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Non-viral siRNA Delivery Systems

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ABSTRACT – The emergence of new biological drugs based on RNA interference (RNAi) technology has been one of the most attractive issues in the field of gene therapy for years. However, the use of siRNA therapeutics in clinical settings is still limited due to lack of appropriate delivery systems for the highly charged macromolecular drug. In this review, recent development of major non-viral siRNA delivery systems, including lipid, liposome, polymer, and peptide-based carriers, is to be summarized.

Key words – siRNA, non-viral gene delivery, lipid, polymer, peptide

Recently, small interfering RNA (siRNA) has attracted much attention not only from academia but from pharmaceutical and medical industry due to its enormous potential for diseases that do not have effective way of treatment with conventional small molecule-based drugs. Once delivered inside the cells, siRNA, double stranded RNA composed of 21-25 nucleotides, can induce the evolutionary conserved mechanism called RNA interference by which the expression of a specific gene can be effectively silenced at the post-transcriptional level (Caplen et al., 2001; Hannon and Rossi, 2004; McManus and Sharp, 2002). An RNA-enzyme complex called RNA-induced silencing complex (RISC) mediates the gene silencing mechanism with high sequence specificity even at picomolar concentrations. Since the first clinical trial for RNAi drug was approved for the treatment of age-related macular degeneration (AMD) in 2004 (Kaiser et al.), several RNAi drug candidates have been in the clinical trials. However, even enthusiastic supports of the technology recognize major huddles that should be overcome for the successful entry of the RNAi drugs into clinical settings. One of the biggest challenges of the field is to deliver the siRNA therapeutic drug to its target mRNA (Jeong et al., 2007b). Since it is composed of nucleic acids, siRNA is inherently vulnerable to the attack of several extracellular nucleases when it is administered in the blood stream. In addition, the highly negatively charged macromolecule may not readily get an entry to the cells (Dorsett and Tuschl, 2004). Therefore, new modality for efficient siRNA delivery should be carefully designed to address the problems. A lot of non-viral siRNA delivery techniques based on lipid (Landen et al., 2005; Santel et al., 2006; Yano et al., 2004; Zimmermann et al., 2006), polymer (Kim et al., 2006b; Urban-Klein et al., 2005), and peptide (Mok and Park, 2008a) carries has been suggested for the improved intracellular delivery of siRNA. Except for neutral liposomes that physically encapsulate siRNA in their aqueous cavity, most of the carriers have multiple positive charges so that they can form stable nano-sized complexes with sRNA via electrostatic interactions (Jeong et al., 2007b). The resulting complexes provide not only efficient protection of siRNA from the nucleases but enhanced cellular uptake by the mechanism called endocytosis. Further modifications on the non-viral carriers have carried out to provide the carriers with tissue targeting ability, biodegradability, and prolonged circulation property. This short review focuses on the recent development of non-viral siRNA delivery systems based on lipids, polymers, and peptides.

Lipid-based Delivery Systems for siRNA

Liposomes have been used for the delivery of genetic materials for almost 30 years since the first demonstration by their ability to transport the preproinsulin gene to the liver. Since then, various types of liposomes, either neutral or cationic, were developed and used for the delivery of nucleic acids both in vitro and in vivo (Soriano et al., 1983).

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Barriers to liposomal delivery of siRNA

Endocytosis

Since the siRNA is negatively charged at physiological conditions, it is not easy to cross the cell membrane by simple diffusion (Lu et al., 2009), which leads to the development of various approaches to facilitate the uptake of siRNA into the target cells. These include i) encapsulation of siRNA into nanoparticles or liposomes, ii) ligand conjugation to siRNA, and iii) fusion of the delivery system with the cell membrane to release the contents into the cytosol. Lu et al., have shown that over 90% of siRNA-DharmaFECT liposome complexes enter the cells via endocytosis, including clathrin-mediated or non clathrin-mediated endocytosis (Lu et al., 2009). Felgner et al., suggested that the positively charged liposomes adhere to the negatively charged plasmid DNA to form a complex and this complex fuse with the negatively charged cell membranes, resulting in the internalization of plasmid DNA into the cells (Felgner and Ringold, 1989). As the siRNA is much smaller (< 13 kDa) than the plasmid DNA (>200 kDa) in size, its complex with cationic liposomes would be capable of fusing with cell membranes and subsequently releasing the siRNAs into the cytosol.

Endosomal escape

As the main delivery mechanism of siRNA/liposome complexes is endocytosis which leads to endosome within a cell, it is necessary to develop a tool to escape the delivered siRNA from the endosome into the cytosol. One suggested way is through “proton-sponge effect”, which acts as follows: endosome is acidified after internalization and amine groups, if any, in the delivery system with pKa between 5 and 7 are protonated, followed by the influx of additional protons and chloride into the endosomes and creating osmotic imbalance; water enters into the endosomes to balance out, causing endosome to inflate until it ruptures (Sonawane et al., 2003).

Neutral liposomes

Traditionally, neutral liposomes were widely used for *in vitro* and *in vivo* delivery of chemical drugs, such as doxorubicin or amphotericin B, though their use as carriers of nucleic acid was limited due to the low encapsulation efficiency of nucleic acids and instability in serum. However, with the realization that significant toxicities were associated with cationic liposomes, neutral liposomes have re-emerged as promising carriers of nucleic acids including siRNA. Several studies have shown that neutral liposomes could be used for the in vivo delivery of focal adhesion kinase-siRNA for the treatment of ovarian carcinoma, (Halder et al., 2006), EphA2-siRNA for ovarian cancer (Landen et al., 2005), interleukin-8-siRNA for ovarian cancer (Merritt et al., 2008), and protease-activated receptor-1-siRNA for melanoma (Villares et al., 2008).

Although efficient encapsulation of siRNA within neutral liposomes and the stability in serum were the initial limitations to the use of these liposomes, these limitations have been overcome, to a certain extent, by development of new strategies. Freeze-thawing allows a high encapsulation of siRNA within neutral liposomes and coating the surface of these liposomes with hyaluronic acid stabilizes these liposome/siRNA complexes in the bloodstream. Peer et al. have shown that cyclin D1-siRNA in β–antibody conjugated neutral liposomes was stably directed to leukocyte which were activated by inflammatory signals due to colitis or malignancies (Peer et al., 2008). Interestingly, they used protamine to complex with cyclin D1-siRNA before encapsulation into the liposomes and the liposomes were sterically stabilized with hyaluronic acid to increase the stability in the serum. Systemic injection of cyclin D1-siRNA/liposomes complexes which were targeted to the activated leukocytes reversed experimentally induced colitis in mice by suppressing leukocyte proliferation and T helper cell cytokine expression. This study revealed that cyclin D1 was among the potential anti-inflammatory targets for siRNA therapy, and suggested that this therapy may not only be applicable toward colitis but also some malignancies.

Cationic liposomes

In 1989 Felgner and Ringold reported that cationic liposomes can combine quickly with negatively charged nucleic acids to form so-called “lipoplexes” and the ease of preparation of these lipoplexes has enabled many researchers to study the delivery of genes of interest both *in vitro* and *in vivo* (Felgner and Ringold, 1989). So far, more than a dozen cationic phospholipids are commercially available and more is coming. These include, but not limited to, Lipofectamine 2000 (Invitrogen), DOTAP (Roche Applied Science), Oligofectamine, (Invitrogen), DMRIE-C (Invitrogen), X-tremeGene (Roche), siPORT NeoFx (Ambion) , RNAifect (Qiagen) and GeneSilencer (Genlantis)).

