

Nanotechnology for *In vivo* Targeted siRNA Delivery

James E. Dahlman^{*,§,||,a}, Kevin J. Kauffman^{||,||,a}, Robert Langer^{*,§,¶,||},
Daniel G. Anderson^{*,§,¶,||,1}

^{*}The Institute for Medical Engineering and Science, Massachusetts Institute of Technology, Cambridge, MA, USA

[§]Harvard-MIT Division of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, MA, USA

[¶]Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA, USA

^{||}David H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA, USA

¹Corresponding author: E-mail: dgander@mit.edu

Contents

| | |
|------------------------------------------------------|----|
| 1. RNA Potently Modifies Gene Expression | 38 |
| 2. Targeting Strategies | 40 |
| 2.1 Passive Targeting | 40 |
| 2.2 Active Targeting | 42 |
| 3. Targeting the Liver | 46 |
| 3.1 Liver Physiology Promotes Targeting | 46 |
| 3.2 Passive Liver Targeting | 46 |
| 3.2.1 Lipidoid LNPs | 48 |
| 3.2.2 Amino Lipid LNPs | 50 |
| 3.2.3 Endocytosis of Lipidoid and Amino Lipid LNPs | 53 |
| 3.3 Active Liver Targeting | 53 |
| 4. Targeting Primary Tumors and Metastasis | 56 |
| 4.1 Tumor Physiology can Promote or Inhibit Delivery | 56 |
| 4.2 Active Tumor Targeting | 58 |
| 5. Endothelial Cell Targeting | 60 |
| 6. Future Perspectives | 62 |
| References | 64 |

Abstract

Small interfering RNAs (siRNAs) can specifically inhibit gene expression. As a result, they have tremendous scientific and clinical potential. However, the use of these molecules in patients and animal models has been limited by challenges with delivery. Intracellular RNA delivery is difficult; it requires a system that protects the siRNA from degradative nucleases in the bloodstream, minimizes clearance by the reticuloendothelial

^a These authors contributed equally to this work.

system, maximizes delivery to the target tissue, and promotes entry into, and out of, an endocytic vesicle. Despite these barriers, recent data suggest that RNA may be targeted to cells of interest *in vivo*. Herein we outline strategies for targeted siRNA delivery, and describe how these strategies may be improved.



1. RNA POTENTLY MODIFIES GENE EXPRESSION

Once primarily viewed as an intermediary between DNA and protein, RNA is now known to actively regulate gene expression by interacting with DNA, other RNAs, and proteins (Rinn & Chang, 2012; Sabin, Delás, & Hannon, 2013; Ulitsky & Bartel, 2013). Because many of these regulatory functions are dictated by sequence-specific interactions between the RNA sequence and its target, RNAs can precisely modify gene expression and downstream cellular behavior. One well-known example of RNA-mediated gene regulation is RNA interference (RNAi), an endogenous mechanism that reduces protein expression by inhibiting translation of mRNA (Bumcrot, Manoharan, Koteliansky, & Sah, 2006). RNAi is induced by short interfering RNAs (siRNAs) and microRNAs (miRNAs). These small RNAs, which can be introduced into the cytoplasm endogenously by transcription or exogenously through transfection, discourage translation by guiding a protein complex called RISC (RNA-induced silencing complex) to a complementary sequence on the target mRNA (Rana, 2007). While the RNAi pathway has been studied closely for over 10 years, more recent evidence suggests that RNA–RNA interactions can regulate genes through non-RNAi mechanisms. For example, circular RNAs (circRNAs) can sequester miRNAs by binding to them in the cytoplasm (Jeck & Sharpless, 2014).

RNAs can also interact directly with DNA and protein; as a result, RNA-mediated gene regulation does not require RNA–RNA interactions (Sabin et al., 2013). Long noncoding RNAs (lncRNAs) can affect genomic stability by concurrently interacting with DNA and protein complexes that modify the epigenetic state of the cell. For example, an lncRNA named Tsix binds PRC2, a protein complex that modifies histones; another domain of the lncRNA binds to the target DNA, thereby “silencing” DNA expression by recruiting PRC2 to modify chromatin (Sabin et al., 2013). Similarly, RNAs derived from bacterial clustered regularly interspaced palindromic repeats (CRISPRs) can bind to Cas9, a nuclease that induces a double stranded cut in DNA (Sander & Joung, 2014). Once bound to Cas9, the RNA guides the nuclease to a complementary DNA sequence. The result is targeted genomic modification mediated by the DNA–RNA–protein

complex. More simply, RNAs can simultaneously bind two separate proteins and bring them together to activate downstream signaling. These, and other mechanisms reviewed elsewhere, provide strong evidence that RNAs play a fundamental role in cellular function (Rinn & Chang, 2012; Sabin et al., 2013; Ulitsky & Bartel, 2013).

As biologists continue to uncover RNAs that promote health and disease, the number of clinical applications requiring therapeutic RNA delivery will expand. However, to date, effective therapeutic RNA delivery has been limited to siRNAs targeted to hepatocytes of the liver (Kanasty, Dorkin, Vegas, & Anderson, 2013). Therapeutic siRNA delivery has reduced pathological protein in patients with liver diseases including TTR-amyloidosis and familial hypercholesterolemia (Coelho et al., 2013; Fitzgerald et al., 2014). One study showed that nanoparticle-mediated delivery of siRNA targeting TTR reduced serum TTR in humans by nearly 90% after a systemic injection (Coelho et al., 2013). A related formulation reduced low density lipoprotein (LDL) by 57% for one individual in the trial after silencing PCSK9, a gene involved in lipid transport (Fitzgerald et al., 2014). Additional clinical trials that use the same delivery vehicles are planned for other liver diseases, since the biophysical characteristics of the nanoparticles used in these studies do not change with the siRNA sequence. This effect is also illustrated by broad application of the liver-targeting nanoparticle C12-200, which is currently being evaluated for clinical use (Love et al., 2010).

While these nanoparticles convincingly demonstrate that siRNA can affect liver disease in mice, nonhuman primates, and humans, significant needs in the RNA delivery field remain unmet. Most notably, highly efficient delivery to cells outside the liver, and the delivery of longer RNAs to any tissue has remained challenging (Dahlman et al., 2014; Kanasty et al., 2013). Highly efficient *in vivo* delivery requires the material to perform several difficult functions. Without eliciting an unwanted immune response, the material must locate and transfect the target cell in a highly complex and heterogeneous microenvironment (Whitehead, Dahlman, Langer, & Anderson, 2011; Whitehead, Langer, & Anderson, 2009). This requires that the material maximizes interactions with the cell of interest while minimizing similar interactions with nontarget cells and the reticuloendothelial system. A substantial amount of material is typically lost through these unwanted interactions, most notably those interactions with the kidney, liver, and immune system. If the RNA avoids these tissues and reaches the cell of interest, it must get both into and out of an endosome. Even this endocytotic process is inefficient; only 1–2% of siRNA endocytosed by

hepatocytes *in vivo* eventually reached the cytoplasm (Gilleron et al., 2013). The rest of the material was degraded or recycled out of the cell.

The potential for targeted drug delivery vehicles to address important clinical problems has inspired many labs to design nanomaterials for targeted siRNA delivery. For the remainder of this publication, we define targeted delivery systems as those that preferentially transfect certain cells after administration *in vivo*. Delivery can be achieved by active mechanisms (e.g., targeting ligands) or passive mechanisms (e.g., modifying biophysical nanoparticle characteristics like size and charge) (Figure 3.1). As described below, specific strategies within these subsets, each with their own advantages and disadvantages, can be applied to improve siRNA delivery *in vivo*.



2. TARGETING STRATEGIES

2.1 Passive Targeting

As soon as a nanoparticle is injected *in vivo*, it interacts with its environment. If a particle is injected intravenously, the system is initially exposed to blood and endothelial cells that line the vasculature. By contrast, subcutaneous injection exposes the material to the local microenvironment, lymphatic system, and capillary beds near the injection site. These immediate local interactions, and those experienced by the particle as it is transported around the body, can influence where the material is delivered, how well it is delivered, and whether the system induces an unwanted immune response. Put more directly, there is increasing evidence that interactions between particles and the natural physiology of the body can have a substantial effect on the pharmacokinetic profile of the delivery system (Akinc et al., 2010; Monopoli, Aberg, Salvati, & Dawson, 2012; Tenzer et al., 2013).

