Breaking down the barriers: siRNA delivery and endosome escape

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Summary

RNA interference (RNAi)-based technologies offer an attractive strategy for the sequence-specific silencing of disease-causing genes. The application of small interfering (si)RNAs as potential therapeutic agents requires safe and effective methods for their delivery to the cytoplasm of the target cells and tissues. Recent studies have shown significant progress in the development of targeting reagents that facilitate the recognition of and siRNA delivery to specific cell types. However, most of these delivery approaches are not optimized to enable the intracellular trafficking of the siRNAs into the cytoplasm where they must associate with the RNA-induced silencing complex (RISC) to direct the cleavage of mRNAs bearing complementary binding sites. In particular, the trafficking of siRNAs from endosomes into the cytoplasm represents a major rate-limiting step for many delivery approaches. This Commentary focuses on novel strategies designed to enhance endosomal escape and thereby increase the efficacy of siRNA-mediated gene silencing.

Key words: RNA interference, Gene silencing, siRNA delivery, siRNA trafficking

Introduction

RNA interference (RNAi) is a highly conserved process of post-transcriptional gene silencing that is triggered by small, double-stranded (ds) regulatory RNA molecules (Fire et al., 1998; Zamore et al., 2000). In the RNAi pathway, endogenously expressed long dsRNAs, derived from either convergent transcription or hairpin-structured RNAs, are processively cleaved by the RNase III family member, Dicer, into short (~21-nt) duplexed RNAs, termed small interfering (si)RNAs (Fig. 1) (Okamura et al., 2008a; Okamura et al., 2008b). These siRNAs have a characteristic 19-nt duplexed region with 5′-terminal phosphate groups and 2-nt 3′ overhangs (Bernstein et al., 2001; Zamore et al., 2000). Dicer acts in concert with a dsRNA-binding protein – R2D2 in *Drosophila* and HIV-transactivating response RNA-binding protein (TRBP) in mammalian cells – which binds to the more thermodynamically stable end of the duplexed RNA. This interaction helps to determine the orientation of siRNA loading onto Argonaute 2 (Ago2), the catalytic core of the RNA-induced silencing complex (RISC) (Chendrimada et al., 2005; Gregory et al., 2005; Matranga et al., 2005; Tomari et al., 2004). Once assembled in RISC, the passenger strand of the siRNA duplex is cleaved and released, leaving the guide strand to direct the activated RISC to the complementary sequence in the target mRNA. This leads to the endonucleolytic cleavage of the target mRNA and the silencing of gene expression. The discovery by Tuschl and colleagues that the introduction of chemically synthesized siRNAs into mammalian cells efficiently induced sequence-specific inhibition of gene expression propelled RNAi-based silencing approaches to the forefront of modern molecular biological research (Elbashir et al., 2001). In addition, it became evident that harnessing these endogenous pathways could prove to be an effective mechanism for the targeted silencing of disease-causing genes.

Two principal approaches have been used to harness the RNAi machinery for the silencing of gene expression: treatment with synthetic siRNA molecules or the expression of short-hairpin (sh) RNAs that are processed intracellularly into active siRNAs (Fig. 1). This latter mechanism is based on the ability of Dicer to recognize hairpin-structured RNAs that resemble intermediates of the microRNA (miRNA) pathway (Dykxhoorn and Lieberman, 2005; Dykxhoorn and Lieberman, 2006). Although it is effective, the stable overexpression of shRNAs has, in some cases, been associated with cytotoxic effects resulting from outcompetition of endogenous molecules for limited amounts of RNAi pathway components (An et al., 2006; Grimm et al., 2006). In addition, toxicities (such as increased oncogenic potential) associated with the integration into the host genetic material of retroviral-based vectors, a commonly used approach for the stable delivery of shRNAs to many cell types, have raised concerns about the safety of these delivery systems (Noguchi, 2003). By contrast, chemically synthesized siRNAs can efficiently silence gene expression without altering the host genetic material. In addition, the delivery of siRNAs can be altered based on the specific clinical needs, and the treatment can be discontinued, as warranted, without long-term effects.

RNAi-based approaches have been used extensively for the targeted silencing of gene expression in a variety of contexts, including inhibition of pathogenic genes. However, the application of siRNAs as potential therapeutic silencing agents requires the development of reagents that can effectively direct siRNAs to the cytoplasm of the appropriate target cells in clinically relevant doses. To be effective, these approaches must be optimized at all stages of the delivery process, from target-cell recognition to uptake into RISC and mRNA cleavage. This Commentary focuses on recent efforts to optimize the intracellular trafficking of siRNAs.