Several studies have shown that these carriers are effective carriers not only for in vitro but for in vivo delivery of siRNA. Pirollo et al. targeted several different xenografts with HER2-siRNA in complex with 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) liposomes (Pirollo et al., 2007). These liposomes contain histidine-lysine peptides on their surface to facilitate their endosomal escape as well as transferrin receptor (TfR) antibody. Although the HER2-siRNA/immunoliposomes

Table I. *Representative Examples of Cationic Lipids*

Transfection reagents	Composition	Reference
Lipofectamine 2000	DOSPA:DOPE-3:1 mixture)	(Dalby et al., 2004)
Lipofectin	DOTMA:DOPE=1:1	(Troussard et al., 2003)
DMRIE-C	DMRIE:Cholesterol=1:1	(Leu et al., 2003)
DOTAP	Cationic lipid	(Leirdal and Sioud 2002)
siPORT Lipid	Cationic + neutral lipid mixture	(Grayson et al., 2006)
GeneSilencer	Cationic lipid mixture	(Grayson et al., 2006)
TransGene	DOPE + proprietary material	(Potente et al., 2003)
CDAN/DOPE	CDAN:DOPE=45:55	(Spagnou et al., 2004)
LithoP pLitho	Lithocholic acid derivatives 3, 5	(Lorenz et al., 2004)
RPR209120/DOPE	RPR209120B:DOPE:DOPE-lissamine rhodamine B =20:20:0.4	(Khoury et al., 2006)
DPPC-DPPG	DPPG:DPPC:DPPE-PEG2000 =77:18:5	(Schiffelers et al., 2004)

complexes significantly inhibited the growth of pancreatic cancer in a xenograft model, combining gemcitabine with these lipoplexes resulted in a synergistic effect and inhibited tumor growth almost completely.

DOTAP/cholesterol liposomes were known to be the most effective for delivery of nucleic acids in vivo with several other equivalent phospholipids. However, a liposome comprised of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and cardiolipin in complex with luciferase plasmid DNA administered intravenously resulted in tumors expressing 7-times higher levels of luciferase than did DOTAP/cholesterol lipoplexes (Chien et al., 2005). The cardiolipin analog of the liposome was significantly less toxic than the DOTAP/cholesterol liposome: whereas two-thirds of the mice died when injected with 100 mg/kg of DOTAP/cholesterol liposomes, no mice died at a similar dose with the cardiolipin-containing liposome. In addition, c-Raf siRNA in complex with the cardiolipid-containing liposome resulted in about 50% inhibition of breast cancer xenografts compared to the liposome- control mismatch siRNA group. The significant inhibition of tumor growth with c-Raf-1 siRNA, the lower toxicity of the lipid, and greater *in vivo* transfection efficiency with the cardiolipin-containing liposomes suggest that further studies of these liposomes are needed

Unlike the lipoplexes discussed earlier, Li et al., prepared nanoparticles in which the siRNA (targeting luciferase) was encapsulated within liposomes (Li et al., 2008). Minimizing direct interactions between siRNA and cationic lipids may reduce induction of immune responses. They mixed the siRNA with basic protamine, followed by encapsulation within DOTAP/cholesterol (1:1, molar ratio) liposomes to obtain LPD (liposome-protamine-DNA) nanoparticles. The positive charge on liposomes from the DOTAP lipids may promote the interaction with the negatively charged cell membranes, thereby increasing endocytosis of LPD nanoparticles. After single intravenous injection, the targeted Luciferase-siRNA nanoparticle down-regulated luciferase levels by 80% in lung metastatic model compared to the targeted control siRNA nanoparticle. Moreover, cytokine induction was minimal with this targeted LPD nanoparticles, particularly when protamine/siRNA or DOTAP/siRNA complexes would induce strong cytokine responses.

Some representative examples of cationic lipids are listed in Table I.

Other lipid-based delivery systems

Recently, Akinc et al. reported that a combinatorial library of lipid-like materials, termed “lipidoids”, were synthesized and used for the delivery of RNAi (Akinc et al., 2008), where new chemical methods were developed to synthesize rapidly a large library of over 1,200 structurally diverse lipidoids. From this library, they identified lipidoids that facilitates high levels of specific gene silencing of endogenous genes when formulated with siRNA. The safety and efficacy of lipidoids were evaluated in three animal models including mice, rats, and nonhuman primates.

Another example of liposomes for siRNA delivery is the stable nucleic acid-lipid particle or “SNALP”. SNALP nanoparticles are PEGylated liposomes with low cationic lipid content that encapsulate nucleic acids, including siRNA, within the lipid bilayer. Morrissey et al., showed that systemic delivery of siRNA-SNALP complexes which targeted HBV RNA can inhibit the HBV replication effectively (Morrissey et al., 2005). It showed that three daily intravenous injections (3

mg/kg) reduced HBV levels by 10-fold for 7 days. Moreover, Zimmerman et al., used the apolipoprotein B-siRNA-SNALP nanoparticles in non-human primates (Zimmermann et al., 2006), where almost 90% of apolipoprotein B mRNA expression in the liver of nonhuman primates was silenced with a single injection of apolipoprotein B-siRNA-SNALP nanoparticles.

Polymer-based Delivery Systems for siRNA

Synthetic and natural polymers have also been used for designing gene delivery systems. Most polymeric carriers have well-defined structure and physic-chemical properties and high degree of molecular diversity which allows additional modifications. Functional moieties such as a targeting ligand that improves specificity toward a target tissue and units for facilitated endosomal escape were popularly introduced for enhanced therapeutic effect of siRNA drug.

Polyethylenimine (PEI)-based carriers for siRNA delivery

Among the polymeric gene carriers, PEI is one of the most popularly employed synthetic polymers. PEI has primary, secondary, and tertiary amines with different dissociation constants (pKa) which can be protonated at different pHs. This property of PEI allows proton buffering in the acidic endosomal compartment, leading to facilitated endosomal escape. PEI also demonstrated an excellent condensing property for several nucleic acid drugs, including plasmid DNA and siRNA, providing efficient protection from nucleases (Urban-Klein et al., 2005). When administered systemically and intraperitoneally to tumor-bearing mice, PEI complexed with siRNA targeting an epidermal growth factor receptor (HER-2) showed a significant reduction of tumor growth through gene-specific silencing of HER-2 protein (Urban-Klein et al., 2005). Fas is a membrane receptor known to be closely related with an incidence of diabetes. PEI used as a carrier for siRNA targeting Fas gene expression exhibited efficient silencing of the target gene in vitro as well as in vivo experiments (Jeong et al.). In a cyclophosphamide-induced accelerated diabetes animal model, systemic injection of PEI/Fas siRNA complexes through tail vein of the diabetes-prone mice (NOD) led to significant delay in the diabetes occurrence. The study suggested that Fas expression in the early stage of diabetes seems to important role in the occurrence of diabetes. Additional stability of siRNA/PEI nano-complexes in the presence of serum proteins could be introduced by modifying PEI with a hydrophilic polymer such as PEG (Malek et al., 2008; Merkel et al., 2009) and hyluronic acid (Jiang et al., 2009). PEI modified with PEG showed changes in biodistribution profiles in the major organs, including liver, lung, kidney, and spleen, and improved pharmacokinetic behavior after a systemic administration (Malek et al., 2009).