Enabling natural physiology to passively target siRNA *in vivo* is a promising therapeutic strategy for two reasons. First, passive targeting systems do not contain extraneous active targeting ligands like antibodies, aptamers, or small molecules. This may simplify the synthesis, formulation, and characterization of the delivery system, and thereby reduce batch-to-batch variability. Second, there are many well-characterized differences in physiology that can be exploited for RNA delivery. One such example is the differential structure and function of endothelial cells that line blood vessels throughout the body (Aird, 2006, 2007). Endothelial cells were once considered passive conduits for oxygen and nutrients, but are now known to actively modify metabolism, the immune response, endocytosis, and inflammation by secreting factors and expressing cell-surface receptors (Hagberg

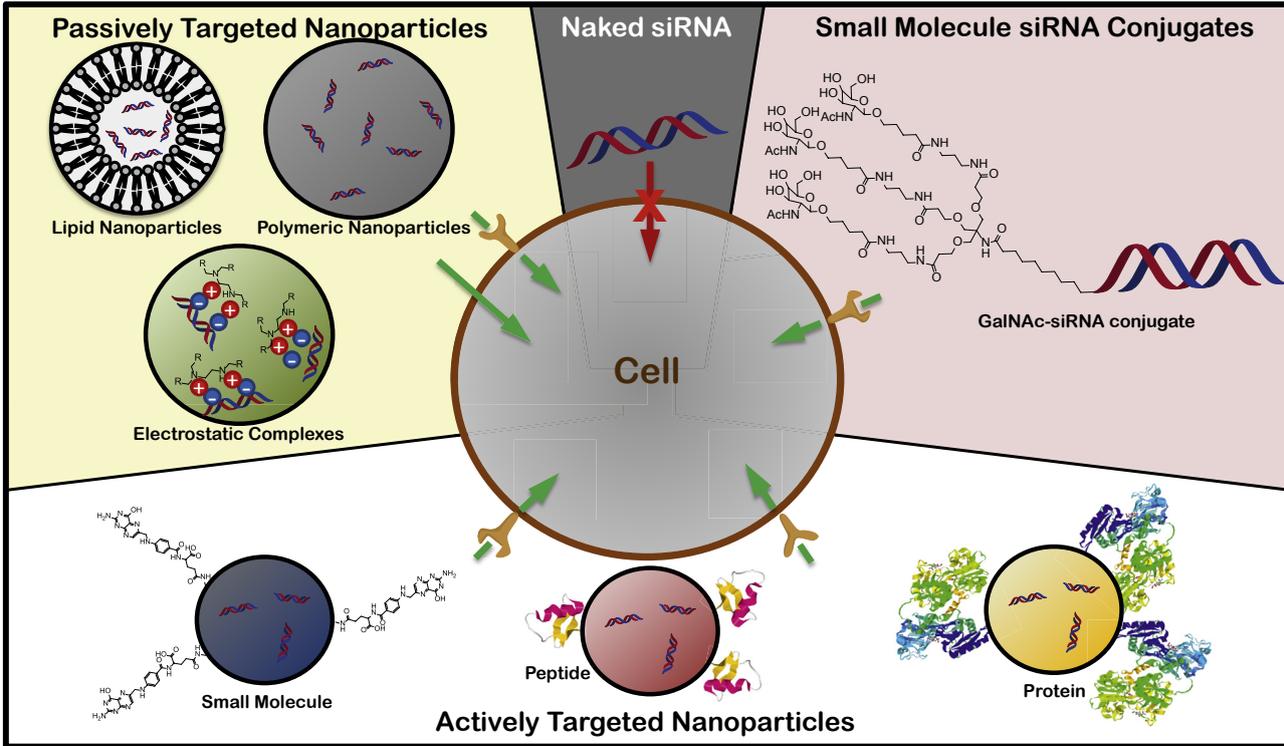


Figure 3.1 Strategies to improve siRNA delivery. siRNAs, which are large, hydrophilic, and anionic, cannot easily cross the cell membrane by themselves. siRNA delivery can be improved with nanoparticles or conjugates that actively target ligands on the outside of the cell, or by nanoparticles that use natural interactions with the body (e.g., serum proteins) to passively target the cell of interest. (See the color plate.)

et al., 2012, 2010; Pober & Sessa, 2007). In addition to playing a critical role in health and disease, endothelial cells are functionally heterogeneous. The structure, function, and gene expression of these cells vary across different tissues, and within a given tissue (Aird, 2006, 2007). These differences can promote delivery to specific tissues; for instance, delivery to hepatocytes is enhanced by regions of endothelial cells which contain gaps, while delivery to neurons and glial cells in the brain is limited by the tight and continuous barrier of endothelial cells lining the blood–brain barrier. In this same way, natural routes of clearance that increase blood flow to the liver can promote delivery to hepatocytes. The same mechanisms designed to remove and concentrate toxins from the blood can be exploited to concentrate nanoparticles in cells of interest (Akinc et al., 2010).

2.2 Active Targeting

Active targeting systems utilize ligands like proteins or small molecules to bind specific receptors on a target cell surface. The binding can either stabilize the particle on the outside of the cell or trigger receptor-mediated endocytosis and internalization. While many different targeting ligands can be used for targeted siRNA delivery, most scientists have utilized three types of ligands: small molecules, peptides, and proteins (Figure 3.1). Small molecule targeting ligands are molecules with a distinct chemical structure and a molecular weight generally less than 1 kDa. These compounds can mimic natural biomolecules and are synthesized by traditional organic chemistry techniques. Peptide and protein targeting ligands are made of amino acids; peptides consist of less than approximately 50 amino acids while proteins are composed of many more, up to tens of thousands of amino acids. Peptide- and protein-mediated targeting requires precise three-dimensional folding that results from secondary and tertiary protein structures.

Active targeting requires the use of a molecule that binds to a cognate receptor on a target cell. Targeting molecules can be attached to the siRNA directly, however, the synthesis of these siRNA conjugates is challenging. Effective synthesis of siRNA conjugates requires chemical synthesis schemes that meet three criteria. First, reaction conditions that degrade or denature the siRNA or ligand must be avoided. Second, conjugating the siRNA and targeting ligand together cannot reduce the efficacy of either component: the siRNA must still be incorporated into RISC and the ligand must still have specificity for its target receptor. Third, the reaction should generate the highest yield possible so that expensive and inefficient purification is minimized.

One limiting factor in the synthesis of siRNA conjugates is the stability of the siRNA in different chemical reactions. The siRNA must not be denatured during the reaction, since siRNA must remain as a duplex to be properly loaded into RISC and subsequently silence genes (Fire et al., 1998). To maintain RNA integrity, siRNA conjugation reactions should be run in conditions that avoid high temperatures, harsh solvents, and high concentrations of reactive intermediates. One method to avoid denaturing the double stranded RNA during synthesis is to perform conjugation chemistry on the sense strand and then duplex with the antisense strand later. Sense strand modifications have been made to both the 3' and 5' end of the siRNA. However, further work is required to understand whether the location of the targeting ligand on the sense strand affects RISC loading and mRNA silencing. Conjugations to the antisense strand should be avoided, since the antisense strand should not have steric hindrances which prevent hybridization with the target mRNA in the RNAi pathway. It is also very important to chemically modify the RNA nucleotides and phosphodiester bonds, as unmodified siRNAs can both induce an immune response and be easily degraded by endogenous ribonucleases (Whitehead et al., 2011). It is common to replace the 2'-hydroxy group on some riboses in the sequence with a 2'-O-methyl group or 2'-fluoro and/or to replace one or several phosphodiester bonds with phosphorothioate bonds, although many more modifications have been reported with varying degrees of success (Deleavey & Damha, 2012). These internal modifications are crucial for *in vivo* experiments as they can dramatically both decrease immunogenicity and increase serum stability of the siRNA.

Selecting the right solvent to ligate small molecules to the siRNA can be a particularly important decision. siRNA is soluble in aqueous conditions and precipitates in solutions with too much organic solvent. At the same time, many small molecules commonly linked to siRNA, like cholesterol and folate, are hydrophobic, and therefore require an organic solvent to solubilize. Researchers have overcome this problem using two strategies: first, small molecules and siRNA have been solubilized and reacted in a mixture of water and organic solvent like dimethylsulfoxide or acetonitrile. Second, researchers have attached small molecules during the oligonucleotide synthesis process. For example, cholesterol was conjugated to siRNA by initiating the siRNA synthesis on a controlled-pore glass solid support carrying a cholesterol-aminocaproic acid-pyrrolidine linker; this linker placed a cholesterol on the 3' end of the sense strand (Soutschek et al., 2004). However, these techniques are limited by the fact that only certain small molecules

are soluble in solvents with aqueous and organic components, many labs do not have access to oligonucleotide synthesis machines, and the solid support method requires optimization for each desired targeting ligand. Reaction conditions that do not affect double stranded siRNA still might denature small molecules, proteins, or oligonucleotides. For example, solvents with aqueous and organic components may differentially attract hydrophobic and hydrophilic regions of a protein, resulting in protein denaturing and loss of function. It is also critical that any modifications to the targeting ligand do not change its ability to bind its receptor. As a result, the active site of the ligand should be identified and conjugations should be performed as far away from this area as possible. Because conjugation reactions change for each targeting ligand, reactions that universally promote conjugation remain an important unmet need.

Despite the strict criteria associated with these reactions, several schemes that successfully conjugate biomolecules have been described (Hermanson, 1996) (Figure 3.2). Many of these reactions utilize common biological functional groups, including amines, carboxylic acids, and thiols. Importantly, siRNAs with these functional groups on the 3' or 5' of the sense strand can be purchased from commercial vendors. A bifunctional crosslinker is then used to connect the functional group on the RNA to a different functional group on the targeting ligand. In one example, primary amines are reacted with N-hydroxysuccinimide (NHS) esters to form an amide bond that is stable in physiological pH for several hours. The NHS esters can be generated from carboxylic acids using 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) chemistry. Another reaction that is commonly used takes place between thiols and maleimide groups; the reaction forms thioether bonds that are stable in physiological pH. Conjugates can also be formed using the highly efficient reaction that takes place between streptavidin and biotin conjugation. This reaction may not be appropriate for smaller targeting ligands, however, because the size of the 53-kDa streptavidin protein can sterically interfere with the targeting ligand. These bioconjugation techniques have been complemented by new approaches that rely on highly efficient and mild “click chemistry.” One type of promising click reaction used for siRNA small molecule conjugation links an azide group with an alkyne using a copper catalyst (Yamada et al., 2011). This reaction generates a stable and biologically inert triazole linkage, and can be even performed without the need for a toxic copper catalyst if the alkyne is replaced with a strained cyclooctyne group (Chang et al., 2010). The reaction is rapid and robust, bioorthogonal, and can take place in an aqueous solvent at room temperature.

