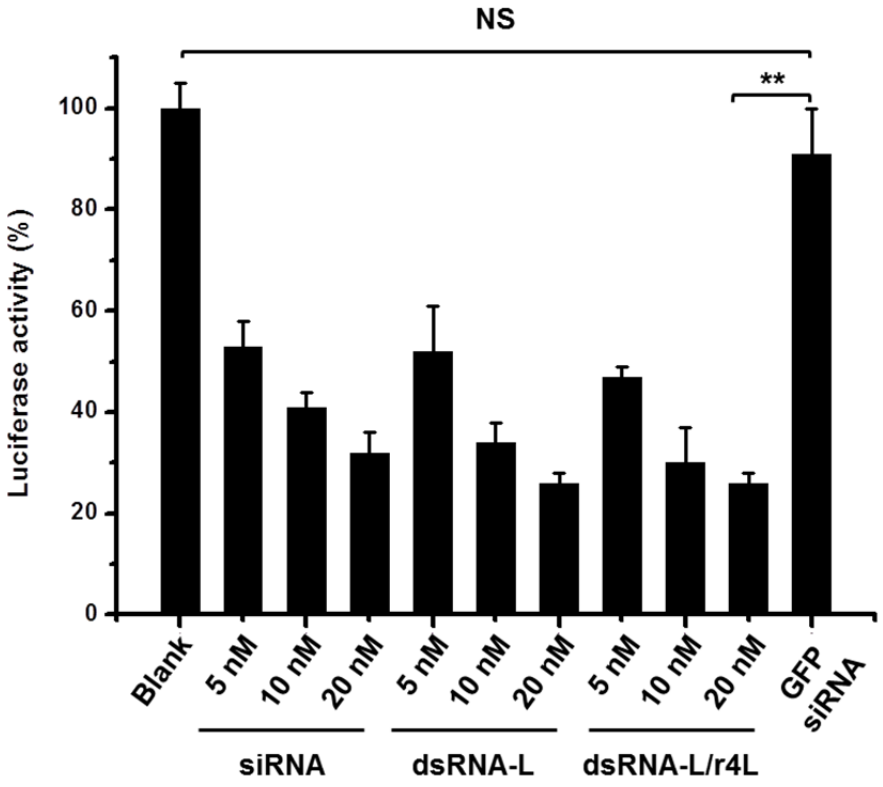

Figure 3.4 Concentration-dependent RNAi effect of dsRNA/PNA-peptide hybrids in CT-26 cells. Luciferase activity was measured for 48 hour after treatment with the hybrids. GFP siRNA (20 nM) was used as a negative control. **: $p < 0.01$, NS: Not significant.

3-2-5. Inhibition of cathepsin B in CT-26 cells by inhibitor

To further obtain the evidence of the cathepsin B-responsive RNAi effect, I examined the effect of cathepsin B inhibitor on the RNAi effect of the hybrid. First I confirmed the effect of a cathepsin B inhibitor (CA-074 Me)[24] by using commercial cathepsin B fluorescent probe, Magic red cathepsin B substrate, which becomes fluorescence after hydrolysis by cathepsin B in lysosome.[25] As shown in Figure 3.5, untreated cells showed a strong red fluorescence in the cytosol resulting from the cathepsin B probe. However, the red fluorescence became weak

with increasing the concentration of the inhibitor. Thus, cathepsin B activity can be inhibited by CA-074 Me in CT-26 cells. Then I examined the inhibitory effect of cathepsin B on the RNAi effect of the hybrid. As shown in Figure 3.6, the suppression of the luciferase activity by dsRNA-L/r4L hybrid was weakened with increasing concentration of the inhibitor. At the highest concentration of the inhibitor (10 μM), the luciferase activity recovered to almost same level with the negative control, dsRNA-L/r4L-N. These results are another evidence that the RNAi effect of dsRNA-L/r4L hybrid was triggered by the cellular cathepsin B as depicted in Figure 2.2.