Cyclodextrin-polymer based siRNA delivery

A class of nonionic cyclic polysaccharide, cyclodextrin, was used as a core material for gene delivery. A polycation-containing cyclodextrin which elicited no significant cytotoxicity in the cells and the animals can condense siRNA to form tight nanoparticles. Transferrin(Tf) was additionally introduced to the polycation-cyclodextrin system as a targeting ligand for cancer. Systemic administration of the Tf-conjugated polycation-cyclodextrin/siRNA nanoparticles demonstrated marked reduction in tumor growth (Bartlett and Davis, 2008; Bartlett et al., 2007). The nanoparticular formulation consisting of a cyclodextrin-containing polycation, PEG as a stabilizing agent, and human Tf as a targeting moiety (Davis, 2009) was tested in human subject having solid tumor (Davis et al.). The systemically delivered siRNA targeting M2 subunit of ribonucleotide reductase (RRM2) led to reduction of corresponding mRNA and protein. The main mechanism of the silencing was revealed to be RNAi mediated by a sequence specific cleavage of RRM2 mRNA.

siRNA delivery using reducible polymers

A polymer-mediated gene delivery system should provide protection of siRNA from the action of extracellular nucleases and mediate effective transport of siRNA into target cells. Once localized inside the cell, the polymeric carrier should lead to rapid endosomal escape of siRNA, after which the dissociation of siRNA from the complex should be followed in the cytosolic space where RNAi mechanism takes place. The cytosolic release of siRNA from the complex can be facilitated by employing polymers containing reducible linkages such as disulfide (S-S) bond that is readily degraded in the reductive cytoplasm owing to the presence of excess amount of intracellular reduced glutathione (GSH). A poly(amido ethylenimine), poly(TETA-CBA) was synthesized via Michael-type addition polymerization between cystamine bisacrylamide (CBA) and triethetyl tetramine (TETA) and used for cytosolic siRNA delivery (Jeong et al., 2007a). After transfection with the polymer, fluorescently labeled siRNA was distributed over the cytoplasm, whereas particular aggregates were observed with non-degradable control polymer, PEI. The cytosolic distribution of siRNA seems to facilitate the access of siRNA to RISC, resulting in enhanced RNAi-mediated silencing of target gene. Similarly, reducible poly(disulfide amines) grafted

with arginine also demonstrated the efficient unloading of siRNA in the cytoplasm and effective inhibition of target gene (VEGF) expression (Kim et al., 2009).

Alternative strategies for polymeric siRNA delivery

Natural polymers such as chitosan and atelocollagen were also showed promise for siRNA delivery. Chitosan can be obtained from deacetylation of chitin consisting of N-acetyl-D-glucosamine units, linked via (1-4) glycosidic bonds (Okamoto et al., 2002). Chitosan was used for the delivery of siRNA to the lung epithelium through intranasal administration (Howard et al., 2006). Atelocollagen obtained by the removal of telopeptides by protease treatment was also employed in local and systemic delivery of siRNA targeting myostatin, leading to meaningful increment of muscle mass (Kinouchi et al., 2008).

Direct conjugation of polymer or small molecules can be another promising approach for enhanced siRNA delivery. The conjugate generally synthesized by covalently attaching small molecules such as cholesterol and α-tocopherol and polymers such as PEG to the sense strand of siRNA, which does not hamper the silencing activity of the siRNA. This approach has been considered attractive since various molecular entities that can act as a stabilizer and targeting ligand can be used as a conjugation partner without serious loss of activity of siRNA. In addition, the resulting conjugates are expected to have low cytotoxicity, compared to polycation-based delivery systems. The efficient inhibition of apoB protein in the liver and jejunum was observed after systemic administeration of cholesterol conjugate of siRNA (Soutschek et al., 2004). In a recent report, a covalent conjugate of PEG and siRNA showed improved serum stability and enhanced transfection and inhibition of a target gene in vitro as well as in vivo, when formulated with a polycation (Kim et al., 2006a; Kim 2008).

Peptide-based Delivery Systems for siRNA

Protein transduction domains for siRNA delivery

Cell membrane permeability and transduction ability of full-length HIV-1 TAT protein was discovered in 1988 (Frankel and Pabo, 1988). Continuous studies defined a small region spanning residues of the full-length protein necessary for the internalization. Since the discovery, various protein transduction domains (PTDs), also named cell penetrating peptides (CPPs), have been reported such as, Antennapedia homeodomain protein (Antp), transportan, HSV-1 protein VP22, MPG, model amphipathic peptide (MAP), and poly-arginine (Meade and Dowdy, 2007). PTDs have shown the ability for the intracellular delivery of several cargoes, including, proteins (Lim et al., 2010; Won et al., 2010b), plasmid DNA (Won et al., 2010a), liposomes (Torchilin et al., 2003; Torchilin et al., 2001). Early uptake mechanism studies of PTDs suggest non-traditional endocytosis; however, more recent studies propose the various ways of endocytosis, in particular, macropinocytosis (Wadia et al., 2004). Despite the controversy of internalization mechanism, it is widely accepted that they have the capability to penetrate 100% cells of a given cell culture population (Wadia and Dowdy, 2005). Therefore, the high cellular uptake efficiency provides a great potential to the siRNA delivery via various approaches with several types of PTDs.

Tat peptides for siRNA delivery

Tat peptide was conjugated to PAMAM dendrimers to exam the effect of conjugation on the siRNA delivery efficiency (Kang et al., 2005). PAMAM G5 dendrimers conjugated with a fluorophore (BODIPY) and Tat peptide were prepared, and then complex formation, cellular uptake, intracellular distribution and pharmacological efficacy were examined using antisense or siRNA-targeted MDR1. Both free PAMAM and Tat-PAMAM formed polyplexes with either antisense or siRNA without significant difference in complexation ability and protected siRNA from enzymatic degradation. Although Tat peptide enhanced the cellular uptake of the dendrimers by 20-30% under the test conditions, only a small portion of both antisense and siRNA were released from the dendrimers in the cytoplasm. In the bioactivity tests, Tat-PAMAM failed to further improve the ability of PAMAM for siRNA delivery, and both dendrimers were poorly effective to deliver siRNA, whereas they were partially effective in the antisense delivery. This limited bioactivity of antisense and siRNA delivered by those dendrimers is most likely due to the incomplete release of the nucleotides in the cells.

Direct conjugation of PTDs to siRNA is another approach to improve the stability and bioactivity of siRNA. Moschos et al., investigated the effect of direct conjugation of Tat, penetratin and cholesterol on the siRNA-mediated knockdown of p38 MAP kinase in mouse lung (Moschos et al., 2007). Tat-, penetratin- and cholesterol-conjugated siRNA showed moderate knockdown of the target mRNA without significant cytotoxicity, and the conjugates led to a 30-45% knockdown of p38 MAP kinase mRNA after the intratracheal administration. Unfortunately, both penetration and Tat peptide alone caused an almost similar reduction in p38 MAP kinase mRNA as penetratin- and Tat-siRNA conjugates and penetratin-siRNA further activated innate immune response. These results require the future study regarding the mechanism of cellular uptake of those peptides because the innate immune response may be

due to the different intracellular fates of Tat and penetratin.