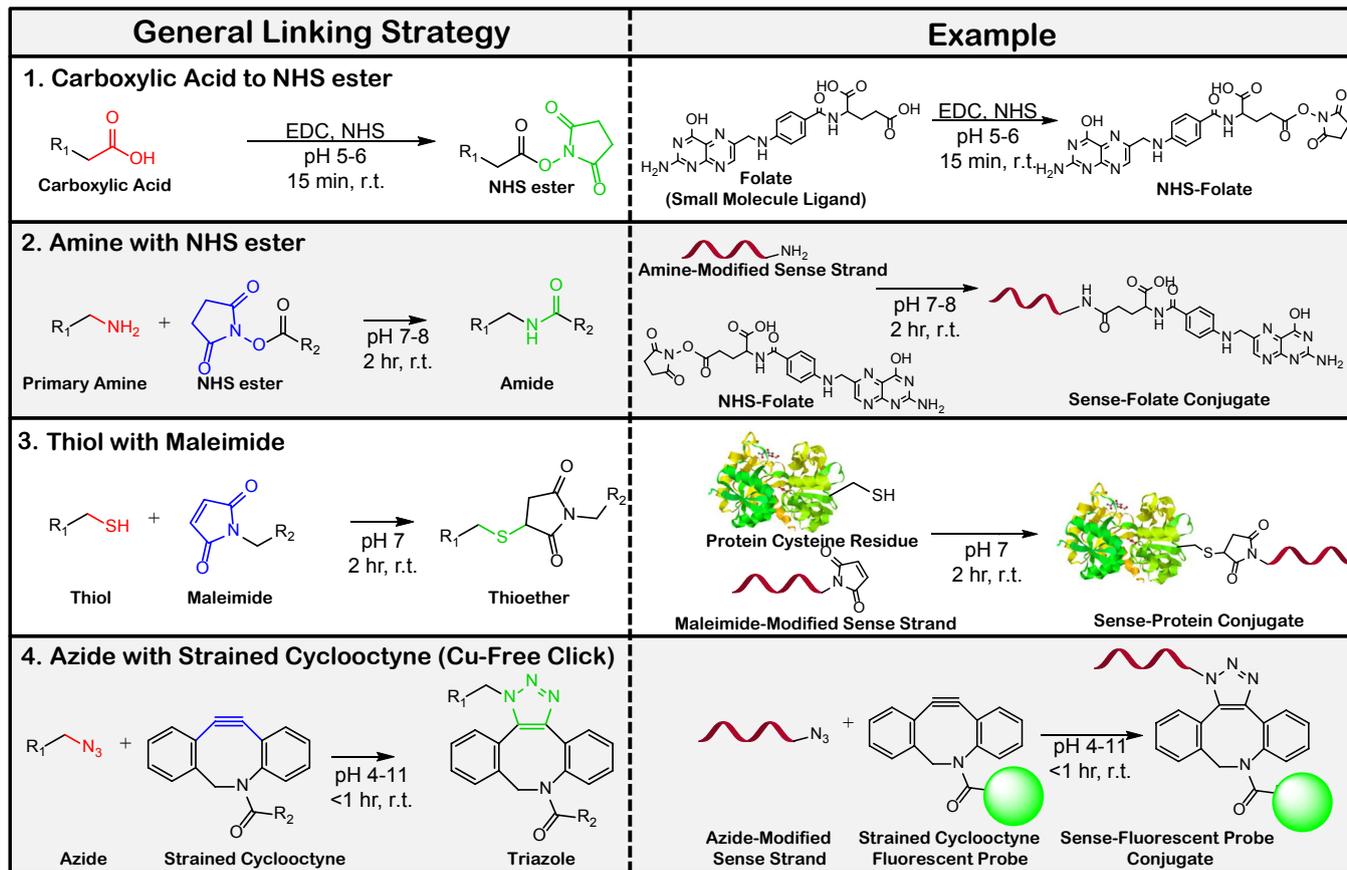


Figure 3.2 Chemical reactions to synthesize active siRNA conjugates. Conjugation reactions must not denature the targeting ligand, affect siRNA stability, or prevent siRNA loading into RISC. Notably, many synthetic schemes in use today require modification for every new targeting ligand. (See the color plate.)



3. TARGETING THE LIVER

3.1 Liver Physiology Promotes Targeting

Dysfunction of the liver can negatively affect metabolism, glycogen storage, hormone secretion, and serum lipid concentrations (Nolan, Damm, & Prentki, 2011; Rader, Cohen, & Hobbs, 2003). As result, this tissue contributes to a myriad of common diseases that may be amenable to genetic therapies, including cancer, diabetes, clotting disorders, and heart disease. To date, the most clinically advanced siRNA therapies have targeted aberrant gene expression in hepatocytes (Kanasty et al., 2013). The relative ease with which hepatocytes have been targeted can be partially attributed to distinct physiological characteristics that promote hepatocyte delivery. The liver is perfused with the hepatic portal vein, which directs blood gastrointestinal tract to the liver. As a result, delivery systems circulating in the blood have excellent access to the liver. Circulating drugs that flow by the liver can extravasate out of the bloodstream into surrounding liver tissue through nanoscale holes (fenestrae) in sinusoidal endothelial cells (Kanasty, Whitehead, Vegas, & Anderson, 2012). The average diameter of these fenestrae, roughly 100–150 nm depending on the animal species, make fenestrated endothelial cells ideal as a way for nanoscale drug delivery systems designed to reach hepatocytes (Wisse, Jacobs, Topal, Frederik, & De Geest, 2008). Finally, liver delivery mediated by lipid nanoparticles (LNPs) and other hydrophobic systems may be enhanced by the natural mechanisms the liver uses to remove circulating lipids from the bloodstream (Akinc et al., 2010).

3.2 Passive Liver Targeting

The most advanced clinical siRNA-delivery systems utilize LNPs which passively target hepatocytes. One disease that has been treated with LNP formulations is familial hypercholesterolemia, an autosomal dominant genetic disorder characterized by elevated LDL cholesterol. This disease, which greatly increases the risk for cardiovascular disease and sudden death, is driven by overactive PCSK9, a gene whose protein binds to and degrades LDL receptors. A Phase I clinical trial studied the tolerability and efficacy of an LNP developed by Alnylam Pharmaceuticals that was formulated with siRNA targeting the PCSK9 gene (ClinicalTrials.gov, 2011; Fitzgerald et al., 2014). This formulation, termed ALN-PCS02, was tolerated at all tested doses. Moreover, at the highest dose (0.4 mg/kg siRNA) (Fitzgerald et al., 2014) an average of 70% reduction in PCSK9 protein and 40% reduction

in LDL cholesterol from baseline was reported. Using another LNP formulation, Alnylam made nanoparticles targeting transthyretin (TTR), a gene whose gain of function mutations cause TTR-amyloidosis, a debilitating and fatal genetic disease characterized by extracellular deposits of insoluble, misfolded proteins. This formulation was well tolerated in Phase I clinical trials (ClinicalTrials.gov, 2012a, 2010), with no drug related serious adverse events reported. The formulation, called ALN-TTR02, was also effective, reducing TTR levels by over 80% with doses between 0.15 and 0.3 mg/kg in humans ([Coelho et al., 2013](http://doi.org/10.1016/j.jheale.2013.05.001)). Robust and durable protein reduction was also observed with this LNP: TTR serum protein decreased between 57% and 67%, 28 days after treatment. ALN-TTR02 has completed Phase II clinical trials (ClinicalTrials.gov, 2012b) and is currently enrolling patients for a Phase III clinical trial (ClinicalTrials.gov, 2013a).