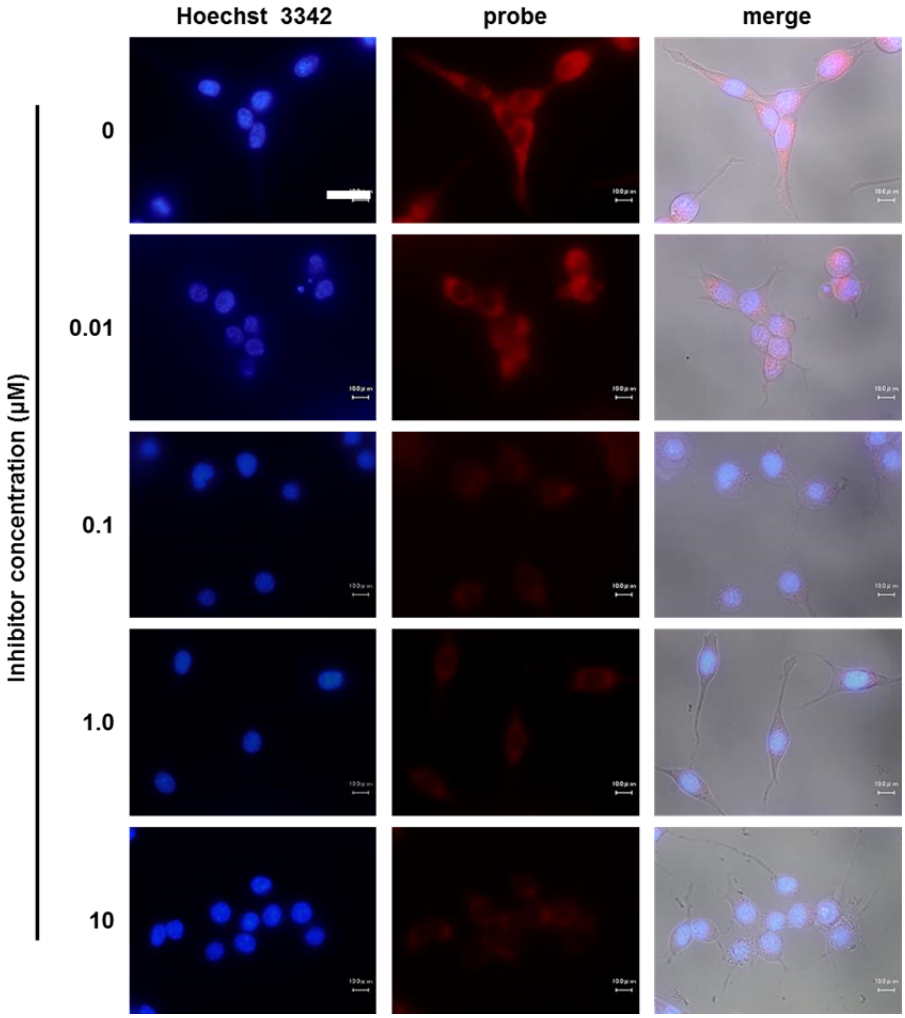

Figure 3.5 Inhibition of cathepsin B in CT-26 cells by inhibitor (CA-074 Me). Inhibitory activity was detected by fluorescent cathepsn B probe (Magic red cathepsin B substrate). The scale bar is 20 μm. Cells were treated with CA-074 Me (0 to 10 μM) for 3 h then incubated with the probe for 1 h.

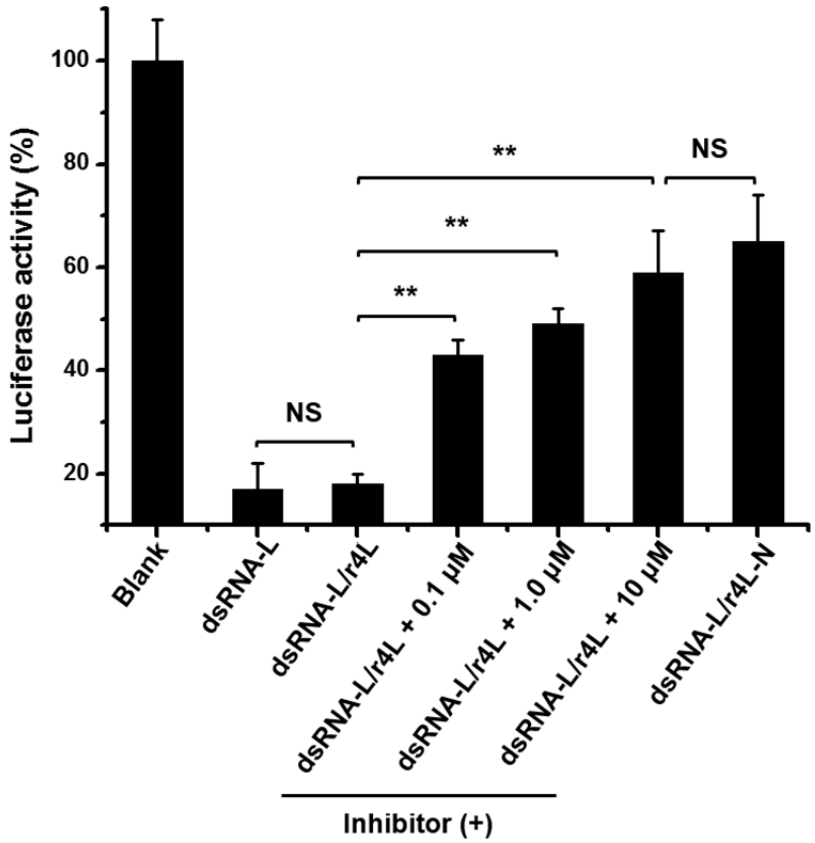

Figure 3.6 Suppression of RNAi effect of dsRNA/r4L hybrid by cathepsin B inhibitor (CA-074 Me) (0.1, 1.0, 10 μM) in CT-26 cells. Luciferase activity was measured for 48 hours after treatment with both the inhibitor and the hybrids. **: $p < 0.01$, NS: Not significant.

3-2-6. RNAi effects of dsRNA/PNA-peptide hybrid in other cell types

dsRNA-L/r4L hybrid showed effective RNAi effect in CT-26 cells. I determined whether RNAi effect of dsRNA-L/r4L hybrid appears in 4T1 cells. The 4T1 cells also have been reported high expression in lysosome.[26] Before dsRNA-L/r4L hybrid transfection, cathepsin B activity was analyzed by cathepsin B probe. Figure 3.7a showed that cathepsin B activity was detected in cytosol. Then RNAi effect of dsRNA-L/r4L hybrid was also confirmed in 4T1 cells. The luciferase activity of dsRNA-L/r4L hybrid was also reduced similar to CT-26

cells while the negative control hybrid did not show such significant reduction (Figure 3.7b). Based on this result, dsRNA/r4L hybrid was effectively activated by cathepsin B.