In vivo delivery

Preclinical experiments have demonstrated the effectiveness of synthetic siRNAs as potent inhibitors of a variety of disease-associated genes. However, the physicochemical properties of siRNAs – including their small size, hydrophilicity, net negative charge and sensitivity to nuclease degradation – pose challenges

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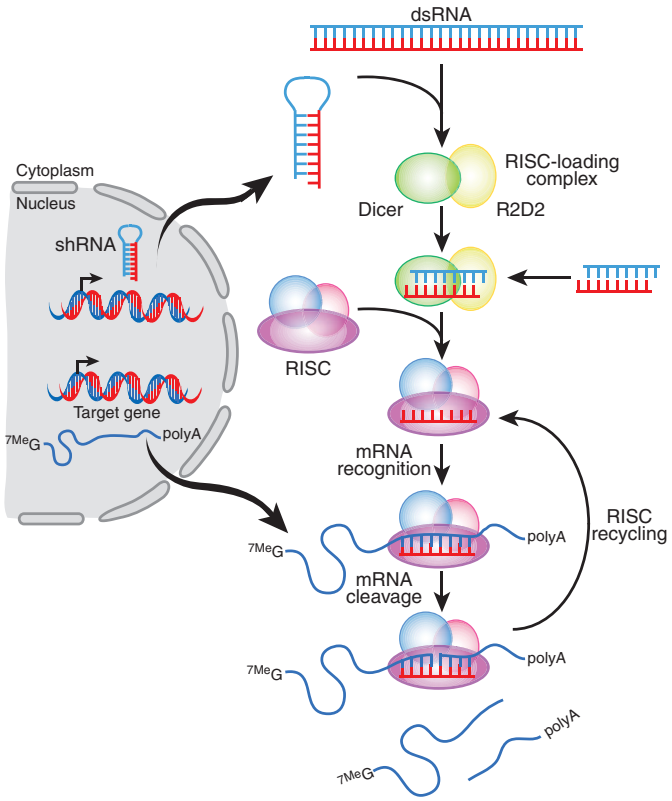

Fig. 1. RNAi-mediated gene silencing. At the center of the RNAi pathway are small siRNAs, the ~21-nt dsRNAs that facilitate the sequence-specific silencing of gene expression. siRNAs can be derived from longer precursor RNA molecules, such as endogenously expressed or exogenously added long dsRNAs and shRNAs expressed from DNA-based vectors. These long dsRNAs and shRNAs are processed in the cytoplasm by the RNase III family member Dicer in concert with the dsRNA-binding protein R2D2 into siRNAs. The resulting siRNAs containing the characteristic 19-nt duplexed region with 2-nt 3′ overhangs and 5′-terminal phosphate groups are taken up by the multi-subunit RNA-induced silencing complex (RISC). The duplexed siRNA bound to the Ago2 protein, the central component of RISC, is unwound and the passenger strand rapidly dissociates. Once unwound, the antisense RNA strand guides RISC to the complementary site in the target mRNA, which engages the endonucleolytic activity of Ago2, resulting in mRNA cleavage. The siRNA-loaded RISC is recycled for several rounds of mRNA cleavage.

for their delivery and uptake into cells, particularly following systemic administration. Therefore, the application of siRNAs as potential therapeutic agents requires delivery approaches that will enhance their pharmacological properties. These delivery approaches aim to: (1) increase the retention time of the siRNAs in the circulatory system by reducing the rate of renal clearance; (2) protect the siRNAs from serum nucleases; (3) ensure effective biodistribution; (4) facilitate targeting to and uptake of the siRNAs into the target cells; and (5) promote trafficking to the cytoplasm and uptake into RISC.