Incredibly, *in vivo* LNP efficiency with hepatocytes has increased by more than 10,000 \times within the last 10 years, as shown in [Figure 3.3](#). This rapid improvement has been catalyzed by high-throughput screening and rational design techniques that generate lipids and lipid-like materials that promote delivery ([Figures 3.4 and 3.5](#)). These LNPs are typically formulated with three components: (1) cationic or ionizable lipids containing a hydrophilic amine group, a hydrophobic carbon tail, and a chemical linker that binds them, (2) lipid-anchored polyethylene glycol (PEG), which “shields” the LNP against nonspecific uptake by macrophages, increases the circulation time *in vivo* and reduces particle aggregation, and (3) cholesterol or other sterol-like molecules,

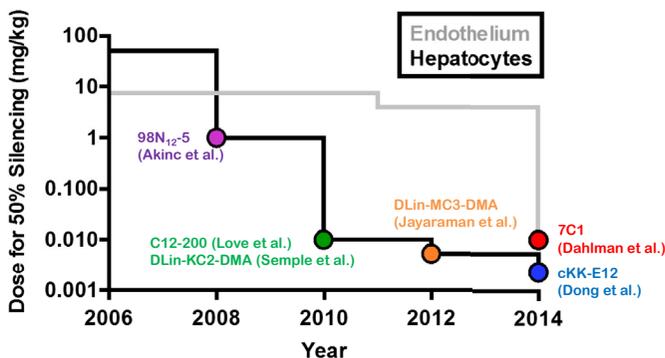


Figure 3.3 The efficacy of hepatocyte-targeting siRNA vehicles has improved dramatically over time. The dose required to reduce target gene expression in hepatocytes by 50% *in vivo* has decreased more than 10,000 \times since 2006. Recently, the efficacy of endothelium-targeting siRNA vehicles has been improved by approximately 1,000 \times with 7C1 nanoparticles ([Dahlman et al., 2014](#)). siRNA delivery to other cell types has remained challenging.

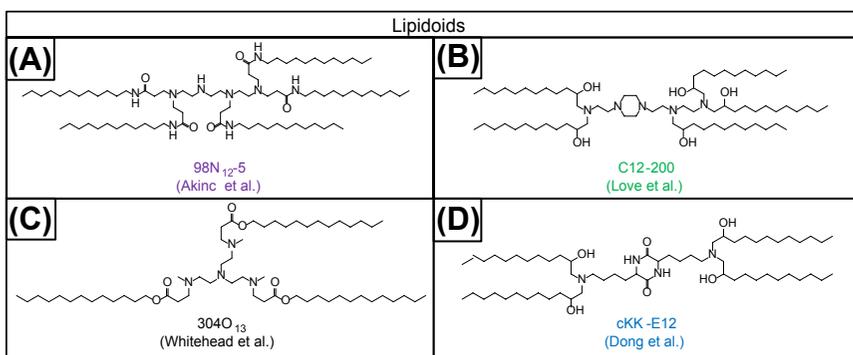


Figure 3.4 Multitailed, lipid-like molecules (lipidoids) have successfully delivered siRNA to hepatocytes. (A) First-generation lipidoid N98₁₂₋₅ (Akinc et al., 2008), (B) Second-generation lipidoid C12-200 (Love et al., 2010), (C) Biodegradable lipidoid 304O₁₃ (Whitehead et al., 2014), and (D) and lipopeptide-inspired lipidoid cKK-E12 (Dong et al., 2014).

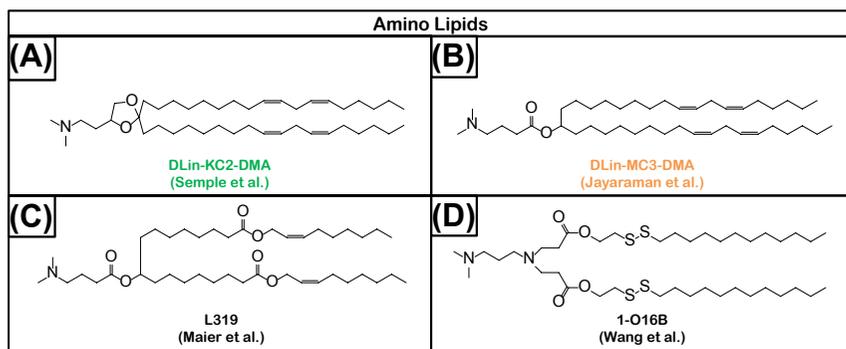


Figure 3.5 Two-tailed amino lipids have also successfully delivered siRNA to hepatocytes. (A) First-generation amino lipid DLin-KC2-DMA (Semple et al., 2010), (B) Second-generation amino lipid DLin-MC3-DMA (Jayaraman et al., 2012), (C) Biodegradable amino lipid L319 (Maier et al., 2013), and (D) Bioreducible amino lipid 1-O16B (Wang et al., 2014).

which increase LNP stability (Allen & Cullis, 2013; Kanasty et al., 2013). These formulation parameters affect physical properties of the LNP, including size, surface charge, and siRNA loading. Moreover, a growing body of work suggests that the ratio of lipid: PEG: cholesterol: siRNA needs to be tuned to optimize RNA delivery (Akinc et al., 2009).

3.2.1 Lipidoid LNPs

Chemically modifying the components of the LNP can drastically influence biodistribution, efficacy, and tolerability *in vivo*. To investigate the relationship

between lipid structure and *in vivo* function, combinatorial chemistry techniques that permit the rapid synthesis of many multitailed, lipid-like structures (termed “lipidoids”) have been developed (Akinc et al., 2008; Dong et al., 2014; Love et al., 2010). Once synthesized, these material libraries are often tested *in vitro* before the best performing LNPs are selected for further testing *in vivo*. Although *in vitro* conditions do not recapitulate the complicated *in vivo* environment (Whitehead et al., 2012), this approach has assisted in the discovery of first, second, and third generation hepatocyte-targeting LNPs that reduce target mRNA expression at doses as low as 1.0, 0.01, and 0.001 mg/kg, respectively (Figure 3.3). Increased efficiency reduces the amount of LNP required for delivery, enables more durable gene silencing, and lessens the likelihood of an unwanted immune response. Over the past several years, the generation of lipidoid libraries has resulted in several lead lipidoid compounds (Figure 3.4) discussed in detail below.

The first lead lipidoid, termed 98N₁₂₋₅ (Figure 3.4(A)), was selected from a library of over 1200 lipidoids (Akinc et al., 2008). The library was synthesized by conjugating small amines to lipids terminated with acrylates or acrylamides via the Michael addition reaction. This reaction was selected because it was robust, required one step, and did not require complicated or expensive purification. 98N₁₂₋₅ LNPs were formed by mixing 98N₁₂₋₅ with PEG, cholesterol, and siRNA. This LNP was formulated with siRNA targeting Factor VII, a blood clotting factor produced specifically by hepatocytes and easily measured in serum. 98N₁₂₋₅ mediated delivery reduced Factor VII serum concentration by over 90% in mice after two daily IV 2.5 mg/kg doses; similarly, when formulated with siRNA targeting apolipoprotein B (ApoB), 98N₁₂₋₅ reduced ApoB mRNA expression by 85% in nonhuman primates after a single IV 6.25 mg/kg injection. In addition to identifying a lead candidate, this high-throughput screening technique revealed structural features that were shared by top-performing compounds in the study. Effective compounds had amide bonds linking the lipid tails and amines, more than two alkyl tails with 8–12 carbons, and at least one secondary amine.

The structure–function relationships from the first lipid library informed the synthesis of second-generation lipids that dramatically improved delivery to hepatocytes *in vivo*. Once again, simple and robust chemistry allowed for the rapid generation of a structurally diverse library that was screened *in vitro* (Love et al., 2010). A lead lipidoid termed C12-200 (Figure 3.4(B)) had an IC₅₀ of approximately 0.01 mg/kg and reduced Factor VII serum in mice by nearly 100% after a 0.1 mg/kg injection. C12-200 was also effective

in nonhuman primates, reducing TTR serum protein by 90% after a single injection of 0.3 mg/kg. C12-200 further enabled highly durable Factor VII protein silencing in mice, and facilitated the first reported multigene *in vivo* knockdown after it was concurrently formulated with five different siRNAs, each targeting a different hepatocyte-specific gene (Love et al., 2010).

To study whether biodegradable linkages could further improve *in vivo* tolerability, another lipidoid library was then synthesized using alkyl acrylate tails, which contain ester groups capable of hydrolysis by liver enzymes, to make biodegradable lipidoids (Whitehead et al., 2014). As predicted, exposing the new biodegradable lipidoid to hydrolytic conditions resulted in degradation into the predicted alkyl-alcohol products. siRNA-loaded LNPs made with the lead degradable lipidoid, 304O₁₃ (Figure 3.4(C)), had an IC₅₀ of 0.01 mg/kg, which is equivalent in potency to C12-200. To determine if enhanced degradability translated to better tolerance *in vivo*, the authors dosed 304O₁₃ and C12-200 LNPs at levels 100-fold their IC₅₀. 304O₁₃ LNPs produced less immune response (lower cytokine levels) than C12-200 at this high dose, and liver histology showed necrosis and inflammation in mice dosed C12-200 at 1 mg/kg but none for 304O₁₃ dosed at 5 mg/kg. Furthermore, the authors identified “efficacy criteria” for this class of lipidoids: potent lipidoids had a tertiary amine with at least three tails of 13 carbons, but the most important LNP parameter was having a surface pKa of at least 5.5.

Inspired by the fact that the liver naturally takes up lipoproteins from the blood, we recently synthesized a library of lipopeptide-based materials (Dong et al., 2014). These biomimetic compounds were synthesized by reacting lipids with different functional groups to the free amine on small peptides. After screening the library *in vitro*, we identified a lead candidate, termed cKK-E12 (Figure 3.4(D)), that reduces FVII serum protein by 50% after a single injection of 0.002 mg/kg cKK-E12 also reduced TTR serum concentration in nonhuman primates by 95% after a single injection of 0.3 mg/kg cKK-E12 selectivity toward hepatocytes and tolerability was also measured *in vivo*; at low doses it did not transfect endothelial cells or immune cells *in vivo*, and resulted in no apparent toxicity. Lipopeptide structure–function relationships were again studied. The most effective compounds commonly had a lysine-based ring structure and a lipid tail between 12 and 14 carbons bound by an epoxide- or aldehyde-terminated lipid.