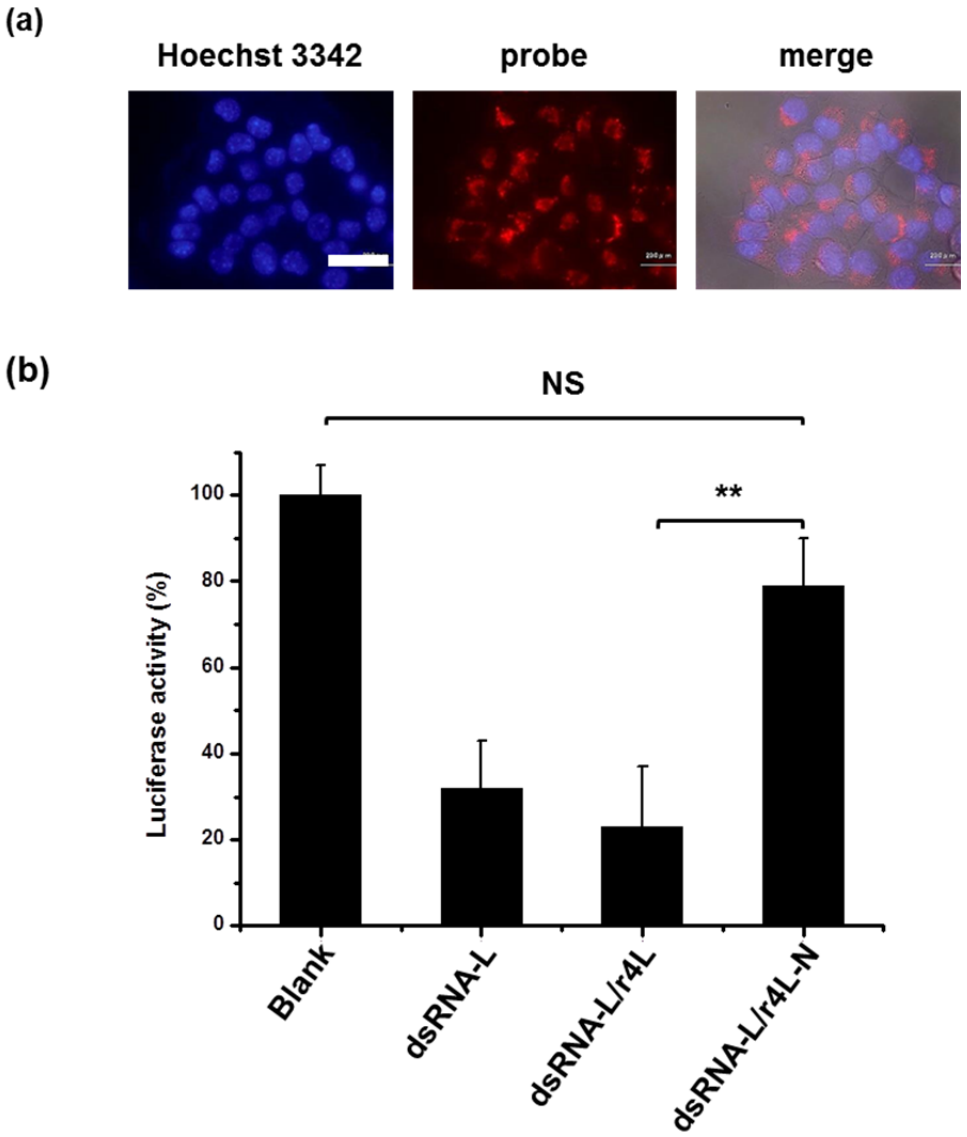

Figure 3.7 Detection of cathepsin B activity (a) and RNAi effect of dsRNA-L/PNA-peptide hybrid in 4T1 cells (b). Lysosomal cathepsin B activity was detected by cathepsin B probe. The scale bar is 20 μm. Luciferase activity was measured for 48 hours after treatment with the hybrids. **: $p < 0.01$, NS: Not significant.

3-3. Summary

In conclusion, the luciferase activity of this hybrid successfully showed the RNAi effect in a cancer cell line with high cathepsin B activity. And the hybrid lacking the cathepsin B cleavage site showed a minimal RNAi effect. These results indicate that transfection of hybrid into cancer cell leads to gene silencing. Our design of the hybrid is universally applicable to any kind of protease by changing the sequence of protease substrate region of the PNA-peptide.

3-4. Experimental Section

Cell culture

CT-26 stably expressing firefly luciferase was kindly provided by Dr. Atsushi Maruyama (Tokyo Institute of Technology) and Dr. Arihiro Kano (Kyushu University).[27] CT-26 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Wako, Osaka, Japan) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (all from Giboco Life Technologies, Grand island, NY, USA) in humidified atmosphere with 5% CO_2 and 95% air at 37 ^{o}C. Also, 4T1 cells that had been harvested from mouse breast cancer and stable expression of firefly luciferase. The 4T1 cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI-1640, Wako, Osaka, Japan) containing 10% FBS.

Cytotoxicity of PNA-peptide

CT-26 cells were seeded in 96-well plates (1×10^3 cells/well) at 37 ^{o}C in DMEM before the assay. After 24 h incubation, PNA-peptide (0 to 50 nM) solution containing Lipofectamine 2000 was added in each well. After 4h, medium containing 10% FBS was replaced and incubated for 20 h. A 10 μL of Cell Counting Kit-8 regent (Dojindo Laboratories, Kumamoto, Japan) were added to each well and were incubated for 2 h. Then, 96-well plate was measured at 450 nm by microplate reader.

Cellular uptake

For fluorescence imaging, CT-26 cells seed in 96-well plates (1×10^3cells/well) at 37 ^oC in DMEM with 10% FBS. After 24 h, CT-26 cells were incubated with dsDNA-F, dsDNA-F/r4L hybrid and Lipofectamine mixed dsDNA-F/r4L hybrid. After 4 h, cells were washed with DPBS and analysed by fluorescence microscope (BZ-8000, Keyence, Osaka, Japan). For flow cytometry analysis, CT-26 cells seed in 24-well plates (2×10^4cells/well) at 37 ^oC in DMEM with 10% FBS for 24h. The cell was washing with DPBS and then the hybrid were adding into wells and incubating at 37 ^oC for 4 h. The fixed cells were washed with serum free medium, trypsinized, and then, the cells were collected by centrifugation. Then, cellular uptake was analyzed by Tali image-based cytometry (Invitrogen, Carlsbad, California, USA).

Lysosomal cathepsin B activity

To monitor cathepsin B activity, CT-26 cells were seed in 96-well glass plates (1×10^4 cells/well) until 80% confluent. After 24 h, CT-26 cellw were incubated for 3 h with 0 to 10 μM cathepsin B inhibitor (CA-074 Me, L-trans-Epoxysuccinyl-Ile-Pro-OMe propylamide, Bachem AG, Bubendorf, Switzerland). Then, Magic red cathepsin B substrate reagent solution (MR-$(RR)_2$, Immuno Chemistry Technologies, Bloomington, MN, USA) was added to each medium at a volume ratio of 1:26, and incubated for 1 h at 37 ^oC. Then, the medium was removed and the cells were rinsed with PBS. The cells were stained with Hoechst 33342 for 10 min and analyzed by Biozero fluorescence microscope (BZ-8000, Keyence, Osaka, Japan).