A variety of approaches have been developed that promote siRNA delivery in vivo, including cationic nanoparticles (Morrissey et al., 2005), lipids and liposomes (Akinc et al., 2008; Wu and McMillan, 2009), antibody (Ab)-fusion molecules [Ab-protamine (Peer et al., 2007; Song et al., 2005) and Ab-poly-arginine (Kumar et al., 2008)], as well as cholesterol- (Soutschek et al., 2004; Zimmermann et al., 2006) and aptamer-conjugated siRNAs (Dassie et al., 2009; McNamara et al., 2006; Zhou et al., 2008) (Fig. 2). On their own, siRNAs fall below the size threshold for renal filtration and are rapidly cleared from the circulatory system. Complexes of siRNAs and the various delivery reagents remain in the circulation for longer, either because they exceed the size cut-off for renal clearance or because the delivery agents promote association with serum proteins (e.g. serum albumin). In addition, the encapsidation of the siRNAs into nanoparticles (using either lipid- or cationic-polymer-based systems) helps to shield them from serum nucleases. Ab-fusion molecules have been used to effectively deliver naked, unmodified siRNAs to specific cell types following intravenous injection (Kumar et al., 2008; Peer et al., 2007; Song et al., 2005). Although the siRNAs are thought to be exposed on the surface of these recombinant Ab-fusion molecules, they were effectively delivered to the target cells, suggesting that complexation with these molecules provides some protection from nucleolytic degradation. The incorporation of chemical modifications to the phosphate backbone, the sugar moiety and the nucleoside bases of the siRNA increases its resistance to degradation by serum nucleases (Behlke, 2008). As some of these modifications are detrimental to the silencing efficacy, however, a balance must be maintained between the incorporation of chemical modifications and the inhibitory activity of the siRNAs (Watts et al., 2008).

An attractive strategy for decreasing the dosage of the siRNAs needed to achieve effective silencing and minimizing off-target silencing in bystander cells (see below) is the use of delivery agents that target the siRNAs to specific cell types and tissues. This has been achieved using Abs or ligands that are fused to highly positively charged peptides or proteins, with which the siRNAs can associate by electrostatic interactions, or by directly conjugating aptamers or ligands to the siRNA. These reagents (Abs, ligands and aptamers) can bind with high affinity to cell-surface molecules and deliver the siRNA specifically to cells expressing these markers. By combining these targeting reagents with nanoparticles (e.g. immunoliposomes containing lipid nanoparticles coated with specific Abs), the quantity of siRNAs delivered and, as a consequence, the efficacy of silencing can be increased (Peer et al., 2008).

Intracellular trafficking of siRNAs

The plasma membrane is a significant barrier for siRNA uptake. Despite their small size, the hydrophilicity and negative charge of siRNA molecules prevent them from readily crossing biological membranes. Therefore, effective siRNA delivery approaches need to overcome this limitation by facilitating cellular uptake. The electrostatic complexation of siRNA molecules with cationic lipids and polymers helps to mask their net negative charge. The net positive charge of these nanoparticles facilitates binding to negatively charged cell membranes following internalization via adsorptive pinocytosis (Zabner et al., 1995). Alternatively, siRNAs can be delivered to specific cell types through the direct conjugation to or association with molecules, including Abs, ligands and aptamers, that recognize specific antigens on the surface of target cells. Upon binding to the cell, these cell-type-specific delivery reagents and the associated siRNAs are taken up by receptor-mediated endocytosis and deposited into endosomes.

Whether they are delivered by cationic lipids, nanoparticles or cell-type-specific delivery reagents, the intracellular trafficking of siRNAs begins in early endosomal vesicles (Fig. 3). These early endosomes subsequently fuse with sorting endosomes, which in turn transfer their contents to the late endosomes. Late endosomal vesicles are acidified (pH 5-6) by membrane-bound proton-pump