3.2.2 Amino Lipid LNPs

Whereas multitailed lipidoids have been generated primarily via high-throughput combinatorial libraries, a second class of lipids, two-tailed

ionizable “amino lipids” (Figure 3.5), have been generated with a rational-design approach. To synthesize the compound DLin-KC2-DMA (Figure 3.5(A)), each section of the lipid (polar head and linker group) was individually optimized by systematically varying parameters like carbon chain length and hydrophilicity (Semple et al., 2010). A previous study had already optimized the tail region of the amino lipid, finding that 18-carbon tails with two-double bonds (“DLin”) had the best gene silencing *in vitro* (Heyes, Palmer, Bremner, & MacLachlan, 2005). LNPs formulated with DLin-KC2-DMA, cholesterol, PEG, and siRNA reduced Factor VII serum protein by 90% after a single injection of 0.1 mg/kg in mice and TTR serum protein by 80% after a single 1.0 mg/kg injection in nonhuman primates (Semple et al., 2010). The IC₅₀ of DLin-KC2-DMA LNPs was 0.01 mg/kg, equivalent to the lipidoid C12-200: interestingly, two different approaches (high-throughput vs rational design) and two different lipid-inspired chemical structures (multitailed lipidoids vs two-tailed amino lipids) resulted in siRNA-delivery compounds with nearly identical potency *in vivo*.

Since the initial report of DLin-KC2-DMA, further work has been performed to improve LNP formulation and characterize the particle. The formulation process used to synthesize DLin-KC2-DMA LNPs has been improved through the use of microfluidic mixing devices, which produces LNPs with more reproducible size, higher siRNA encapsulation efficiency, and larger scale than traditional pipet-mixed particles (Belliveau et al., 2012). Importantly, these LNPs made with microfluidic mixing devices were as potent *in vivo* as particles made with traditional mixing methods. Computational modeling, cryo-TEM, and other techniques used to study the internal structure of these microfluidic-mixed DLin-KC2-DMA LNPs revealed that the nanoparticle structure is made of siRNA complexed to cationic lipid and periodically spaced aqueous regions inside the LNP core (Leung et al., 2012). Since its initial report, DLin-KC2-DMA has also been used to passively deliver siRNA to nonhepatocellular targets. By increasing the size of the LNP from 80 nm diameter to 360 nm diameter (much larger than the 100–150 nm fenestrae in liver endothelium), the authors reduced siRNA delivery to liver and allowed for more selective delivery *in vivo* to antigen-presenting cells like bone marrow macrophages and dendritic cells, which readily endocytose particles less than 10 μm in size (Basha et al., 2011).

Second-generation amino lipid based on DLin-KC2-DMA were then synthesized (Jayaraman et al., 2012). The amine head group and linker section of the amino lipid were further modified to modulate the pKa; 53 new amino lipids with pKa values ranging from 4.17 to 8.12 were generated.

These amino lipids were complexed with siRNA (without PEG, cholesterol, or other excipients) and screened in mice via intravenous injection. The authors found a remarkable correlation between pKa and potency: pKas between 6.2 and 6.5 had the lowest IC₅₀ for all amino lipids tested, and efficacy rapidly decreased below 6.2 and above 6.5. This amino lipid pKa criterion corroborates the pKa criterion found with biodegradable lipiods, which was that pKa must be 5.5 or greater (Whitehead et al., 2014). The lead compound discovered in the *in vivo* screen was called DLin-MC3-DMA (Figure 3.5(B)), which had a pKa of 6.44 and an IC₅₀ of 0.005 mg/kg when incorporated into an LNP formulation with helper lipid, cholesterol, and PEG (Jayaraman et al., 2012). It should be noted that the previously mentioned clinical trials led by Alnylam Pharmaceuticals to treat hypercholesterolemia (ClinicalTrials.gov, 2011) and TTR-amyloidosis (ClinicalTrials.gov, 2012b) (Section 3.2) both use an LNP formulation based on DLin-MC3-DMA.

A biodegradable version of DLin-MC3-DMA was then synthesized by replacing one of the double bonds in the hydrocarbon tail with a degradable ester linkage (Maier et al., 2013). It was found that moving this ester bond farther up the tail toward the headgroup decreased *in vivo* efficacy. The lead biodegradable amino lipid in the initial screen was called L319 (Figure 3.5(C)) and had an IC₅₀ of less than 0.01 mg/kg when formulated in LNPs, similar to its nonbiodegradable counterpart DLin-MC3-DMA. Through pharmacokinetic studies in mice, it was demonstrated that L319 is degraded through ester cleavage into hydrophilic, water-soluble metabolites which are rapidly cleared from plasma and tissues. Furthermore, toxicology studies in rats showed no significant increase in liver toxicology markers alanine transaminase (ALT) and aspartate transaminase (AST) up to a 10 mg/kg dose of L319 LNPs, which is 1000× higher than the IC₅₀, showing a very large therapeutic window; in comparison, nonbiodegradable control LNPs showed elevated ALT and AST levels above 3 mg/kg. Because enzyme profiles differ across species, nonhuman primates were used to confirm L319 LNP efficacy (70% TTR knockdown at 0.3 mg/kg) and rapid clearance of L319 from plasma (30,000× reduction in 24 h) in a higher-order species.

A separate report employed a different strategy to create degradable amino lipids: both ester bonds and disulfide bonds were incorporated into the hydrocarbon tails (Wang et al., 2014). Whereas esters can be hydrolyzed by enzymes in the body, disulfide bonds can be broken into thiols in the reducing environment of the cytoplasm. A small set of amino lipids with these biodegradable and bio-reducible bonds were synthesized, and a lead

candidate named 1-O16B (Figure 3.5(D)) was identified. For the six compounds tested through complexation with siRNA, all six had statistically significant increases in gene knockdown *in vitro* when a disulfide bond was incorporated into the tails. It was hypothesized that this increased efficacy is caused by the disulfide bond reduction in the cytoplasm, triggering siRNA release from the electrostatic complex as the amino lipid degrades more rapidly. Behavior of 1-O16B and similar bio-reducible amino lipids *in vivo* has not yet been reported.

3.2.3 Endocytosis of Lipidoid and Amino Lipid LNPs

LNP-mediated delivery is thought to exploit the natural endocytosis of serum apolipoproteins, however, the precise mechanism governing LNP cellular endocytosis by hepatocytes is poorly understood and likely varies with the chemical nature of a given LNP. Some LNPs use an apolipoprotein-E (ApoE) dependent mechanism for hepatocyte uptake; it is postulated that protein ApoE adsorbs on to the LNP, directs the LNP to hepatocytes, and binds its natural receptor, LDL receptor (LDLR). Once bound to the LDLR receptor, the ApoE and the LNP are concurrently taken into the hepatocyte via receptor-mediated endocytosis. To support this mechanism, LNPs made from DLin-KC2-DMA were injected into wild-type, ApoE^{-/-}, and LDLR^{-/-} mice (Akinc et al., 2010). The LNP was highly potent in normal mice, but did not deliver siRNA in ApoE^{-/-} knockout mice or LDLR^{-/-} knockout mice. Moreover, delivery efficiency was rescued when recombinant ApoE was administered to the ApoE^{-/-} mice. This same methodology revealed that cKK-E12 uptake was also ApoE-dependent. Interestingly, LNPs made from the lipidoid N98₁₂-5 still transfected ApoE^{-/-} mice, suggesting some other form of internalization. Although not conclusive, these studies suggest that endocytosis may be impacted by LNP surface charge; those with near neutral surface charge at physiological pH may be more likely to undergo an ApoE-dependent endocytosis mechanism.

3.3 Active Liver Targeting

Complementing LNPs which passively target the liver are systems that have been designed to actively target hepatocytes by binding to the asialoglycoprotein receptor (ASGPR). This receptor, which is constitutively and specifically expressed on the surface of hepatocytes, has a carbohydrate recognition domain that binds to the monosaccharide galactose and plays an important role in glycoprotein homeostasis (Meier, Bider, Malashkevich, Spiess, & Burkhard, 2000). Binding between galactose and ASGPR is well

characterized: alcohol groups at the 3- and 4- positions of the galactose bind to ASGPR by interacting with a calcium ion in the ASGPR and forming hydrogen bonds with neighboring amino acids (Weis, 1996). This binding changes the configuration of the receptor and triggers receptor-mediated endocytosis. Because this receptor is expressed primarily on hepatocytes, several labs have developed galactose-analog conjugates for the delivery of siRNAs and other therapeutics (Jain, Kesharwani, Gupta, & Jain, 2012). This interest has grown as recent evidence suggests that these analogs can effectively deliver therapeutics to hepatocytes without significantly transfecting Kupffer cells or other tissues *in vivo* (Spiess, 1990).