Transfection

CT-26 cells were seeded in 24-well plates (2×10^4cells/well) at 37 ^oC in DMEM containing 10% FBS and grown to 30-50% confluence. For the inhibition of cathepsin B activity, the cells were washed with PBS and then incubated for 3 h at 37 ^oC in serum-free medium with cathepsin B inhibitor (CA-074 Me, 0 to 10 μM). Transfection was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, California, USA). Before transfection, 20 nM siRNA or 20 nM dsRNA/PNA-peptide hybrid was s added respectively in Opti-MEM (Giboco) and Lipofectamine 2000 was diluted with Opti-MEM. Each solution was incubated for 5 min at room temperature. Then, they were combined and mixed gently and then incubated for 20 min at room temperature. When solution appeared cloudy after 20 min, the solutions were added to each well containing cells and serum-free medium. After 6 h incubation, medium was replaced to DMEM, and the cells were cultured for 42 h. To investigate the RNAi effect, the cells were lysed with 200μL of lysis buffer [20 mM Tris-HCl, 0.05% TritonX-100, 2 mM EDTA (pH 7.5)] for 20min. A 10-μL of the lysate solution was mixed with 40-μL of luciferase assay solution (Promega, Madison, WI, USA) and then measured with a luminometer (GloMax 20/20n, Promega, Madison, WI, USA).

3-5. References

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CHAPTER 4

Apoptosis-Inducing RNAi System

CHAPTER 4
Apoptosis-Inducing RNAi System

4-1. Introduction

The process of cell death is known to be caused by two main methods which are necrosis and apoptosis (Figure 4.1). Necrosis is caused by direct stimulation such as toxic or physical injury and destroyed the cells through an inflammation by swelling.[1,2] By contrast, cell dies by apoptosis or programmed cell death, after growth, cell differentiation.[3,4] Apoptosis which was first by Kerr et al. has characterized the morphological, biochemical, physiological different aspects contrary to necrosis.[5-8] The regulation of apoptosis is important to induce various diseases including cancer.

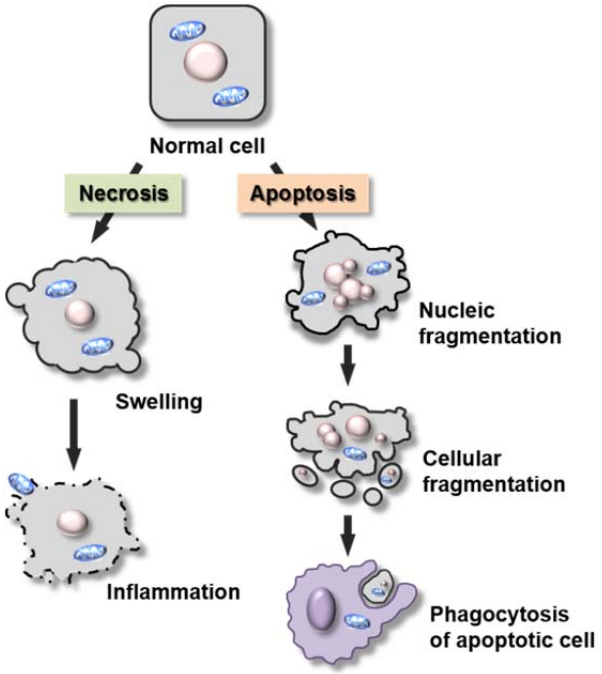

Figure 4.1 Cell death necrosis and apoptosis.

Apoptosis pathway is divided into extrinsic and intrinsic pathway. The extrinsic pathway occurs by cell surface death receptors with specific ligands such as Fas or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL).[9,10] The death receptors bind to extrinsic ligands and then combine death domain (DD) between death receptor and adapter (Figure 4.2). Caspase 8 is activated by binding between adaptor and death domain. Active caspase 8 lead to activation of caspase 3, 6, 7 then, induces apoptosis.[11]

On the other hand, intrinsic pathway is initiated by intracellular stress including DNA damage or hypoxia or radiation in the mitochondria. When the pathway is induced, the mitochondria outer membrane permeabilization (MOMP) is occurred and apoptotic proteins release Cytochrome c (Cyt c) from mitochondria into the cytosol. The release of cytochrome C triggers activation of procaspase and then activates caspase to induce apoptosis.[12,13]