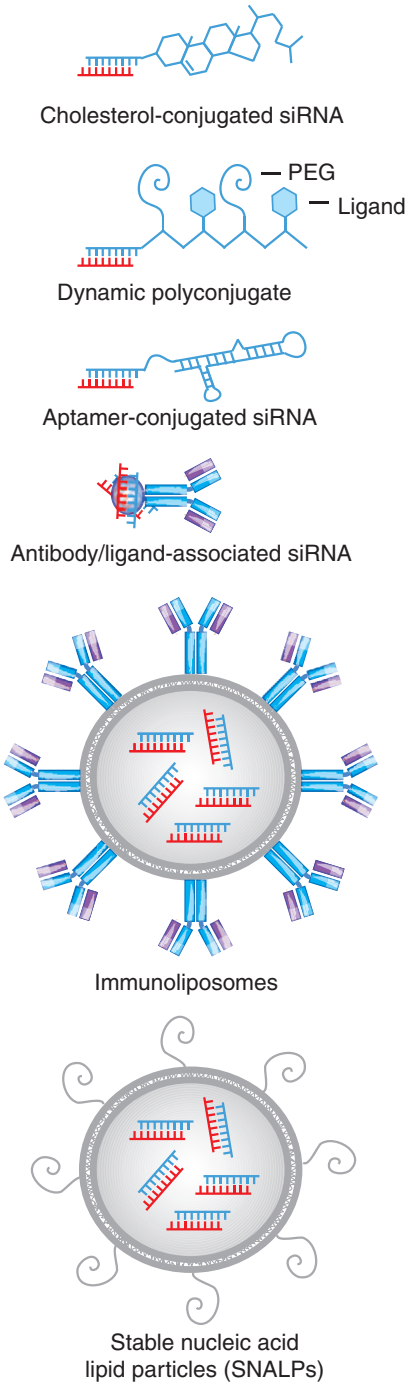

Fig. 2. Strategies for the delivery of siRNA in vivo. A variety of approaches have been used for siRNA delivery: these include the direct conjugation of the siRNA to targeting molecules, such as aptamers, ligands (e.g. cholesterol moiety) or polymers (e.g. dynamic polyconjugates); the association of the negatively charged siRNAs with a positively charged peptide or protein linked to an Ab or ligand; or the encapsulation of the siRNA into lipid or polymer nanoparticles.

ATPases. The endosomal content is then relocated to the lysosomes, which are further acidified (pH ~4.5) and contain various nucleases that promote the degradation of the siRNAs. To avoid lysosomal degradation, siRNAs (free or complexed with the carrier) must escape from the endosome into the cytosol, where they can associate

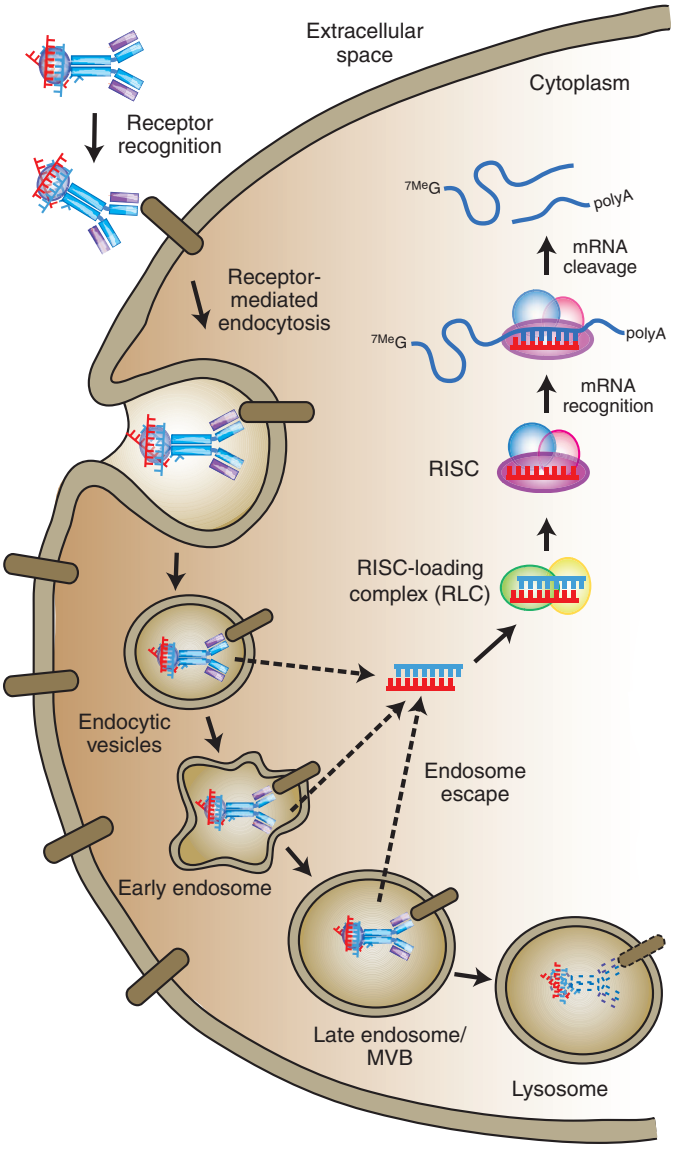

Fig. 3. Uptake and intracellular trafficking of a targeted siRNA delivery vehicle. An Ab-protein fusion molecule and its associated siRNA interacts with cell-surface receptors and is taken up into the target cells by receptor-mediated endocytosis. These endocytic vesicles then fuse to form the early endosome. The contents of the early endosome are trafficked to the late endosome/multivesicular body (MVB) en route to the lysosome, which is the site of hydrolysis of proteins and nucleic acids. To direct target-gene silencing, the siRNAs need to escape from the endosome into the cytoplasm, where they associate with the RNAi machinery and direct the cleavage of the complementary mRNA (see Fig. 1).

with the RNAi machinery. Endosomal escape is a major barrier for efficient siRNA delivery. In the following sections, we focus on strategies that have been developed to promote endosomal release, including the use of fusogenic lipids, polymers with high buffering capacity and membrane-interacting peptides.