Hepatocyte-targeting with galactose and its analogs has improved with our understanding of the mechanisms that govern the interaction between the ligand and its receptor. Early work relied on a cationic polymer polyethyleneimine (PEI) that was conjugated to galactose and complexed with DNA (Kim et al., 2005). This compound improved DNA delivery to hepatocytes *in vivo*, but was limited by the inherent toxicity associated with high molecular weight PEI. To improve selectivity and tolerability, groups utilized the ligand *N*-acetylgalactosamine (GalNAc), a simple derivative of galactose with an acetyl amino group replacing the hydroxyl at the 2-position of the sugar, that binds to the ASGPR receptor with higher selectivity than unmodified galactose (Drickamer, 1996). This engineering approach was used to study whether additional chemical modifications made to the 2- and 6- positions of GalNAc increased the binding affinity to ASGPR. Indeed, when trifluoroacetyl modifications were made to the 2- position GalNAc, the binding affinity for ASGPR increased by more than 50-fold (dissociation constant $K_d = 0.7$ vs $40 \mu\text{M}$, respectively) (Mamidyalu et al., 2012). Because this binding study was performed without RNA, it will be important to understand whether increased affinity is still observed with conjugated siRNA.

The binding of GalNAc to ASGPR has also been improved by increasing the valency of the GalNAc ligand. It has been shown that binding affinity increases when clusters of glycoside receptors are simultaneously bound with an optimal spacing of at least 15 \AA between sugar residues (Rensen et al., 2001). To take advantage of this clustering effect, triantennary GalNAc was synthesized; its ASGPR dissociation constant was 2 nM , 2000-fold lower than the single GalNAc system (Rensen et al., 2001). Triantennary GalNAc conjugated directly to the 3' end of the sense strand of siRNA has been used by Alnylam Pharmaceuticals in clinical trials. The most clinically advanced triantennary GalNAc-siRNA conjugate is ALN-TTRsc, a

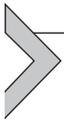
subcutaneously administered therapeutic for treatment of TTR-mediated amyloidosis (Alnylam, 2013). No significant adverse effects were observed in Phase I clinical trials, and TTR serum protein was reduced in patients treated with 2.5 mg/kg dose (Alnylam, 2013; Butler et al., 2014). Increasing the dose to 10 mg/kg resulted in more potent TTR protein reduction; up to 94% protein reduction was measured after a single dose. Although these siRNA doses are much higher than Alnylam's lipid nanoparticle TTR formulation (ALN-TTR02), the direct GalNAc-siRNA conjugates were well tolerated at doses well above those needed for potent silencing. Moreover, these targeted conjugates did not require PEG or cholesterol, and were administered subcutaneously instead of intravenously. This makes patients more likely to tolerate the injection, and in turn, increases the number of clinical indications to which these systems can be applied. Alnylam is currently utilizing the GalNAc conjugates to reduce PCSK9 and antithrombin (AT) to treat familial hypercholesterolemia and hemophilia, respectively.

GalNAc targeting ligands have also been used by the Dynamic Polyconjugate (DPC) system (Rozema et al., 2007; Wong et al., 2012; Wooddell et al., 2013). First-generation DPCs (Rozema et al., 2007) used an amphiphatic polymer made of butyl and amino vinyl ethers (PBAVE) as a reactive backbone to which siRNA, the GalNAc targeting ligand, PEG were attached via acid-labile linkages. While the exact mechanism of action remains unclear, it is hypothesized that these linkages break in the acidic environment of the endosome, allowing the newly "unmasked" amine groups on the PBAVE to help destabilize the membrane and facilitate endosomal escape. First-generation DPCs administered intravenously to mice at a 2.5 mg/kg siRNA dose reduced ApoB by 80–90%. This potent silencing was accompanied by a phenotypic reduction in serum cholesterol (Rozema et al., 2007). Slight but nonsignificant increases of liver enzymes and cytokines were observed during the study; however, the authors concluded DPC was well tolerated. Based on the hypothesis that a biodegradable polymer would decrease toxicity, Merck synthesized a bioreducible variant of the DPC system by incorporating disulfide bonds in the polymer (Parmar et al., 2013). This compound reduced ApoB mRNA expression by 80% in mice after a 3 mg/kg injection.

Recently, it was reported that the PBAVE polymer need not be covalently bound to the siRNA for efficient hepatocyte gene silencing (Wong et al., 2012). When authors co-injected PBAVE and a cholesterol-siRNA conjugate, they found that both systems co-localized to mouse hepatocytes and silenced genes, even when the polymer and siRNA were injected 2 h

apart. Injecting the endosomolytic agent and cholesterol-siRNA improved the efficiency by 500-fold compared to cholesterol-siRNA alone and greatly simplified the formulation process. A second-generation delivery system utilizing a GalNAc conjugated to a small peptide called melittin, a small biodegradable peptide component of bee venom which is thought to disrupt the endosome and enhance endosomal escape. This system delivered siRNA that reduced chronic Hepatitis B virus (HBV) infection in mice (Wooddell et al., 2013). These results demonstrated that melittin promoted delivery without generating anti-melittin antibodies. The successful mouse and nonhuman primate results of this study led to a Phase I clinical study studying the safety and tolerability of a coinjection of GalNAc/masked-melittin conjugates with siRNA-cholesterol conjugates (called ARC-520) to treat chronic hepatitis B infection in humans (ClinicalTrials.gov, 2013b). In March 2014, a Phase IIa clinical trial of ARC-520 was started in patients with chronic HBV (ClinicalTrials.gov, 2014).

GalNAc ligands have also been used to actively target nanoparticles to hepatocytes. Triantennary GalNAc was incorporated into DLin-KC2-DMA nanoparticles and injected *in vivo* (Akinc et al., 2010). This conjugation significantly improved siRNA delivery compared to unmodified DLin-KC2-DMA nanoparticles in ApoE^{-/-} mice, which could not use the endogenous ApoE-dependent endocytosis mechanism and thus forced ASGPR-mediated endocytosis.



4. TARGETING PRIMARY TUMORS AND METASTASIS

4.1 Tumor Physiology can Promote or Inhibit Delivery

The term cancer encompasses hundreds of complicated diseases with distinct presentations, available treatments, and prognoses. At the most basic level, cancers are characterized by aberrant and uncontrolled cell growth. As mutated cells continue to grow more rapidly and die less often than normal cells, the physiology of the resulting primary tumor becomes unstable and heterogeneous. The complicated genetic and phenotypic landscape of primary tumors (and their metastases) often creates an environment that can either promote or prevent the delivery of nanotherapeutics. Tumor vasculature, for instance, can be discontinuous, twisted, and leaky (Carmeliet & Jain, 2011). This occurs because the synthesis of normal functional blood vessels requires an intricate cascade of molecular signals that occur in specific order both in space and time (Herbert & Stainier, 2011). Abnormal signaling that takes place in the tumor prevents the normal signaling cascade

from being completed, leading to the rapid generation of dysfunctional, tortuous vessels. While leakiness may enhance the delivery of some drug delivery systems in certain animal models, there is also strong evidence that suggests just the opposite: because the tumor vessels had been leaky for some time and new cells were being generated rapidly in the tumor, hydrostatic pressure builds up in the tumor microenvironment (Jain, 2005). As a result, nanoparticles are not able to exit tumor vessels. Delivery may also be affected by the immunological state of the tumor. Tumor physiology is pro-inflammatory; as a result, tumors are often filled with immune cells, which can endocytose nanoparticles (Fridman, Pagès, Sautès-Fridman, & Galon, 2012). Finally, the cancer cells and co-opted cells of the tumor microenvironment are often mutated, leading to abnormal cellular function. Cells can undergo mutations that can either promote or inhibit endocytosis and cytoplasmic delivery. Taken together, these factors suggest that the relationship between drug delivery and tumor physiology will remain contentious, and will likely depend on the specific tumor and type and *in vivo* model (Carmeliet & Jain, 2011; Jain, 1994).

Primary tumors can also shed cells into the circulation. A small fraction of these cells can spread to distant organs through a process called metastasis. This process has been difficult to treat therapeutically because surgical resection and localized radiation are often not viable options when the disease has spread. As a result, over 90% of cancer deaths result from metastasis. Metastasis is a relatively complicated, and therefore inefficient, biological process that requires cells to clear a path to the vasculature, enter the circulation, exit the circulation, and proliferate in the secondary site (Chiang & Massagué, 2008; Gray, 2010; Nguyen & Massagué, 2007). Metastasis is initially promoted by primary tumor growth. Rapid growth leads to the formation of dysfunctional and inefficient blood vessels; poor perfusion in the tumor prompts cancer cells to express genes that induce cell motility and anaerobic metabolism (Chiang & Massagué, 2008; Nguyen & Massagué, 2007). In some cases, cancer cells can actively degrade extracellular matrix and epithelial cell–cell junctions in their surrounding microenvironment to clear a path toward nutrient-rich vasculature. Once at the vasculature, the cells can enter the bloodstream by degrading endothelial cell–cell junctions or increasing endothelial cell permeability by releasing molecules like vascular endothelial growth factor. Metastatic cells in the bloodstream can bind circulating platelets and leukocytes to increase survival before they reach their secondary site. At the secondary site, the cells must extravasate out of the circulation, survive in an unfamiliar microenvironment, and

proliferate. Cancer cells exit the circulation in a process akin to the one they use to enter it; they release molecules that induce endothelial cell death and degrade the surrounding extracellular matrix. At that point, the metastatic cells can co-opt natural growth signals in the microenvironment to enhance their own survival and growth. For example, metastatic breast cancer cells embedded in bone marrow express CXCR4 receptors that bind to nearby CXCL12 ligands, leading to metastatic progression (Wang, Loberg, & Taichman, 2006).