Recent study, a Tat-PTD fusion protein with a single double-stranded (ds) RNA-binding domain (DRBD) was constructed to avoid the charge neutralization of PTD after forming complexes with siRNA (Eguchi et al., 2009). Despite the great achievement of PTD-mediated siRNA delivery, their poor transfection efficiency in the primary cells and the charge neutralization still remain to be solved. DRBD has the ability to bind siRNA with high affinity, thereby the cargo siRNA efficiently bind with DRBD motif in the fusion protein, while PTD moiety remains free to interact with cell membrane. PTD-DRBD fusion protein exhibited a higher transfection efficiency and silencing of the target mRNA with negligible cytotoxicity than commercial Lipofection. This recombinant fusion protein could deliver siRNA rapidly and induce RNA interference in various primary cells, including T cells, human umbilical vein endothelial cells and human embryonic stem cells. The results reveal that the PTD-DRBD fusion protein constitutes a promising strategy for the efficient gene silencing in difficult-to-transfect primary cells.

Oligo-arginine-PTDs for siRNA delivery

Nonaarginine (R9), a most well-known PTD among arginine-rich peptides, has been explored to deliver siRNA over the last few years. The first study reported a hydrophobically modified PTD by conjugating cholesteryl to oligo-D-arginine (Chol-R9) (Kim et al., 2006c). The arginine domain provides a binding affinity to siRNA, while cholesteryl moiety interacts with cell membrane for the efficient cellular uptake of the complex. The resulting conjugate was well characterized and the ability for the complex formation was determined using siRNA against VEGF. Chol-R9 exhibited better silencing ability than its counterpart, R9, in *in vitro* tests; hence it suppressed tumor growth by reducing the production of VEGF at tumor region in an animal model. It is clearly demonstrated that Chol-R9 is efficiently deliver siRNA *in vitro* and it maintains the bioactivity of siRNA *in vivo* after local administration. In another study, it was described that unmodified-R9 was capable of delivering siRNA in mammalian cells (Wang et al., 2007). Synthetic-R9 could condense siRNA at the optimized N/P ratio, and RNA interference was confirmed by silencing of EGFP expression in the EGFP stable cells. In similar study, the anti-cancer effects of HER2 siRNA was evaluated using R15 peptide (Kim et al., 2010). Recent attention has strived for the physicochemical characterization of R9/siRNA polyplex in order to control the properties of the polyplexes (Law et al., 2008). Various methods were suggested to characterize the polyplexes. UV and circular dichroism (CD) spectra provide the optimal binding ratio of R9 to siRNA by determination of hypochromicity that represents structural changes in siRNA. Dynamic light scattering (DLS) and Zeta potential measurements are the powerful tool to demonstrate aggregation, hydrodynamic diameter and surface charge of the polyplexes, and salt dissociation can be used to confirm the non-covalent interaction between R9 and siRNA. These techniques can be applied to the development of effective siRNA carrier based on the peptide by controlling the physicochemical properties.

Octaarginine was attached on the surface of liposome (R8-liposomes) in order to deliver HDM2-siRNA (Zhang et al., 2006). The encapsulated siRNA was highly stable and protected from the enzymatic degradation, thereby high transfection efficiencies with low cytotoxicity were observed in three types of lung tumor cells. R8-liposome has advantages over the lipid-based siRNA carriers, such as reduced systemic toxicity, enhanced stability and circulation time, and increased permeability of siRNA into cells. Similar to R8-liposome, Nakamura et al., developed a octaarginine-modified multifunctional envelope-type nano device (R8-MEND) (Nakamura et al., 2007). Although a series of MEND has been developed as a delivery system for pDNA or antisense ODN, it is unknown whether MEND is an appropriate carrier for siRNA. The condensation ability, physicochemical properties, and silencing effect of R8-MEND was examined and compared with the conventional polycations and commercially available transfection agent. In order to prepare R8-MEND, stearyl octaarginine (STR-R8) and siRNA was complexed, thereby the polyplex was added to the lipid film, followed by incubation, sonication and coating. The resulting STR-R8 solution (5 mol% of lipids) was mixed with R8 peptide for the formation of R8-MEND. The rapid release of siRNA from R8-MEND in cytosol and immediate degradation of R8 might lead to high silencing ability and low cytotoxicity, respectively, in comparison with the transfection agent. MEND was further modified with a pH-sensitive fusogenic GALA peptide for the improved siRNA activity in the cells (Hatakeyama et al., 2009). The fusogenic peptide facilitated the endosomal escape of nanoparticles, leading to the enhanced siRNA bioactivity resulting in increased gene silencing.

Recent study demonstrated an application of R7-based siRNA carrier modified with a 14-carbon myristic acid moiety (MPAP) labeled with Cy5.5 fluorescent dye for dual effect of imaging and therapy (Ifediba et al., 2010). Myristoylation provides an increase in the binding affinity of peptide to the lipid bilayers, enhancing cellular uptake of peptide, and BBB permeability *in vivo*. This system was evaluated in the primary astrocytes, neurons, or bEND.3 brain endothelial cells by

determining cytotoxicity, probe internalization using FACS and confocal microscope and silencing of the target mRNA, c-Src. The cellular uptake and distribution in the cells were clearly visualized, while c-Src siRNA silenced c-Src mRNA in all three cell types, and the cells were rescued under ischemic condition by treating c-Src siRNA. Despite the preliminary *in vitro* results of the dual effects, it may be useful in the neurological applications due to the tracking ability and therapeutic efficacy.

Cell-penetrating peptides for siRNA delivery

Attempts to deliver siRNA into cells have discovered various CPPs, among which, bPrPp (1-30), MPGΔ^{NLS}, penetratin, TP10 were evaluated to design a endosomolytic peptide, EB1 (Lundberg et al., 2007). EB1 theoretically become an alpha helix upon protonation in acidic environments because of a substitution of histidine for the certain amino acids in the penetratin. Endocytic pathway is mostly accepted as the main mechanism of internalization for CPPs, excepting MPG and PEP-1. Despite the similar abilities of peptides for condensing siRNA, MPGΔ^{NLS} showed high biological activity of siRNA, while the poor bioactivity was observed from the penetratin and TP10. Interestingly, EB1 exhibited the same silencing ability as MPGΔ^{NLS}, suggesting the notably enhanced bioactivity of siRNA in cytosol, at least in part, resulted from the ease of endosomal escape.

Divita group has designed a new secondary amphipathic CPP (CADY) composed of 20 amino acids (Crombez et al., 2008). CADY originated in PPTG1 peptide, which is suitable for DNA delivery but not for siRNA, was modified by replacing seven residues in PPTG1 peptide with tryptophan and arginine. The high binding affinity (K_d=15.2 nmol/l) of CADY for siRNA was mostly obtained from the arginine residues, whereas tryptophan is involved in the cellular uptake and stabilization of siRNA/peptide polyplex. The modification makes CADY possible to bind, stabilize, and deliver siRNA, resulting in enhanced RNA interference in a broad spectrum of cell types, including primary, suspension, and adherent cell lines. In the more recent study, the cellular uptake mechanism of CADY was investigated (Konate et al., 2010). Upon electrostatic binding, the polyplex initiates conformational changes of unfolded CADY to a helical formation; thereby it interacts with the cell surface molecules, proteoglycans and/or phospholipids. Finally, endosomal pathway-independent membrane disorganization occurs after the interaction of siRNA/CADY with phospholipid phase in the cell membrane.

Cell penetrating peptide derived from low molecular weight protamine (LMWP) was developed for the systemic delivery of siRNA (Choi et al., 2010). LMWP, arginine-rich peptide composed of 14 amino acids (10 arginines), was able to condense and stabilize siRNA. It showed almost similar silencing of the target mRNA and non-toxicity in comparison with Tat peptide in *in vitro* tests. For the treatment of cancer, siRNA-targeted VEGF was administered through the systemic injection after forming the siVEGF/LMWP polyplexes. The systemic administration of polyplexes led to the tumor regression resulted from the reduced VEGF expression in tumor region with negligible systemic side effects.