Strategies for endosomal escape

Fusogenic lipids

When dispersed in water, most bilayer-forming lipids readily adopt the lamellar phase, in which lipid molecules are organized in

extended bilayer sheets surrounded by an aqueous environment. However, there is a class of lipids (e.g. phosphatidylethanolamines or PEs) that assembles in the inverse hexagonal phase, wherein the lipid molecules adopt long, cylindrical, inverted micelle-like structures to form non-bilayer assemblies under physiological conditions (Koltover et al., 1998; Litzinger and Huang, 1992). Unilamellar lipid vesicles tend to adhere to the surface of anionic vesicles without inducing a fusion event. However, lipoplexes (complexes containing cationic lipids and nucleic acids) that adopt an inverted hexagonal phase attach themselves to and rapidly fuse with anionic membranes, resulting in the release of their contents into the cytoplasm (Koltover et al., 1998). The incorporation of these so-called fusogenic lipids, such as dioleoylphosphatidyl-ethanolamine (DOPE), into a lipoplex has been shown to promote endosomal release by increasing the interactions between the liposomal and endosomal membranes (Farhood et al., 1995; Heyes et al., 2005; Litzinger and Huang, 1992). One of the conditions inducing the transition from the lamellar to the fusogenic (inverted hexagonal) phase is unsaturation of the hydrocarbon chains in PE lipid molecules (Heyes et al., 2005; Litzinger and Huang, 1992). MacLachlan and colleagues compared the fusogenicity of lipid nanoparticles on the basis of their enrichment in lipids with different numbers of double bonds in the hydrophobic tails (Heyes et al., 2005). They found that lipoplexes containing unsaturated cationic lipids enhanced luciferase gene knockdown in Neuro2A-G cells that stably express luciferase, compared with those containing saturated cationic lipids. This improvement in efficiency was directly correlated with the extent of unsaturation of the cationic lipid hydrophobic domain and, as a consequence, the fusogenicity of the delivery system. Similarly, Hassani and co-workers incorporated fusogenic lipids with the commercially available lipid-based transfection reagent JetSI^{TM} to increase the efficacy of siRNA delivery (Hassani et al., 2005). By combining JetSI^{TM}, a mixture of cationic lipids specifically prepared for siRNA delivery in vitro, with the fusogenic lipid DOPE, they significantly inhibited the expression of a cotransfected luciferase gene using subpicomolar amounts of siRNA.

pH-sensitive lipoplexes

Lipid-based siRNA delivery vectors face several barriers in vivo when transporting their nucleic acid cargo to target cells. One such barrier is the maintenance of lipoplex stability in the bloodstream. A variety of liposome modifications have been developed to protect positively charged fusogenic liposomes from interactions with serum proteins and macrophages while maintaining the fusogenic character necessary for efficient endosomal escape and intracellular trafficking of the siRNAs. For example, Guo and colleagues designed a biocompatible liposome that is sensitive to small changes in pH (Guo et al., 2003; Guo and Szoka, 2001). They synthesized a hydrophilic, pH-sensitive polyethylene glycol-diortho ester-distearoyl glycerol (POD) polymer, which they used to modify the outer surface of DOPE vesicles. The polymer-liposome conjugate showed good stability in serum, but when the pH decreased, most POD polymer molecules were rapidly hydrolyzed, promoting the aggregation of PE-enriched lipid vesicles and vesicle fusion. Similarly, Thompson and co-workers developed a polyethyleneglycol (PEG)-based cleavable polymeric system containing nonlamellar highly fusogenic PE lipid nanoparticles stabilized by surface-exposed PEG-conjugated vinyl ether lipids (Shin et al., 2003). Hydrolysis of the vinyl ether bond at low pH caused the removal of the PEG polymer and destabilization of the liposomes, allowing their fusion with endosomal membranes.

In another study, efficient delivery of siRNAs to HeLa cells and human umbilical vein endothelial cells (HUVECs) was achieved using pH-sensitive liposomes coated with a cationic block polymer composed of PEG and a polycation polymer (Auguste et al., 2008). Coating the liposome surface with the PEG-b-polycation polymer protected the liposomes from recognition by the immune system, and prevented their degradation in serum. Upon endosomal acidification, the PEG-b-polycation polymer was released from the liposome surface, allowing the cationic liposome to fuse with the anionic endosomal membrane and release its contents into the cytoplasm. When compared with a commercially available transfection reagent (Oligofectamine^{TM}), siRNAs delivered in PEG-b-polycation-coated liposomes more efficiently silenced GFP expression in HeLa cells stably expressing GFP, as well as GAPDH expression in HUVECs.