Each step of metastatic process occurs in a different part of the body (e.g., primary tumor, bloodstream, secondary site), relies on the activation of different genetic programs, and takes place in patients who are already sick and weakened (Schroeder et al., 2012). As a result, siRNA therapies targeting metastatic disease will require highly advanced delivery systems that can be targeted to different parts of the body safely.

4.2 Active Tumor Targeting

Aberrant gene expression in tumors can result in the overexpression of cell-surface receptors that can be actively targeted. One such receptor-used active-targeted tumor delivery is the folate receptor. This receptor is expressed in low levels on healthy cells, but is overexpressed in epithelial cancers (including those of the ovary, colon, lung, prostate, nose, throat, and brain) and hematopoietic malignancies of myeloid origin (including myelogenous leukemias) (Hilgenbrink & Low, 2005). Binding between the folate receptor and its ligand (folate, also termed folic acid) initiates downstream signaling that promotes cell survival and proliferation. The utility of folate as a ligand is enhanced by its γ -carboxylic acid. This carboxylic acid has been directly conjugated to nanoparticles, siRNA, and even DNA origami structures that were simultaneously bound to siRNA (Lee et al., 2012). In all cases, delivery to cells with folate receptors was greater than delivery to cells without the receptor. For example, one study found that mesoporous silica nanoparticles conjugated with folate delivered drugs more effectively to pancreatic xenograft tumors in mice than nanoparticles than without folate (Lu, Li, Zink, & Tamanoi, 2012). Similarly, siRNA that was directly conjugated to folate via a low molecular weight PEG spacer showed increased RNA delivery compared to nonconjugated siRNA (Dohmen et al., 2012).

In addition to the 441-Da folate molecule, the 78-kDa glycoprotein transferrin can also be used in cancer-targeting RNA therapies. The transferrin receptor, which affects ion transportation and cell growth, is

overexpressed on malignant cells (Tortorella & Karagiannis, 2014). Transferrin was used to target a linear, cationic cyclodextrin polymer formulated into nanoparticles which preferentially deliver siRNA to tumors *in vivo* (Bartlett & Davis, 2008; Davis et al., 2010; Gonzalez, Hwang, & Davis, 1999; Heidel, Yu, et al., 2007). The therapeutic target for the incorporated siRNA is the M2 subunit of ribonucleotide reductase (RRM2), an enzyme whose inhibition reduces cancer cell proliferation both *in vitro* and *in vivo* for humans and other species (Heidel, Liu, et al., 2007). The transferrin targeting ligand is crucial for potency: at 2.5 mg/kg dosing in mice, RRM2-siRNA-loaded cyclodextrin nanoparticles formulated with the transferrin ligand slowed tumor growth, while identical particles formulated without the transferrin ligand did not (Bartlett & Davis, 2008). A study was performed in non-human primates to determine the tolerability of these cyclodextrin-based nanoparticles with siRNA (Heidel, Yu, et al., 2007). Doses up to 9 mg/kg appeared to be well tolerated with no detectable toxicity, whereas doses at 27 mg/kg produced a mild immune response, an unsurprising result considering the authors used siRNA which contained no chemical modifications to reduce immunogenicity.

The transferrin-targeted, cyclodextrin-based nanoparticles with RRM2-siRNA (called CALAA-01) were used in clinical trials and produced the first direct evidence of RNAi in humans (ClinicalTrials.gov, 2008; Davis et al., 2010). The nanoparticles were injected IV into patients with solid cancers refractory to standard-of-care therapies at mg/kg on days 1, 3, 8, and 10 of a 21-day cycle. Preliminary results from the Phase I clinical trials showed that the treatment reduced RRM2 mRNA and protein levels and induced mRNA cleavage, as measured by 5'-RLM-RACE. Unfortunately, in 2013 this Phase I clinical trial was terminated (ClinicalTrials.gov, 2008), the reasons for which were not reported.

Active siRNA delivery to ovarian cancer cells has been achieved with siRNA conjugated to tumor-targeting peptides. More specifically, the cyclic nonapeptide Lyp-1, found through an *in vivo* screen of random peptides displayed on phage surfaces, targeted lymphatic endothelial cells in tumors and by selectively binding to HABP1, a mitochondrial protein overexpressed in ovarian and other tumors (Fogal, Zhang, Krajewski, & Ruoslahti, 2008). Lyp-1 has also been modified to increase functionality: dual peptides with Lyp-1 domains and transportin domains were synthesized to promote tumor penetration and membrane transport, respectively (Ren et al., 2012). The tandem peptide was complexed with siRNA targeting ID4, an oncogene that was found to be essential for proliferation in many ovarian cancers

(Ren et al., 2012). The conjugates were then formulated into nanoplexes and injected in the peritoneal cavity or intravenously with ovarian tumor-bearing mice. The particles penetrated deeply into tumor parenchyma, reduced tumor ID4 mRNA expression by 80%, and significantly reduced tumor growth. These delivery systems also distributed to the liver, spleen, and lung. Although intraperitoneal injection showed better accumulation in the ovarian tumor, there was not a significant difference in ID4 knockdown between the two routes of administration.



5. ENDOTHELIAL CELL TARGETING

Endothelial cells, which line the blood vessels that penetrate nearly every tissue in the body, actively influence blood pressure, inflammation, metabolism, angiogenesis, and microenvironmental regulation. As a result, these cells contribute to more disease than any other tissue in the body, including cardiovascular ischemia, cerebrovascular ischemia (e.g., stroke), primary tumor growth, metastasis, diabetes, and chronic inflammation (Kumar, Abbas, Fausto, & Aster, 2009). Because there are many diseases that would benefit from highly efficient endothelial siRNA delivery, a number of delivery systems have been designed to deliver siRNA to endothelial cells *in vivo* (Kaufmann, Ahrens, & Santel, 2010). LNPs made from a cationic lipid, a helper lipid, and PEG were injected intravenously at a dose of 1.88 mg/kg on four consecutive days. Following this total dose of 7.52 mg/kg siRNA, pulmonary and hepatic endothelial cell mRNA and protein decreased significantly (Santel, Aleku, Keil, Endruschat, Esche, Durieux, et al., 2006; Santel, Aleku, Keil, Endruschat, Esche, Fisch, et al., 2006). Second-generation LNPs were then shown to reduce pulmonary endothelial cell mRNA expression after a total dose of 2.8 mg/kg siRNA (Aleku et al., 2008). While these delivery systems effectively targeted pulmonary endothelial cells *in vivo*, they required doses much higher than those required for potent hepatocyte silencing (0.01 mg/kg), as shown in Figure 3.3.

Inspired by the highly efficient hepatocyte delivery, a library of low molecular lipid-polymer materials was developed for endothelial delivery. Formulations were first screened them for their ability to reduce target mRNA expression in multiple cell lines, including two endothelial cell lines. Lead candidates were screened *in vivo*, leading to selection of a compound termed 7C1, a low molecular weight compound made by conjugating C₁₅ epoxide-terminated lipids and extremely low

molecular weight ($M_N = 600$ Da) branched PEI (Dahlman et al., 2014). 7C1 nanoparticle formulation was optimized to ensure particles were small, stable, and repeatedly silenced target mRNA *in vivo*. For example, seven batches of 7C1 nanoparticles were made with either extrusion or microfluidic mixing, and injected intravenously. Particles made with microfluidic mixing silenced mRNA expression much more consistently than those made with extrusion. The 7C1 formulation was then made with different 7C1: cholesterol: PEG molar ratios; unlike liver-targeting compounds, 7C1 did not require cholesterol to maximize mRNA silencing. The optimized 7C1 formulations potently reduced target mRNA expression in pulmonary, cardiovascular, and renal endothelial cells *in vivo* after injections of 0.017, 0.04, and 0.08 mg/kg, respectively. These compounds did not reduce target mRNA expression in pulmonary immune cells, systemic immune cells, or hepatocytes *in vivo*. The precise mechanism governing the preferential targeting of 7C1 particles to endothelial cells is currently unclear, but may be related to interactions with serum proteins. 7C1 delivery also reduced target mRNA expression for over 21 days following one injection, and to simultaneously deliver siRNAs targeting five different genes concurrently *in vivo*.

The functional effect of 7C1-mediated endothelial siRNA delivery was confirmed in animal models of vascular permeability, emphysema, primary tumor growth, and metastasis (Dahlman et al., 2014). However, in all these cases, a single siRNA was used to elicit a desired phenotype. Because multiple small RNAs can be formulated into a single 7C1 nanoparticle, we investigated whether targeted combination therapy could reduce disease progression and extend survival in a genetically engineered mouse model of nonsmall cell lung cancer (NSCLC) (Xue et al., 2014). A clinically relevant spontaneous tumor model in which lung epithelial cells simultaneously express tumorigenic Kras^{G12D} was investigated. These so-called KP tumors are extremely aggressive, and mimic both human lung cancer progression and response to therapeutics (Meylan et al., 2009). 7C1 was first formulated with a therapeutic miRNA called miR-34a that is downregulated in NSCLC. miR-34a replacement significantly reduced tumor growth without inducing measurable increases in serum cytokine expression. 7C1 nanoparticles were then formulated with siRNA targeting Kras, an oncogene that drives tumor progression and metastasis. siKras therapy significantly slowed tumor growth, and increased tumor apoptosis. The combination therapy with both miR-34a and siKras resulted in tumor regression and significantly extended

survival on its own and when used in concert with cisplatin, a first-line NSCLC therapy (Xue et al., 2014).