Reducible peptides for siRNA delivery

Reducible peptides with internal disulfide linkages have been considered as a promising non-viral carrier for siRNA due to the cytoplasm-sensitive reducibility and high stability in the extracellular spaces. Histidine-based reducible peptide composed of repeating units of $CH_6K_2H_6C$ monomer (His6 RPC) was prepared for siRNA delivery as well as pDNA delivery (Stevenson et al., 2008). In similar study, the histidine-rich peptide (HRP) was copolymerized with nuclear localization sequence (NLS) into five different compositions of HRP-NLS copolypeptide (Rahbek et al., 2008). In order to prepare reducible copolypeptides, CKH_3KH_3KC for HRP and $CGAGPK_3RKVC$ for NLS were synthesized, and the reducible copolypeptides with various molecular weights were prepared by oxidative polymerization of the cysteine sulfhydryl groups. The resulting copolypeptides have the potential to the siRNA and pri-miRNA delivery in terms of the ability to silence the nuclear compartment necessary for transcription and utilize endogenous miRNA biogenesis pathway. For the enhanced charge density, endosomal escape and rapid dissociation in cytoplasm, fusogenic KALA peptide was reducibly copolymerized into cross-linked KALA (cl-KALA) via DMSO-mediated oxidative polymerization of cysteine sulfhydryl groups at the both ends of KALA peptide (C-KALA-C) (Mok and Park, 2008b). cl-KALA showed no superior gene silencing to its counterpart, KALA, whereas it exhibited less cytotoxicity and more stable polyplexes with siRNA. Tanaka et al., developed a disulfide cross-linked stearoyl peptide consisted of arginine and histidine (STR-$CH_2R_4H_2C$) (Tanaka et al.). The condensation ability for siRNA, cellular uptake and transfection efficiency of the peptide were compared with its non-reducible counter peptide (STR-$GH_2R_4H_2G$) and commercially available transfection agent, LipoTrust. STR-$CH_2R_4H_2C$ exhibited a comparable and improved silencing with LipoTrust and STR-$GH_2R_4H_2G$, respectively, and slightly better tumor suppressive effects than LipoTrust in tumor model.

Other peptides for siRNA delivery

MPG, a peptide-based gene carrier derived from the HIV-1 gp41 protein and NLS from the SV40 T antigen, has been explored to prove the effectiveness for siRNA delivery. Two types of MPG, MPG and MPGΔ^{NLS}, were prepared to investigate the mechanism of internalization and evaluate the siRNA delivery in the cells (Simeoni et al., 2003). The slightly higher silencing of the MPGΔ^{NLS} than that of MPG peptide might be likely due to the NLS, and the internalization mechanism was determined to be independent from the endosomal pathway. Veldhoen et al., prepared a modified MPG peptide (MPGα) by substituting six amino acids in the hydrophobic part of the MPG peptide (Veldhoen et al., 2006). High tendency toward the helical conformation is a main difference of MPGα from the parent peptide, while the physicochemical properties required for the complexation were maintained. However, the vesicular escape remains the main obstacle for the MPGα-mediated siRNA delivery because the high vesicular accumulation of the polyplex was observed upon cellular uptake. MPG peptide was shortened into MPG-8 composed of 21 amino acids by removing 6 amino acids (G^1, L^3, S^{13}, A^{17}, G^{20} and V^{27}), substitution of tryptophan (W) for 2 amino acids (F^7 and A^{11}) and maintaining the cysteamide group at the C-terminus of MPG (Crombez et al., 2009).

Dendritic poly(L-lysine) with 6th generation (KG6) was developed as a peptide-based gene carrier for DNA and siRNA delivery (Inoue et al., 2008). Similar to PAMAM dendrimer, KG6 has advantages including controllable molecular weight, shape, ease of surface modification, and a lot of amine groups on its surface (128 amine groups on KG6). Despite the high transfection efficiency for DNA and cellular uptake, KG6 exhibited poor silencing of the target mRNA. A combination of KG6 with Endo-Porter (EP), a weak-base amphiphilic peptide, was suggested to improve the activity of siRNA in the cells. The combined peptide enabled the effective silencing of the target mRNA with low cytotoxicity. Recently, KG6 was applied to the treatment of hypercholesterolemia by delivering siRNA-targeted ApoB (Watanabe et al., 2009). Leng et al., developed a series of branched peptide carrier consisted of histidine and lysine (HK) for DNA and siRNA delivery (Leng et al., 2005). Four and eight terminal branches (H^3K4b, H^2K4b, HK4b, and H^3K8b) were evaluated for the siRNA delivery. H^3K4b exhibited slightly better reduction in β-galactosidase activity among the four terminal branches, whereas H^3K8b showed significantly reduced target activity in comparison with lipofectamine, suggesting the high potency of H^3K8b peptide as a siRNA carrier.

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References

Akinc, A., Zumbuehl, A., Goldberg, M., Leshchiner, E.S., Busini, V., Hossain, N., Bacallado, S.A., Nguyen, D.N., Fuller, J., Alvarez, R., Borodovsky, A., Borland, T., Constien, R., de Fougerolles, A., Dorkin, J.R., Narayanannair Jayaprakash, K., Jayaraman, M., John, M., Koteliansky, V., Manoharan, M., Nechev, L., Qin, J., Racie, T., Raitcheva, D., Rajeev, K.G., Sah, D.W., Soutschek, J., Toudjarska, I., Vornlocher, H.P., Zimmermann, T.S., Langer, R., Anderson, D.G., 2008. A combinatorial library of lipid-like materials for delivery of RNAi therapeutics. Nat Biotechnol 26, 561-569.

Bartlett, D.W., Davis, M.E., 2008. Impact of tumor-specific targeting and dosing schedule on tumor growth inhibition after intravenous administration of siRNA-containing nanoparticles. Biotechnol Bioeng 99, 975-985.

Bartlett, D.W., Su, H., Hildebrandt, I.J., Weber, W.A., Davis, M.E., 2007. Impact of tumor-specific targeting on the biodistribution and efficacy of siRNA nanoparticles measured by multimodality in vivo imaging. Proc Natl Acad Sci U S A 104, 15549-15554.

Caplen, N.J., Parrish, S., Imani, F., Fire, A., Morgan, R.A., 2001. Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. Proc Natl Acad Sci U S A 98, 9742-9747.

Chien, P.Y., Wang, J., Carbonaro, D., Lei, S., Miller, B., Sheikh, S., Ali, S.M., Ahmad, M.U., Ahmad, I., 2005. Novel cationic cardiolipin analogue-based liposome for efficient DNA and small interfering RNA delivery in vitro and in vivo. Cancer Gene Ther 12, 321-328.

Choi, Y.-S., Lee, J.Y., Suh, J.S., Kwon, Y.-M., Lee, S.-J., Chung, J.-K., Lee, D.-S., Yang, V.C., Chung, C.-P., Park, Y.-J., 2010. The systemic delivery of siRNAs by a cell penetrating peptide, low molecular weight protamine. Biomaterials 31, 1429-1443.

Crombez, L., Aldrian-Herrada, G., Konate, K., Nguyen, Q.N., McMaster, G.K., Brasseur, R., Heitz, F., Divita, G., 2008. A New Potent Secondary Amphipathic Cell-penetrating Peptide for siRNA Delivery Into Mammalian Cells. Mol Ther 17, 95-103.