In a similar manner, Carmona and colleagues developed liposomes composed of DOPE lipid and an aminoxy cholesteryl lipid, which enables attachment of biocompatible polymers, such as PEG (Carmona et al., 2009). This bioconjugation gives rise to oxime bonds, which are stable at neutral pH, but hydrolyzable at pH 5.5 and lower. The pH sensitivity of the siRNA carrier is needed for the PEG polymer desorption that leads to nanoparticle destabilization and, consequently, escape from the early endosome. The delivery of siRNAs targeting hepatitis B virus sequences with lipid nanoparticles resulted in significant suppression of viral replication in mouse hepatocytes. In fact, siRNA-mediated silencing of HBV replication using these lipid carriers more effectively inhibited viral proliferation than treatment with lamivudine, a licensed HBV drug (Carmona et al., 2009).

pH-sensitive polyplexes

A number of pH-sensitive, endosomal buffering polymers have been developed as nonviral vectors to improve the efficiency of siRNA delivery. The high efficiency of nucleic acid delivery by protonable cationic polymers has been mainly attributed to their high buffering capacity, which has been hypothesized to mediate endosomal release by acting as ‘proton sponges’. In this approach, proton absorbance by buffering polymers prevents acidification of endosomal vesicles, thereby increasing the ATPase-mediated influx of protons and counter ions (which enter the vesicles to balance the proton flux). Increased counter ion concentrations inside the endosome lead to osmotic swelling, endosomal membrane rupture, and the eventual leakage of the polyplex (cationic polymer complexed with nucleic acid) into the cytosol (Sonawane et al., 2003). Polyethyleneimine (PEI) is one of the most widely used cationic polymers for nucleic acid delivery (Read et al., 2005; Richards Grayson et al., 2006; Urban-Klein et al., 2005). The positively charged amine groups of the polymers facilitate their interaction with negatively charged nucleic acid molecules, and also enable endosomal release through their action as proton sponges. However, treatment with PEI can have toxic side effects that are directly related to the polymer size, which has impeded its application for therapeutic drug delivery.

Wang and co-workers designed a series of biodegradable amphiphilic polymers with tunable pH sensitivity. These systems relied on structural changes to the polymer that occur at low endosomal pH to initiate disruption of the endosomal membrane. A multifunctional polymeric siRNA carrier developed by this group [1,4,7-triazanonylimino-bis[N-(oleicyl-cysteinyl-histinyl-1-

aminoethyl)propionamide] (THCO)] was composed of a proton-sponge domain, a hydrophobic domain and cysteine residues capable of polymerization (Wang et al., 2008). The presence of various protonable amino groups with various pK_as permitted the complexing of siRNA molecules and provided a high buffering capacity. These nanoparticles showed pH-sensitive membrane-disruption activity under acidic conditions. The hydrophobic portion of the particle improved the stability of the carrier by forming a compact structure with the nucleic acids embedded within it. In addition, reduction of disulfide bonds by cytosolic glutathione resulted in dissociation of the nanoparticles and siRNA release into the cytoplasm. The surface of the nanoparticles could also be modified with various molecules (e.g. PEG). The high efficiency of siRNA delivery did not diminish when the nanoparticles were coated with PEG. In fact, PEGylated nanoparticles were more efficient for siRNA delivery and siRNA-mediated silencing than commercially available transfection reagents. The authors of this study went further, by improving the pharmacokinetic properties of the pH-sensitive nanoparticles for siRNA delivery. Recently, they developed a multifunctional carrier [(1-aminoethyl)imino-bis[N-(oleicyl-cysteinyl-histynyl-1-aminoethyl)propionamide (EHCO)] for targeted delivery of therapeutic siRNA to tumor cells (Wang et al., 2009a; Wang et al., 2009b). The EHCO polymer contains a variety of protonable amino groups that act as proton sponges. The presence of thiol groups in EHCO allows facile attachment of various ligands. To that end, the authors modified the nanoparticles with a PEG-bombesin peptide as a target-recognition component. The addition of bombesin, a tumor-specific peptide, improved siRNA delivery to cancer cells that overexpressed the bombesin receptor. Systemic administration of therapeutic siRNAs that target hypoxia-inducible factor 1α (HIF1α) using these nanoparticles resulted in significant inhibition of tumor growth in nude mice implanted with human glioma U87 cells (Wang et al., 2009b).