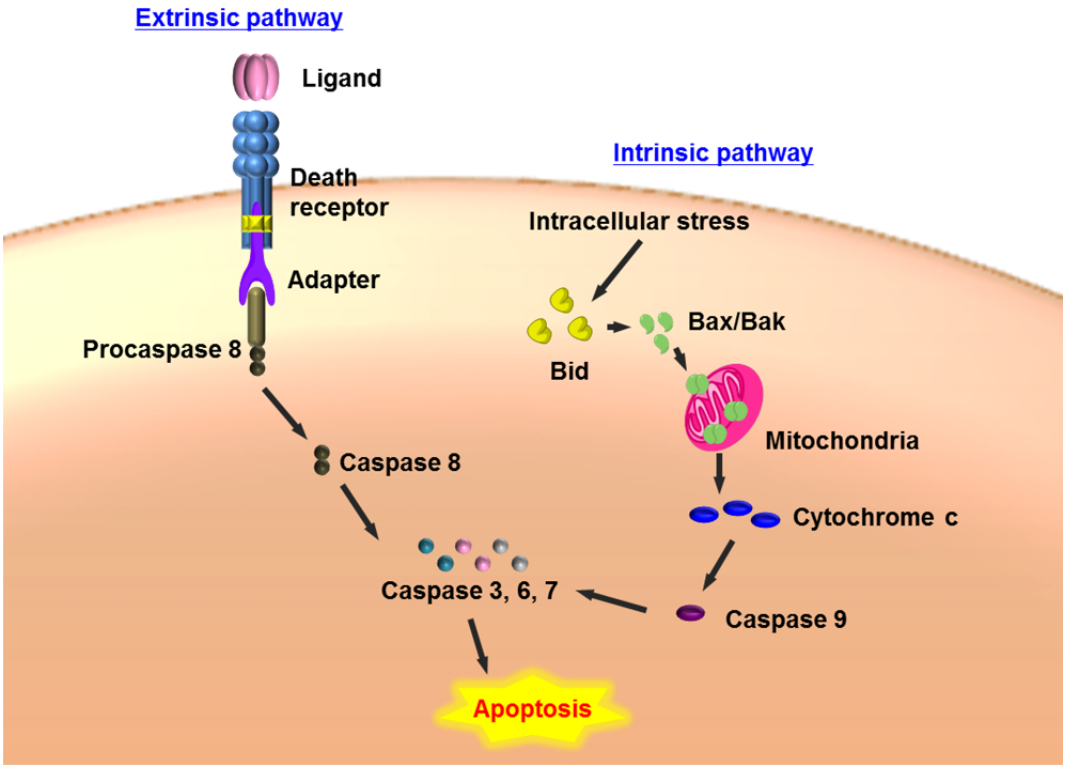

Figure 4.2 Apoptosis pathway is divided into 2 pathways. Extrinsic pathway is triggered by binding between ligand and death receptor and then caspase is activated. Intrinsic pathway is initiated by various intracellular stresses. Caspase 9 is activated by released of cytochrome c from mitochondria, and then apoptosis occurs.

The regulation of pro-apoptotic occurs via B-cell lymphoma-2 (bcl-2) family of proteins which are known as essential regulators of apoptosis. The bcl-2 proteins were first discovered as proto-oncogene in B-cell lymphomas.[14] Intrinsic apoptosis induced signal is triggered by the bcl-2 family proteins that play a role as inhibition (anti-apoptotic) or promotion (pro-apoptotic) of cell death.[15]

Pro-apoptotic proteins that include Bid, Bim, Bax and Bak play roles in promoting apoptosis and exist in cytosol. When cells are exposed to apoptosis factor, pro-apoptotic proteins are transferred from mitochondria to cytosol and inhibit the activation of bcl-2 or bcl-X_L. Then it causes a depolarization of mitochondria membrane and releases Cytochrome c.[16-18] On the other hand, anti-apoptosis such as bcl-2 and bcl-X_L has been reported to inhibit apoptosis. These suppress mitochondria membrane depolarization in the outer membrane of mitochondria, and promote oxidative phosphorylation of membrane. For this process, the release of cytochrome c is inhibited and activation of caspase which is final protease in apoptosis is also prevented.[16-18]

Overexpression of bcl-2 related anti-apoptosis can potentially lead to cancer and the expression is increased by mutation.[19,20] For downregulation of bcl-2, several researchers have demonstrated decreasing bcl-2 expression using RNAi.[21,22] Some studies have demonstrated that bcl-2 specific siRNA induces apoptosis and tumor growth is delayed in vivo by using animal model.[21-25]

Previous study, dsRNA/PNA-peptide hybrid was developed for cancer-specific activation of RNAi. In this chapter, I propose apoptosis inducing RNAi system based on dsRNA/PNA-peptide hybrid. The mechanism of RNAi system is similar to dsRNA/PNA-peptide hybrid in chapter 2. First, the apoptosis-inducing RNAi system which contains protease-responsive PNA-peptide conjugates is cleaved by cathepsin B in lysosomes and then edited by dicer. Thus, the RNAi system will induce the apoptosis by suppression of bcl-2 protein (Figure 4.3).

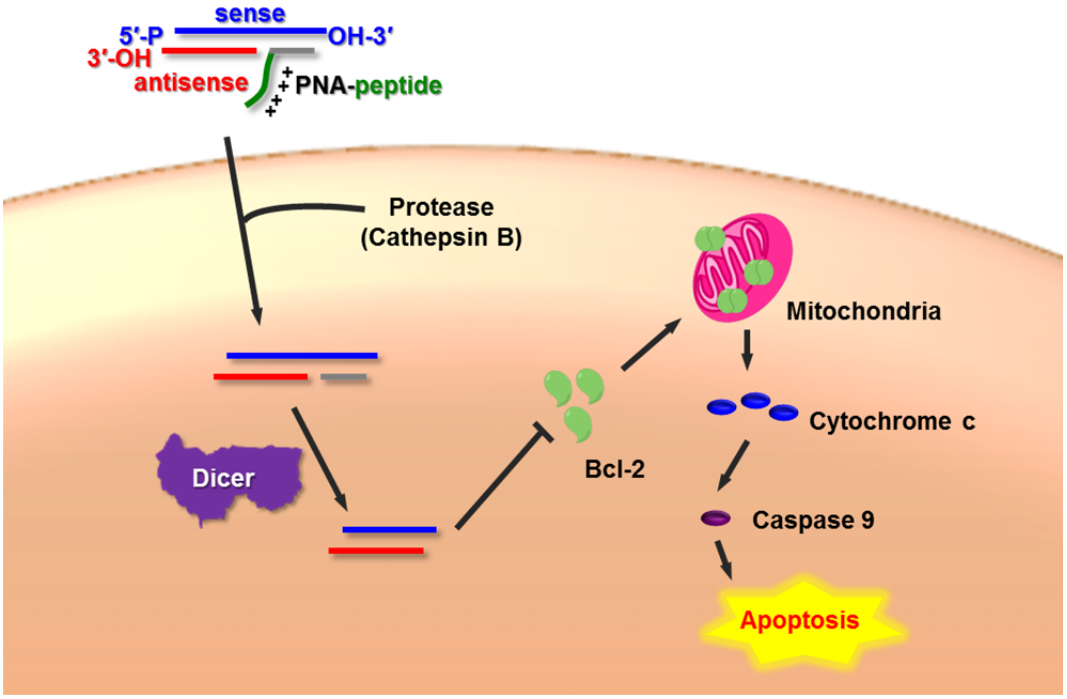

Figure 4.3 Mechanism of apoptosis induced RNAi system. The bcl-2 siRNA/PNA-peptide hybrid inhibits expression of bcl-2 protein after cleaving PNA-peptide by cathepsin B. Then, Non-activation of bcl-2 expression induces apoptosis.