Crombez, L., Morris, M.C., Dufort, S., Aldrian-Herrada, G., Nguyen, Q., Mc Master, G., Coll, J.-L., Heitz, F., Divita, G., 2009. Targeting cyclin B1 through peptide-based delivery of siRNA prevents tumour growth. Nucl. Acids Res. 37, 4559-4569.

Dalby, B., Cates, S., Harris, A., Ohki, E.C., Tilkins, M.L., Price, P.J., Ciccarone, V.C., 2004. Advanced transfection with Lipofectamine 2000 reagent: primary neurons, siRNA, and high-

throughput applications. Methods 33, 95-103.
Davis, M.E., 2009. The first targeted delivery of siRNA in humans via a self-assembling, cyclodextrin polymer-based nanoparticle: from concept to clinic. Mol Pharm 6, 659-668.
Davis, M.E., Zuckerman, J.E., Choi, C.H., Seligson, D., Tolcher, A., Alabi, C.A., Yen, Y., Heidel, J.D., Ribas, A., Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. Nature 464, 1067-1070.
Dorsett, Y., Tuschl, T., 2004. siRNAs: applications in functional genomics and potential as therapeutics. Nat Rev Drug Discov 3, 318-329.
Eguchi, A., Meade, B.R., Chang, Y.-C., Fredrickson, C.T., Willert, K., Puri, N., Dowdy, S.F., 2009. Efficient siRNA delivery into primary cells by a peptide transduction domain-dsRNA binding domain fusion protein. Nat Biotech 27, 567-571.
Felgner, P.L., Ringold, G.M., 1989. Cationic liposome-mediated transfection. Nature 337, 387-388.
Frankel, A.D., Pabo, C.O., 1988. Cellular uptake of the tat protein from human immunodeficiency virus. Cell 55, 1189-1193.
Grayson, A.C., Ma, J., Putnam, D., 2006. Kinetic and efficacy analysis of RNA interference in stably and transiently expressing cell lines. Mol Pharm 3, 601-613.
Halder, J., Kamat, A.A., Landen, C.N., Jr., Han, L.Y., Lutgendorf, S.K., Lin, Y.G., Merritt, W.M., Jennings, N.B., Chavez-Reyes, A., Coleman, R.L., Gershenson, D.M., Schmandt, R., Cole, S.W., Lopez-Berestein, G., Sood, A.K., 2006. Focal adhesion kinase targeting using in vivo short interfering RNA delivery in neutral liposomes for ovarian carcinoma therapy. Clin Cancer Res 12, 4916-4924.
Hannon, G.J., Rossi, J.J., 2004. Unlocking the potential of the human genome with RNA interference. Nature 431, 371-378.
Hatakeyama, H., Ito, E., Akita, H., Oishi, M., Nagasaki, Y., Futaki, S., Harashima, H., 2009. A pH-sensitive fusogenic peptide facilitates endosomal escape and greatly enhances the gene silencing of siRNA-containing nanoparticles in vitro and in vivo. Journal of Controlled Release 139, 127-132.
Howard, K.A., Rahbek, U.L., Liu, X., Damgaard, C.K., Glud, S.Z., Andersen, M.O., Hovgaard, M.B., Schmitz, A., Nyengaard, J.R., Besenbacher, F., Kjems, J., 2006. RNA interference in vitro and in vivo using a novel chitosan/siRNA nanoparticle system. Mol Ther 14, 476-484.
Ifediba, M.A., Medarova, Z., Ng, S.-w., Yang, J., Moore, A., 2010. siRNA Delivery to CNS Cells using a Membrane Translocation Peptide. Bioconjugate Chemistry 21, 803-806.
Inoue, Y., Kurihara, R., Tsuchida, A., Hasegawa, M., Nagashima, T., Mori, T., Niidome, T., Katayama, Y., Okitsu, O., 2008. Efficient delivery of siRNA using dendritic poly(l-lysine) for loss-of-function analysis. Journal of Controlled Release 126, 59-66.
Jeong, J.H., Christensen, L.V., Yockman, J.W., Zhong, Z., Engbersen, J.F.J., Kim, W.J., Feijen, J., Kim, S.W., 2007a. Reducible poly(amido ethylenimine) directed to enhance RNA interference. Biomaterials 28 1912-1917.
Jeong, J.H., Kim, S.H., Lee, M., Kim, W.J., Park, T.G., Ko, K.S., Kim, S.W., Non-viral systemic delivery of Fas siRNA suppresses cyclophosphamide-induced diabetes in NOD mice. J Control Release 143, 88-94.
Jeong, J.H., Kim, S.W., Park, T.G., 2007b. Molecular design of functional polymers for gene therapy. Progress in Polymer Science 32, 1239-1274.
Jiang, G., Park, K., Kim, J., Kim, K.S., Hahn, S.K., 2009. Target specific intracellular delivery of siRNA/PEI-HA complex by receptor mediated endocytosis. Mol Pharm 6, 727-737.
Kaiser, P.K., Symons, R.C., Shah, S.M., Quinlan, E.J., Tabandeh, H., Do, D.V., Reisen, G., Lockridge, J.A., Short, B., Guerciolini, R., Nguyen, Q.D., RNAi-based treatment for neovascular age-related macular degeneration by Sirna-027. Am J Ophthalmol 150, 33-39 e32.
Kang, H., DeLong, R., Fisher, M., Juliano, R., 2005. Tat-Conjugated PAMAM Dendrimers as Delivery Agents for Antisense and siRNA Oligonucleotides. Pharmaceutical Research 22, 2099-2106.
Khoury, M., Louis-Plence, P., Escriou, V., Noel, D., Largeau, C., Cantos, C., Scherman, D., Jorgensen, C., Apparailly, F., 2006. Efficient new cationic liposome formulation for systemic delivery of small interfering RNA silencing tumor necrosis factor alpha in experimental arthritis. Arthritis Rheum 54, 1867-1877.
Kim, S.H., Jeong, J.H., Kim, T.I., Kim, S.W., Bull, D.A., 2009. VEGF siRNA delivery system using arginine-grafted bioreducible poly(disulfide amine). Mol Pharm 6, 718-726.
Kim, S.H., Jeong, J.H., Lee, S.H., Kim, S.W., Park, T.G., 2006a. PEG conjugated VEGF siRNA for anti-angiogenic gene therapy. J Control Release 116, 123-129.
Kim, S.H., Jeong, J. H., Lee, S. H., Kim, S. W., Park, T. G. , 2008. Local and systemic delivery of VEGF siRNA using polyelectrolyte complex micelles for effective treatment of cancer. J Control Release in press.
Kim, S.H., Mok, H., Jeong, J.H., Kim, S.W., Park, T.G., 2006b. Comparative evaluation of target-specific GFP gene silencing efficiencies for antisense ODN, synthetic siRNA, and siRNA plasmid complexed with PEI-PEG-FOL conjugate. Bioconjug Chem 17, 241-244.
Kim, S.W., Kim, N.Y., Choi, Y.B., Park, S.H., Yang, J.M., Shin, S., 2010. RNA interference in vitro and in vivo using an arginine peptide/siRNA complex system. Journal of Controlled Release 143, 335-343.
Kim, W.J., Christensen, L.V., Jo, S., Yockman, J.W., Jeong, J.H., Kim, Y.-H., Kim, S.W., 2006c. Cholesteryl Oligoarginine Delivering Vascular Endothelial Growth Factor siRNA Effectively Inhibits Tumor Growth in Colon Adenocarcinoma. Mol Ther 14, 343-350.
Kinouchi, N., Ohsawa, Y., Ishimaru, N., Ohuchi, H., Sunada, Y., Hayashi, Y., Tanimoto, Y., Moriyama, K., Noji, S., 2008. Atelocollagen-mediated local and systemic applications of myostatin-targeting siRNA increase skeletal muscle mass. Gene Ther 15, 1126-1130.
Konate, K., Crombez, L., Deshayes, S.b., Decaffmeyer, M., Thomas, A., Brasseur, R., Aldrian, G., Heitz, F., Divita, G., 2010.