The proton-absorbing properties of various polyamidoamine dendrimers, which are symmetrically and repeatedly branched polymers, have also been used for siRNA delivery (Patil et al., 2008; Patil et al., 2009). Polyethyleneimine and polyamidoamine dendrimers show high transfection efficiencies owing to their excellent buffering capacity, which is attributable to the high concentration of tertiary amine groups in these molecules. The neutral surface of internally cationic polyamidoamine dendrimers has a significant role in controlling the morphology of the polyplex and helps to protect negatively charged siRNA molecules by condensing them inside the dendrimer particle. A lesser degree of quaternization of amine groups in the dendrimer has been shown to improve siRNA delivery to cells, although it did not significantly increase gene silencing (Patil et al., 2009). The presence of dense peripheral groups also facilitates the attachment of target-recognition elements. For example, modification of the dendrimer with luteinizing hormone-releasing hormone (LHRH) peptide, which targets LHRH receptors expressed on the plasma membrane of many types of cancer cells, improved the cellular uptake of the particles. This method of siRNA delivery was well tolerated by the cells and resulted in a substantial decrease in target gene expression (Patil et al., 2009).

In addition, a pH-responsive siRNA carrier that contains a diblock copolymer composed of a positively charged interior and an ampholytic exterior has been developed. The positively charged block, which is composed of dimethylaminoethyl methacrylate (DMAEMA), facilitates siRNA condensation, whereas the ampholytic block, composed of DMAEMA, propylacrylic acid (PAA) and butyl methacrylate (BMA), enables endosomal escape (Convertine et al., 2009). The presence of the hydrophobic BMA domain in this polyplex enhances intracellular siRNA delivery, leading to a significant siRNA-mediated knockdown of *GAPDH* in HeLa cells.

Fusogenic proteins

As an alternative strategy, short peptides that are capable of translocating throughout biological membranes – so-called cell penetrating peptides (CPPs) – have been used to modify lipoplexes or polyplexes to improve endosomal release of siRNAs (Endoh and Ohtsuki, 2009). Harashima and coworkers constructed a multifunctional envelope-type nano device (MEND) for the efficient delivery of nucleic acids to cells (Hatakeyama et al., 2007; Kogure et al., 2007; Kogure et al., 2004). MEND consists of nucleic acid molecules entrapped inside a liposome that has been enriched with fusogenic lipids. To improve targeting and the efficiency of siRNA delivery, the surface of MEND was modified with PEG, targeting ligands and protein transduction domain peptides. More recently, this siRNA carrier has been improved by incorporating a fusogenic PEG-peptide-DOPE (PPD) construct and a pH-sensitive fusogenic GALA peptide (Hatakeyama et al., 2009). The peptide sequence in PPD constructs can be cleaved by matrix metalloproteinases that are specifically secreted by cancer cells, enhancing the delivery of siRNA complexed with this carrier to tumor cells (Hatakeyama et al., 2007). Modification of MEND with both a PPD construct and a fusogenic GALA peptide resulted in a highly efficient siRNA-mediated gene knockdown of luciferase expression in the human fibrosarcoma cell line HT1080 stably expressing luciferase (HT1080-luc) and in HT1080-luc tumor cells subcutaneously implanted into immunocompromised mice.

Another membrane-destabilization mechanism that has been extensively studied for gene-delivery purposes takes advantage of the pore-forming ability of viroporins. The main action of these highly hydrophobic proteins is to create channels and facilitate ion flow across biological membranes (Gonzalez and Carrasco, 2003). For example, peptides derived from the endodomain of the HIV gp41 envelope glycoprotein (sequence corresponding to residues 783-806 of gp160) form pores in the cell membrane by adopting an amphipathic α-helical structure (Costin et al., 2007). Modification of a cationic polymer, PEI, with a lytic peptide from the endodomain of HIV gp41 (HGP) showed enhanced intracellular trafficking of carriers composed of PEI and HGP (Kwon et al., 2008). The ability of HGP to enhance PEI-mediated siRNA delivery was gauged in HeLa cells. siRNA delivered with PEI-HGP-based carriers were significantly more efficient at silencing GAPDH expression compared with siRNA delivered via unmodified polyplexes. Furthermore, confocal microscopy imaging supported the hypothesis that the addition of HGP improves the endosomal escape of the delivery vehicle.

Finally, influenza-derived fusogenic peptide diINF-7 has also been shown to enhance endosomal release of siRNA carriers in a study that tested siRNA-mediated silencing of the genes encoding the epidermal growth factor receptor (EGFR) and K-Ras oncogenes (Oliveira et al., 2007b). The addition of diINF-7 fusogenic peptide to a commercially available transfection reagent, Lipofectamine™, strongly promoted the escape of siRNA from endosomes, resulting in a greater than twofold increase in the knockdown of EGFR expression in human epidermoid carcinoma A431 cells compared with Lipofectamine™ alone. Similarly, a 3.5-fold increase in knockdown of the K-Ras oncogene was observed in C26 murine

colon carcinoma cells in the presence of the diINF-7 peptide Lipofectamine-based carrier.