4-2. Results and Discussion

4-2-1. Formation of bcl-2 siRNA/PNA-peptide hybrid

The sequences of bcl-2 siRNA and PNA-peptide were summarized in Table 4.1. The PNA-peptide used the same sequences with Chapter 2. The bcl-2 siRNA was selected from human bcl-2 mRNA (accession No. M14745, starting position from 74 to 92).[21] The formation of bcl-2 siRNA/r4L hybrid was verified by polyacrylamide gel electrophoresis

(PAGE). As shown in Figure 4.4, bcl-2 siRNA/r4L hybrid showed a band like dsRNA/r4L hybrid in chapter 2. The low mobility of bcl-2 siRNA/r4L hybrid could be also explained by large molecular weight and cationic charge of r4L.

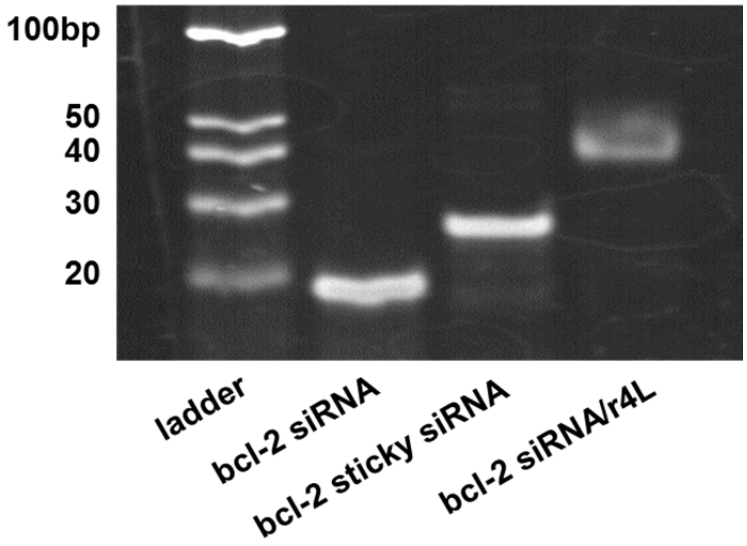

Figure 4.4 Formation of bcl-2 siRNA/r4L hybrid confirmed by PAGE.

Table 4.1 Sequences of bcl-2 siRNA, bcl-2 sticky siRNA and PNA-peptide (X: 8-amino-3,6-dioxaoctanoic acid, FAM: 6-carboxyfluorescein).

Sample	Sequences
bcl-2 ssRNA	5′-GUGAUGAAGUACAUCCAUUdTdT-3′
bcl-2 sssRNA	5′-GUGAUGAAGUACAUCCAUUGAUGAUGAUdGdA-3′
bcl-2 sasRNA	3′-dTdTCACUACUUCAUGUAGGUAA-5′
ssDNA-L	5′-CTTACGCTGAGTACTTCGAAATGTCGATGATGATGA-3′
asDNA-FAM	3′-FAM-TTGAATGCGACTCATGAAGCT -5′
r4L	C-NH_2-rararara-X-GLFG-X-CTACTACTACT-H-N
r4L-N	C-NH_2-rararara-X- X -X-CTACTACTACT-H-N

4-2-2. Cellular uptake of bcl-2 siRNA/PNA-peptide hybrid in PC-3 cells

Prior to detection of apoptosis, I examined cellular uptake of bcl-2 siRNA/r4L hybrid with fluorescein-labeled DNA in PC-3 cells. After adding each sample, the results were analyzed by fluorescence imaging and image-based cytometry. As shown in Figure 4.5a, in case of dsDNA/r4L mixed lipofectamine (LF) showed a strong green fluorescence in the cytosol. However, dsDNA and dsDNA/r4L hybrid have a weak green fluorescence. This indicated that dsDNA/r4L hybrid can be efficiently taken up in the present of lipofectamine. And it showed the same results in the image-based cytometry (Figure 4.5b).