Insight into the Cellular Uptake Mechanism of a Secondary Amphipathic Cell-Penetrating Peptide for siRNA Delivery. Biochemistry 49, 3393-3402.

Landen, C.N., Jr., Chavez-Reyes, A., Bucana, C., Schmandt, R., Deavers, M.T., Lopez-Berestein, G., Sood, A.K., 2005. Therapeutic EphA2 gene targeting in vivo using neutral liposomal small interfering RNA delivery. Cancer Res 65, 6910-6918.

Law, M., Jafari, M., Chen, P., 2008. Physicochemical characterization of siRNA-peptide complexes. Biotechnology Progress 24, 957-963.

Leirdal, M., Sioud, M., 2002. Gene silencing in mammalian cells by preformed small RNA duplexes. Biochem Biophys Res Commun 295, 744-748.

Leng, Q., Scaria, P., Zhu, J., Ambulos, N., Campbell, P., Mixson, A.J., 2005. Highly branched HK peptides are effective carriers of siRNA. The Journal of Gene Medicine 7, 977-986.

Leu, Y.W., Rahmatpanah, F., Shi, H., Wei, S.H., Liu, J.C., Yan, P.S., Huang, T.H., 2003. Double RNA interference of DNMT3b and DNMT1 enhances DNA demethylation and gene reactivation. Cancer Res 63, 6110-6115.

Li, S.D., Chono, S., Huang, L., 2008. Efficient gene silencing in metastatic tumor by siRNA formulated in surface-modified nanoparticles. J Control Release 126, 77-84.

Lim, K.S., Won, Y.-W., Park, Y.S., Kim, Y.-H., 2010. Preparation and functional analysis of recombinant protein transduction domain-metallothionein fusion proteins. Biochimie 92, 964-970.

Lorenz, C., Hadwiger, P., John, M., Vornlocher, H.P., Unverzagt, C., 2004. Steroid and lipid conjugates of siRNAs to enhance cellular uptake and gene silencing in liver cells. Bioorg Med Chem Lett 14, 4975-4977.

Lu, J.J., Langer, R., Chen, J., 2009. A novel mechanism is involved in cationic lipid-mediated functional siRNA delivery. Mol Pharm 6, 763-771.

Lundberg, P., El-Andaloussi, S., Sutlu, T., Johansson, H., Langel, U., 2007. Delivery of short interfering RNA using endosomolytic cell-penetrating peptides. FASEB J. 21, 2664-2671.

Malek, A., Czubayko, F., Aigner, A., 2008. PEG grafting of polyethylenimine (PEI) exerts different effects on DNA transfection and siRNA-induced gene targeting efficacy. J Drug Target 16, 124-139.

Malek, A., Merkel, O., Fink, L., Czubayko, F., Kissel, T., Aigner, A., 2009. In vivo pharmacokinetics, tissue distribution and underlying mechanisms of various PEI(-PEG)/siRNA complexes. Toxicol Appl Pharmacol 236, 97-108.

McManus, M.T., Sharp, P.A., 2002. Gene silencing in mammals by small interfering RNAs. Nat Rev Genet 3, 737-747.

Meade, B.R., Dowdy, S.F., 2007. Exogenous siRNA delivery using peptide transduction domains/cell penetrating peptides. Advanced Drug Delivery Reviews 59, 134-140.

Merkel, O.M., Beyerle, A., Librizzi, D., Pfestroff, A., Behr, T.M., Sproat, B., Barth, P.J., Kissel, T., 2009. Nonviral siRNA delivery to the lung: investigation of PEG-PEI polyplexes and their in vivo performance. Mol Pharm 6, 1246-1260.

Merritt, W.M., Lin, Y.G., Spannuth, W.A., Fletcher, M.S., Kamat, A.A., Han, L.Y., Landen, C.N., Jennings, N., De Geest, K., Langley, R.R., Villares, G., Sanguino, A., Lutgendorf, S.K., Lopez-Berestein, G., Bar-Eli, M.M., Sood, A.K., 2008. Effect of interleukin-8 gene silencing with liposome-encapsulated small interfering RNA on ovarian cancer cell growth. J Natl Cancer Inst 100, 359-372.

Mok, H., Park, T.G., 2008a. Self-crosslinked and reducible fusogenic peptides for intracellular delivery of siRNA. Biopolymers.

Mok, H., Park, T.G., 2008b. Self-crosslinked and reducible fusogenic peptides for intracellular delivery of siRNA. Biopolymers 89, 881-888.

Morrissey, D.V., Lockridge, J.A., Shaw, L., Blanchard, K., Jensen, K., Breen, W., Hartsough, K., Machemer, L., Radka, S., Jadhav, V., Vaish, N., Zinnen, S., Vargeese, C., Bowman, K., Shaffer, C.S., Jeffs, L.B., Judge, A., MacLachlan, I., Polisky, B., 2005. Potent and persistent in vivo anti-HBV activity of chemically modified siRNAs. Nat Biotechnol 23, 1002-1007.

Moschos, S.A., Jones, S.W., Perry, M.M., Williams, A.E., Erjefalt, J.S., Turner, J.J., Barnes, P.J., Sproat, B.S., Gait, M.J., Lindsay, M.A., 2007. Lung Delivery Studies Using siRNA Conjugated to TAT(4860) and Penetratin Reveal Peptide Induced Reduction in Gene Expression and Induction of Innate Immunity. Bioconjugate Chemistry 18, 1450-1459.

Nakamura, Y., Kogure, K., Futaki, S., Harashima, H., 2007. Octaarginine-modified multifunctional envelope-type nano device for siRNA. Journal of Controlled Release 119, 360-367.

Okamoto, Y., Watanabe, M., Miyatake, K., Morimoto, M., Shigemasa, Y., Minami, S., 2002. Effects of chitin/chitosan and their oligomers/monomers on migrations of fibroblasts and vascular endothelium. Biomaterials 23, 1975-1979.

Peer, D., Park, E.J., Morishita, Y., Carman, C.V., Shimaoka, M., 2008. Systemic leukocyte-directed siRNA delivery revealing cyclin D1 as an anti-inflammatory target. Science 319, 627-630.

Pirollo, K.F., Rait, A., Zhou, Q., Hwang, S.H., Dagata, J.A., Zon, G., Hogrefe, R.I., Palchik, G., Chang, E.H., 2007. Materializing the potential of small interfering RNA via a tumor-targeting nanodelivery system. Cancer Res 67, 2938-2943.

Potente, M., Fisslthaler, B., Busse, R., Fleming, I., 2003. 11,12-Epoxyeicosatrienoic acid-induced inhibition of FOXO factors promotes endothelial proliferation by down-regulating p27Kip1. J Biol Chem 278, 29619-29625.

Rahbek, U.L., Howard, K.A., Oupicky, D., Manickam, D.S., Dong, M., Nielsen, A.F., Hansen, T.B., Besenbacher, F., Kjems, J., 2008. Intracellular siRNA and precursor miRNA trafficking using bioresponsive copolypeptides. The Journal of Gene Medicine 10, 81-93.