Photosensitive molecules

Photochemical internalization (PCI) technology has also been used to enhance the release of endocytosed macromolecules to the cytosol (Berg et al., 1999). This technology is based on the activation of endocytosed photosensitive molecules (photosensitizers) by light to induce the release of endocytic vesicle contents before they are transferred to the lysosome. Photosensitizers are amphiphilic componds that, when exposed to light, become excited and stimulate the formation of reactive oxygen species, primarily singlet oxygen (Pass, 1993). Because singlet oxygen has a very short life and limited range of destruction, it damages the endosomal membrane allowing release of its therapeutic contents without affecting the cellular organelles (Moan and Berg, 1991). PCI is also site-specific, because it is limited to the illumination area only. Efficient light-directed delivery of siRNA to the cytosol has been shown by Oliveira and colleagues. The authors used PCI to facilitate endosomal escape of siRNA targeting *EGFR* (Oliveira et al., 2007a). Addition of a photosensitizer [meso-tetraphenylporphine with two sulfonate groups on adjacent phenyl rings ($TPPS_{2a}$)] to Lipofectamine™ increased the silencing efficiency tenfold compared with this transfection reagent alone.

Perspectives

As RNAi-based gene-silencing approaches rapidly make their way into the clinic, there are many issues to consider with respect to siRNA delivery. The accessibility of the target organ influences the delivery mechanism used; siRNA-based therapies that target easily accessible organs and tissues (e.g. eye, airway and lungs) have made the most rapid progress. However, the application of therapeutic RNAi strategies for the targeted silencing of gene expression to most tissues and organs requires systemic delivery approaches. Significant progress has been made in the development of cell-type-specific delivery approaches that reduce the dose of siRNA needed for effective silencing and the potential for off-target gene silencing in bystander cells and tissues.

However, the targeted delivery of siRNAs to the cell types and tissues of interest is only one step in the delivery process. Once the siRNAs arrive at the target cell(s), they must be trafficked into the cytoplasm and taken up by RISC to exert their silencing function. Every step in this process requires optimization to maximize silencing potential while minimizing cost and the potential for off-target effects. These off-target effects include the unintentional targeting of mRNAs bearing limited complementarity to the siRNA and siRNA sequence motifs that stimulate aspects of the innate immune response through engagement of Toll-like receptors (TLRs) (reviewed in Behlke, 2008; Robbins et al., 2009; Ui-Tei et al., 2008). Escape from the endosome represents one of the key rate-limiting steps in the delivery process. We have discussed here several approaches to enhance the endosomal release of siRNAs, including fusogenic lipids, fusogenic peptides and pH-sensitive lipoplexes and polyplexes. Regardless of the approach, an ideal siRNA-delivery strategy should balance the stability needed to maintain the integrity of the delivery reagent in the circulatory system with the need to effectively enter the target cell and gain access to the cytoplasm. This goal has encouraged the development of increasingly sophisticated, multifunctional delivery vehicles that incorporate cell-targeting reagents as well as molecules that promote endosomal escape. This is aptly demonstrated by the dynamic polyconjugate system developed by Rozema and colleagues, with which they achieved siRNA-mediated silencing specifically in hepatocytes in vivo (Rozema et al., 2007) (Fig. 2). They developed multifunctional polymer-containing components designed to maximize each step in the delivery process – from target cell recognition (by using the targeting ligand N-acetylgalactosamine) to endosomal escape [by using the amphipatic poly(vinyl ether), PBAVE, which acts as an endosomolytic agent] and silencing (by using the appropriate siRNA). This system is referred to as a dynamic polyconjugate because each component is revealed as needed. The N-acetylgalactosamine is present for delivery of the siRNA to hepatocytes but is released from the polymer in the acidic environment of the endosome. At the same time, the endosomolytic agent (PBAVE) is revealed through the dissociation of a PEG-shielding group, promoting endosomal release of the conjugated siRNA. The siRNA molecules are attached to PBAVE through a disulfide linkage, which can be easily cleaved in the reductive environment of the cytosol, freeing the siRNA to interact with RISC and direct cleavage of the target mRNA. These types of siRNA delivery vehicles will become more important as RNAi-based silencing approaches move closer to being a clinical reality.

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