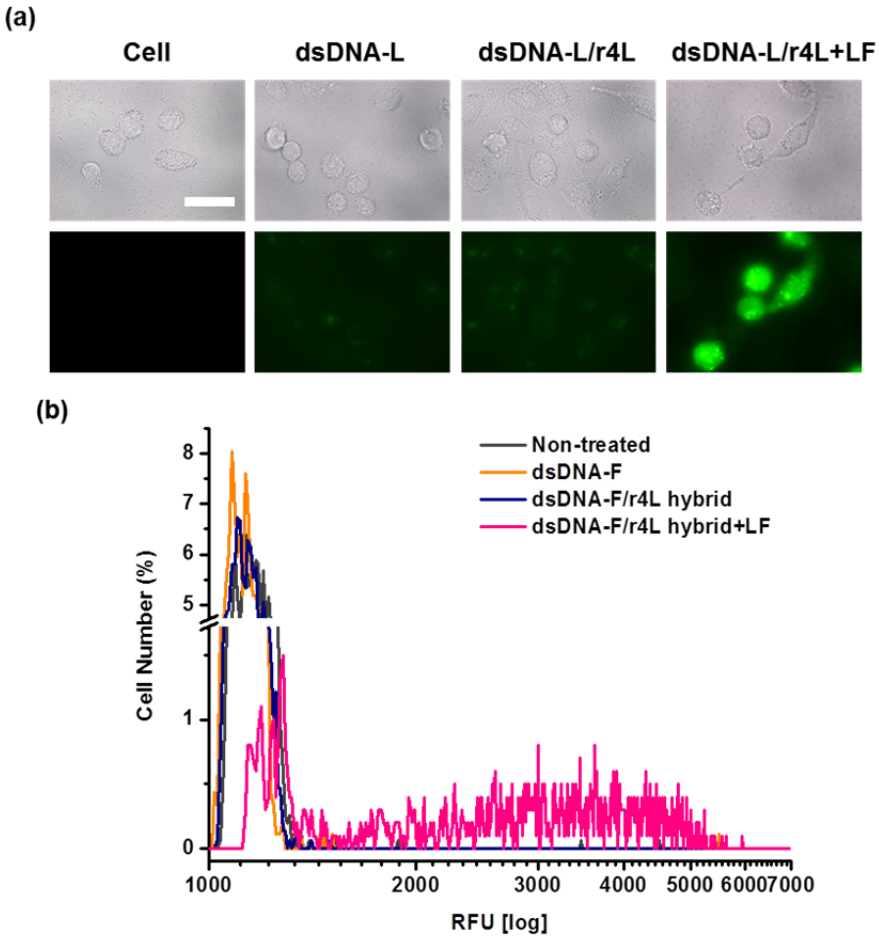

Figure 4.5 Cellular uptake of the fluorescein-labeled dsDNA/r4L hybrid was detected by fluorescence microscope (a) and image-based cytometry (b) in PC-3 cells. Fluorescence is 6-carboxyfluorescein (λex/λem: 495/517nm). The scale bar is 20 μm.

4-2-3. Cell viability of bcl-2 siRNA/PNA-peptide hybrid in PC-3 cells

I evaluated the cell death induced by bcl-2 siRNA/PNA-peptide hybrid based on WST-8 assay. The cell viability was evaluated after 48 hours from the addition of the hybrid with Lipofectamine 2000 regent in PC-3 cells. PC-3 cells have been reported to highly express cathepsin B and was used to perform the siRNA induced cell death by targeting bcl-2.[26,27] As shown in Figure 4.6, dsRNA/r4L hybrid showed marked cell death comparing with negative control dsRNA/r4L-N hybrid at both concentrations. The level of cell death in dsRNA/r4L hybrid is almost same with siRNA. In the case of 800 nM, the statistically significant difference between dsRNA/r4L hybrid and dsRNA/r4L-N hybrid was observed ($p < 0.05$). Thus, the cell death induced by dsRNA/r4L hybrid will be due to the activation of RNAi by cellular cathepsin B, which leads to apoptosis of PC-3 cell.

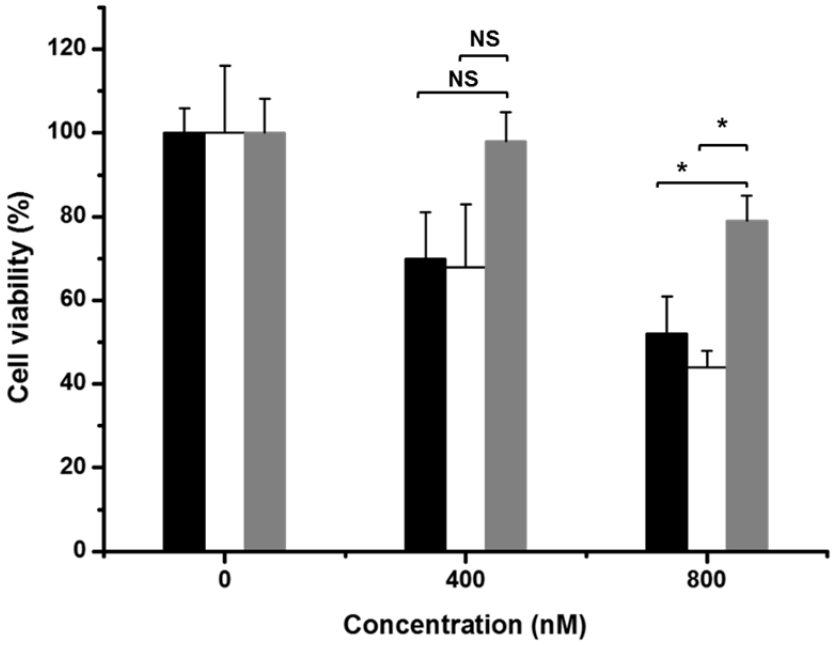

Figure 4.6 Cell viability of bcl-2 siRNA/PNA-peptide hybrid in PC-3 cells (n=3). The bcl-2 siRNA and bcl-2 siRNA/PNA-peptide hybrid were treated for 48 hours with Lipofectamine 2000 regent (Black bar: bcl-2 siRNA, white bar: bcl-2 siRNA/r4L hybrid, gray bar: bcl-2 siRNA/r4L-N hybrid). *: $0.01 < p < 0.05$, NS: Not significant.

4-3. Summary

In this chapter, I designed the bcl-2 siRNA/PNA-peptide hybrid for cathepsin B-induced cell death. I found that PNA-peptide was formed with bcl-2 siRNA by PAGE. And, the hybrid showed high cellular uptake in presence of lipofectamine. The hybrid showed cell death as same as siRNA, which was not observed in negative control dsRNA/r4L-N hybrid. These results suggest that apoptosis of the cell was induced because the hybrid inhibits expression of bcl-2. The RNAi-induced cell death was not so significant as observed in our experiment. To raise the efficacy of cancer cell death induced by siRNA, several groups combined anticancer drug (doxorubicin).[26,27] Such combination cancer therapy may work to raise the efficacy in our system.

4-4. Experimental Section

Concentration determination of PNA-peptide

Concentration of PNA-peptide are calculated by each base extinction coefficient at 260nm ($\varepsilon_{PNA,A}$ = 13700 M^{-1} cm^{-1}, $\varepsilon_{PNA,G}$ = 11700 M^{-1} cm^{-1}, $\varepsilon_{PNA,C}$ = 6600 M^{-1} cm^{-1}, $\varepsilon_{PNA,T}$ = 8600 M^{-1} cm^{-1}).[28]

Gel retardation assay

Bcl-2 siRNA and PNA-peptides (3 μM) in PBS were heated to 90 ^{o}C for 1 min for annealing, then gradually cooled to room. The resulting bcl-2 siRNA/PNA-peptide hybrid was electrophoresed in a 40% polyacrylamide gel in TBE buffer (Sigma Aldrich, St. Louis, MO, USA). During the electrophoresis, the gel temperature was ice-cooled to avoid the dissociation of the hybrid. RNAs and hybrids were visualized by soaking the gel in distilled water containing SYBR Gold nucleic acid gel stain (Molecular Probe, Inc., Eugene, Oreg.).