Santel, A., Aleku, M., Keil, O., Endruschat, J., Esche, V., Durieux, B., Loffler, K., Fechtner, M., Rohl, T., Fisch, G., Dames, S., Arnold, W., Giese, K., Klippel, A., Kaufmann, J., 2006. RNA interference in the mouse vascular endothelium by systemic administration of siRNA-lipoplexes for cancer therapy. Gene Ther 13, 1360-1370.

Schiffelers, R.M., Woodle, M.C., Scaria, P., 2004. Pharmaceutical prospects for RNA interference. Pharm Res 21, 1-7.

Simeoni, F., Morris, M.C., Heitz, F., Divita, G., 2003. Insight into the mechanism of the peptide-based gene delivery system MPG: implications for delivery of siRNA into mammalian cells. Nucl. Acids Res. 31, 2717-2724.

Sonawane, N.D., Szoka, F.C., Jr., Verkman, A.S., 2003. Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine-DNA polyplexes. J Biol Chem 278, 44826-44831.

Soriano, P., Dijkstra, J., Legrand, A., Spanjer, H., Londos-Gagliardi, D., Roerdink, F., Scherphof, G., Nicolau, C., 1983. Targeted and nontargeted liposomes for in vivo transfer to rat liver cells of a plasmid containing the preproinsulin I gene. Proc Natl Acad Sci U S A 80, 7128-7131.

Soutschek, J., Akinc, A., Bramlage, B., Charisse, K., Constien, R., Donoghue, M., Elbashir, S., Geick, A., Hadwiger, P., Harborth, J., John, M., Kesavan, V., Lavine, G., Pandey, R.K., Racie, T., Rajeev, K.G., Rohl, I., Toudjarska, I., Wang, G., Wuschko, S., Bumcrot, D., Koteliansky, V., Limmer, S., Manoharan, M., Vornlocher, H.P., 2004. Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. Nature 432, 173-178.

Spagnou, S., Miller, A.D., Keller, M., 2004. Lipidic carriers of siRNA: differences in the formulation, cellular uptake, and delivery with plasmid DNA. Biochemistry 43, 13348-13356.

Stevenson, M., Ramos-Perez, V., Singh, S., Soliman, M., Preece, J.A., Briggs, S.S., Read, M.L., Seymour, L.W., 2008. Delivery of siRNA mediated by histidine-containing reducible polycations. Journal of Controlled Release 130, 46-56.

Tanaka, K., Kanazawa, T., Ogawa, T., Takashima, Y., Fukuda, T., Okada, H., Disulfide crosslinked stearoyl carrier peptides containing arginine and histidine enhance siRNA uptake and gene silencing. International Journal of Pharmaceutics In Press, Corrected Proof.

Torchilin, V.P., Levchenko, T.S., Rammohan, R., Volodina, N., Papahadjopoulos-Sternberg, B., D'Souza, G.G.M., 2003. Cell transfection in vitro and in vivo with nontoxic TAT peptide-liposome-DNA complexes. Proceedings of the National Academy of Sciences of the United States of America 100, 1972-1977.

Torchilin, V.P., Rammohan, R., Weissig, V., Levchenko, T.S., 2001. TAT peptide on the surface of liposomes affords their efficient intracellular delivery even at low temperature and in the presence of metabolic inhibitors. Proceedings of the National Academy of Sciences of the United States of America 98, 8786-8791.

Troussard, A.A., Mawji, N.M., Ong, C., Mui, A., St -Arnaud, R., Dedhar, S., 2003. Conditional knock-out of integrin-linked kinase demonstrates an essential role in protein kinase B/Akt activation. J Biol Chem 278, 22374-22378.

Urban-Klein, B., Werth, S., Abuharbeid, S., Czubayko, F., Aigner, A., 2005. RNAi-mediated gene-targeting through systemic application of polyethylenimine (PEI)-complexed siRNA in vivo. Gene Ther 12, 461-466.

Veldhoen, S., Laufer, S.D., Trampe, A., Restle, T., 2006. Cellular delivery of small interfering RNA by a non-covalently attached cell-penetrating peptide: quantitative analysis of uptake and biological effect. Nucl. Acids Res. 34, 6561-6573.

Villares, G.J., Zigler, M., Wang, H., Melnikova, V.O., Wu, H., Friedman, R., Leslie, M.C., Vivas-Mejia, P.E., Lopez-Berestein, G., Sood, A.K., Bar-Eli, M., 2008. Targeting melanoma growth and metastasis with systemic delivery of liposome-incorporated protease-activated receptor-1 small interfering RNA. Cancer Res 68, 9078-9086.

Wadia, J.S., Dowdy, S.F., 2005. Transmembrane delivery of protein and peptide drugs by TAT-mediated transduction in the treatment of cancer. Advanced Drug Delivery Reviews 57, 579-596.

Wadia, J.S., Stan, R.V., Dowdy, S.F., 2004. Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. Nat Med 10, 310-315.

Wang, Y.-H., Hou, Y.-W., Lee, H.-J., 2007. An intracellular delivery method for siRNA by an arginine-rich peptide. Journal of Biochemical and Biophysical Methods 70, 579-586.

Watanabe, K., Harada-Shiba, M., Suzuki, A., Gokuden, R., Kurihara, R., Sugao, Y., Mori, T., Katayama, Y., Niidome, T., 2009. In vivo siRNA delivery with dendritic poly(l-lysine) for the treatment of hypercholesterolemia. Molecular BioSystems 5, 1306-1310.

Won, Y.-W., Kim, H.A., Lee, M., Kim, Y.-H., 2010a. Reducible Poly(oligo-D-arginine) for Enhanced Gene Expression in Mouse Lung by Intratracheal Injection. Mol Ther 18, 734-742.

Won, Y.-W., Kim, J.-K., Cha, M.-J., Hwang, K.-C., Choi, D., Kim, Y.-H., 2010b. Prolongation and enhancement of the anti-apoptotic effects of PTD-Hsp27 fusion proteins using an injectable thermo-reversible gel in a rat myocardial infarction model. Journal of Controlled Release 144, 181-189.

Yano, J., Hirabayashi, K., Nakagawa, S., Yamaguchi, T., Nogawa, M., Kashimori, I., Naito, H., Kitagawa, H., Ishiyama, K., Ohgi, T., Irimura, T., 2004. Antitumor activity of small interfering RNA/cationic liposome complex in mouse models of cancer. Clin Cancer Res 10, 7721-7726.

Zhang, C., Tang, N., Liu, X., Liang, W., Xu, W., Torchilin, V.P., 2006. siRNA-containing liposomes modified with polyarginine effectively silence the targeted gene. Journal of Controlled Release 112, 229-239.

Zimmermann, T.S., Lee, A.C., Akinc, A., Bramlage, B., Bumcrot, D., Fedoruk, M.N., Harborth, J., Heyes, J.A., Jeffs, L.B., John, M., Judge, A.D., Lam, K., McClintock, K., Nechev, L.V., Palmer, L.R., Racie, T., Rohl, I., Seiffert, S., Shanmugam, S., Sood, V., Soutschek, J., Toudjarska, I., Wheat, A.J., Yaworski, E., Zedalis, W., Koteliansky, V., Manoharan, M., Vornlocher, H.P., MacLachlan, I., 2006. RNAi-mediated gene silencing in non-human primates. Nature 441, 111-114.