Cell culture

Using PC-3 cells that had been harvested from the human prostate cancer and stably expression of firefly luciferase were kindly provided by Japanese Collection of Research Bioresources (JCRB) Cell Bank (Osaka, Japan). The PC-3 cells were cultured in Kaighn's Modification of Ham's F-12 Medium (Ham’s F-12K, Wako, Osaka, Japan) containing 7% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL

amphotericin B (all from Giboco Life Technologies, Grand island, NY, USA) in humidified atmosphere with 5% CO_2 and 95% air at 37 ^oC.

Cellular uptake

Prior to transfection of bcl2-siRNA/r4L hybrid, intracellular localization of PNA-peptide with 6-FAM labeled dsDNA (dsDNA-F, 100 nM) were measured by fluorescence imaging and image-based cytometry. For fluorescence imaging, PC-3 cells seed in 96-well plates (1×10^4cells/well) at 37^{o}C in Ham's F12-K medium with 7% FBS. After 24h, PC-3 cells were incubated with dsDNA-F, dsDNA-F/r4L hybrid and Lipofectamine 2000 (Invitrogen, Carlsbad, California, USA) mixed dsDNA-F/r4L hybrid. After 4h, cells were washed with DPBS and analysed by fluorescence microscope (BZ-8000, Keyence, Osaka, Japan). For image-based cytometry analysis, PC-3 cells seed in 24-well plates (2×10^4cells/well) at 37^{o}C in Ham's F12-K medium with 7% FBS for 24h. The cell was washing with DPBS and then the hybrid were adding into wells and incubating at 37 ^oC for 4 h. The fixed cells were washed with serum free medium, trypsinized, and then, the cells were collected by centrifugation. Then, cellular uptake was analyzed by Tali image-based cytometry (Invitrogen, Carlsbad, California, USA).

Cytotoxicity of Bcl-2 siRNA/PNA-peptide hybrid

PC-3 cells were seeded in 96-well plates (1×10^3 cells/well) at 37 ^oC in Ham's F12-K medium before the assay. After 24 h incubation, bcl-2 siRNA, bcl-2 siRNA/PNA-peptide hybrid (0, 400, 800 nM) solution containing Lipofectamine 2000 was added in each well. After 4h, half of the culture medium was replenished with new medium every day. After 2day,

the cells were washed with PBS and replaced with medium containing 7% FBS. A 10 μL of Cell Counting Kit-8 regent (Dojindo Laboratories, Kumamoto, Japan) were added to each well and were incubated for 2 h. Then, 96-well plate was measured at 450 nm by microplate reader.

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CHAPTER 5

Conclusions

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Conclusions

In this study, I have developed novel siRNA system based on PNA-peptide conjugates and this system has been applied to the intracellular cathepsin B-responsive RNAi in cancer cell line. Various characteristics of the RNAi system are investigated in test tube.

Key concepts extracted from each chapter are as follows.

Chapter 2: The PNA-peptides were designed to form hybrid with dsRNA for cancer-specific RNAi effect. I found that PNA-peptide was cleaved by cathepsin B at a sequence composed of natural amino acids. In contrast negative control PNA-peptide was not cleavage by cathepsin B. In the view of the thermal stability, I measured thermal denaturalization profiles of the hybrids to determine their melting temperatures (T_m). The T_m value between dsRNA-L and r4L was determined to be 52 ^oC. Thus, dsRNA-L/r4L hybrid was expected to be suitable for RNAi evaluation because its melting temperature was more than 10 ^oC higher than the physiological temperature. And dsRNA-L/r4L hybrid indicated having resistance against dicer because of the peptide region of PNA-peptide.

Chapter 3: The suppression of gene expression of the cancer protease-responsive RNAi system was evaluated in CT-26 cells with expression of protease, cathepsin B. The dsRNA-L/r4L hybrid showed significant RNAi effect, while dsRNA-L/r4L-N hybrid showed limited RNAi effect. Also, the hybrid-induced RNAi was weakened with increasing concentration of a cathepsin B inhibitor (CA-074 Me). Moreover, the hybrid showed the RNAi effect in other

cancer cell line with high expression cathepsin B (4T1 cells). These results showed that the RNAi effect of the hybrid is induced by intracellular cathepsin B activity.

Chapter 4: The apoptosis-inducing RNAi system using bcl-2 siRNA and PNA-peptide conjugates was proposed for cancer therapy. This system also formed PNA-peptide conjugate with bcl-2 siRNA and the hybrid was taken up by PC-3 cell with a help of lipofectamine. The bcl-2 siRNA/PNA-peptide hybrid showed significant cell death, while such cell death was not observed in negative control hybrid. These results suggest that apoptosis was induced by suppression of bcl-2 expression.

I confirmed that novel siRNA system using PNA-peptide conjugates shows cancer-specific RNAi effect. For more clear response to cathepsin B, longer PNA will be needed. Although the expression level of cathepsin B in cancer cell is reported to be higher than normal cell, the normal cell shows expression of some amount of cathepsin B, which will lead to RNAi activation even in normal cell. Thus, to raise the discrimination efficacy of our RNAi system, combining with conventional active targeting to cancer will be useful. To combining the active targeting ability to our RNAi system, both direct modification of ligand to PNA-peptide conjugate and encapsulation of our RNAi system into synthetic carrier will be possible. Further, our RNAi system should be evaluated by a directly injection with transfection reagent in tumor tissue for anticancer therapy. Although several researches showed the inhibition of tumor growth and reduction of tumor size by bcl-2 siRNA, combination of bcl-2 siRNA with anticancer drug or other apoptosis-induced siRNA demonstrated more effective treatment than bcl-2 siRNA alone.[1-4] Thus, such combination will also improve our RNAi system for anti-cancer effect.

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Accomplishments

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Symposium

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