6. FUTURE PERSPECTIVES

Delivery systems that have been designed over the past 10 years have significantly improved the likelihood that successful siRNA therapies will be approved for clinical use. Many of the most clinically advanced delivery systems target hepatocytes. As a result, patients with a myriad of liver diseases stand to benefit from RNAi therapies. These advances are particularly exciting given the number of severe genetic liver diseases driven by a small number of genes that significantly diminish the quality of life (e.g., TTR-amyloidosis, hemophilia, and porphyria). Successful long-term inhibition of these genes may effectively cure these diseases. Because the biodistribution, pharmacokinetics, and safety profile of the administered drugs will not vary significantly with siRNA sequence, the same delivery vehicles may be used to treat liver diseases that are driven by a specific collection of genes with RNA combination therapies. Finally, as delivery systems that are administered subcutaneously continue to improve, siRNA therapies may also be used to treat more common diseases like hypercholesterolemia. For instance, inactive forms of ApoC3 or PCSK9 may significantly reduce serum cholesterol concentrations, and resultant cardiovascular disease. Pharmaceutical companies are developing antibody therapies that block the extracellular component of these targets. However, if subcutaneous conjugate delivery systems are shown to be safe and effective, these blockbuster antibodies may need to compete with siRNA therapies that “delete” both the extracellular and intracellular components of the protein target.

As RNA therapies become more commonplace in the clinic, they may be used in concert with traditional drugs. This effect is most easily illustrated in cancer: tumors undergo a number of genetic changes that decrease the efficacy of administered drugs. siRNA therapies targeting these resistance pathways can be designed to improve outcomes, for example, by knocking out efflux proteins that pump chemotherapeutics out of the cell. Similarly, cancer cells that are affected by a small molecule inhibiting one pathway survive by reverting to another distinct pathway. Rational combination therapies could use siRNAs that knockdown the second pathway, and increase the likelihood the cancer cell will undergo apoptosis.

While a majority of the most advanced siRNA-delivery systems currently target the liver, there are innumerable patients that would benefit

from efficient delivery to nonliver tissues. It is likely that significant advances in delivery vehicles targeting almost every other tissue in the body will be required before the same clinical success is observed outside the liver. For example, there are already many well-known genetic diseases that affect the function of skeletal muscle (muscular dystrophies), cardiomyocytes (cardiomyopathies), and neurons (Huntington's disease). Many patients afflicted with these disorders would likely experience a dramatic improvement in their quality of life if appropriate delivery vehicles are discovered. However, testing for effective *in vivo* delivery using traditional molecular biology techniques can be time-consuming, difficult, and expensive. As a result, new assays designed to easily measure nonliver delivery in a meaningful way will need to be developed to efficiently screen for lead candidates.

Nonliver delivery may be further improved by a more complete understanding of the physiology that promotes disease. For instance, a number of neurological disorders are characterized by inflammatory signaling that results in dysfunctional and leaky vasculature. This pathological change may be used to differentially deliver siRNA to regions affected by disease. In the same way, diseases that result in differential metabolism and subsequent changes in lipid uptake may be targeted by conjugating siRNA to the lipids that are taken up by diseased cells. Although not all diseases will result in physiological changes that promote delivery, many new strategies for specific passive targeting may be uncovered by understanding disease physiology more completely.

Techniques that helped dramatically improve the efficiency of liver-targeting LNPs may be applied to next-generation conjugates targeting nonliver tissues. Many of the most successful LNP formulations were discovered by screening large numbers of compounds that were synthesized using high-throughput chemistry. These chemical synthesis schemes were robust, and did not require purification steps. As a result, thousands of materials could be synthesized with relative ease. By contrast, the chemistry required to conjugate different materials directly to siRNA has remained slow, complicated, and expensive. New chemical synthetic schemes will need to be developed so that large material libraries of conjugates can be easily synthesized and tested.

Advances in nanoparticle- and conjugate-based delivery systems are sure to affect the future of RNA-based medicine. Because RNAs can bind to nearly every type of biomolecule in a cell, the number of diseases that can be impacted by their regulation is likely to increase rapidly. As a result, delivery systems which deliver siRNA may be exploited to deliver other small

RNAs, while completely new systems may be required to deliver larger RNAs. In just over 10 years, our understanding of RNAs has made it clear that they will continue to play an increasingly important role in medicine, as long as we can deliver them safely *in vivo*.

REFERENCES

- Aird, W. (2007). Phenotypic heterogeneity of the endothelium: II. Representative vascular beds. *Circulation research*, *100*, 174–190.
- Aird, W. C. (2006). Mechanisms of endothelial cell heterogeneity in health and disease. *Circulation Research*, *98*(2), 159–162.
- Akinc, A., Goldberg, M., Qin, J., Dorkin, J. R., Gamba-Vitalo, C., Maier, M., et al. (2009). Development of lipidoid–siRNA formulations for systemic delivery to the liver. *Molecular Therapy*, *17*(5), 872–879.
- Akinc, A., Querbes, W., De, S., Qin, J., Frank-Kamenetsky, M., Jayaprakash, K. N., et al. (2010). Targeted delivery of RNAi therapeutics with endogenous and exogenous ligand-based mechanisms. *Molecular Therapy*, *18*(7), 1357–1364.
- Akinc, A., Zumbuehl, A., Goldberg, M., Leshchiner, E. S., Busini, V., Hossain, N., et al. (2008). A combinatorial library of lipid-like materials for delivery of RNAi therapeutics. *Nature Biotechnology*, *26*(5), 561–569.
- Aleku, M., Schulz, P., Keil, O., Santel, A., Schaeper, U., Dieckhoff, B., et al. (2008). Atu027, a liposomal small interfering RNA formulation targeting protein kinase N3, inhibits cancer progression. *Cancer Research*, *68*(23), 9788–9798.
- Allen, T. M., & Cullis, P. R. (2013). Liposomal drug delivery systems: from concept to clinical applications. *Advanced Drug Delivery Reviews*, *65*(1), 36–48.
- Alnylam. (2013). *RNAi therapeutics using conjugate delivery Platform*. Retrieved from: <http://www.alnylam.com/web/wp-content/uploads/2013/10/ALNY-ConjugateUpdate-OTS2013.pdf>.
- 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. *Biotechnology and Bioengineering*, *99*(4), 975–985.
- Basha, G., Novobrantseva, T. I., Rosin, N., Tam, Y. Y. C., Hafez, I. M., Wong, M. K., et al. (2011). Influence of cationic lipid composition on gene silencing properties of lipid nanoparticle formulations of siRNA in antigen-presenting cells. *Molecular Therapy*, *19*(12), 2186–2200.
- Belliveau, N. M., Huft, J., Lin, P. J., Chen, S., Leung, A. K., Leaver, T. J., et al. (2012). Microfluidic synthesis of highly potent limit-size lipid nanoparticles for *in vivo* delivery of siRNA. *Molecular therapy. Nucleic Acids*, *1*, e37.
- Bumcrot, D., Manoharan, M., Koteliansky, V., & Sah, D. W. Y. (2006). RNAi therapeutics: a potential new class of pharmaceutical drugs. *Nature Chemical Biology*, *2*(12), 711–719.
- Butler, J. S., Chan, A., Charisse, K., Costelha, S., Fishman, S., Concalves, P., et al. (2014). *Pre-clinical evaluation of RNAi therapeutics for the treatment of ATTR: An update*. Retrieved from: <http://www.alnylam.com/web/assets/Preclinical-Evaluation-of-RNAiTx-for-TTR-Poster-ISA2014.pdf>.
- Carmeliet, P., & Jain, R. K. (2011). Molecular mechanisms and clinical applications of angiogenesis. *Nature*, *473*(7347), 298–307.
- Chang, P. V., Prescher, J. a, Sletten, E. M., Baskin, J. M., Miller, I. a, Agard, N. J., et al. (2010). Copper-free click chemistry in living animals. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(5), 1821–1826.
- Chiang, A. C., & Massagué, J. (2008). Molecular basis of metastasis. *The New England Journal of Medicine*, *359*(26), 2814–2823.

- ClinicalTrials.gov. (2008). NCT00689065: Safety study of CALAA-01 to treat solid tumor cancers. Retrieved from: <http://www.clinicaltrials.gov/show/NCT00689065>.
- ClinicalTrials.gov. (2010). NCT01148953: Trial to evaluate safety and tolerability of ALN-TTR01 in transthyretin (TTR) amyloidosis.
- ClinicalTrials.gov. (2011). NCT01437059: Trial to evaluate safety and tolerability of ALN-PCSO2 in subjects with elevated LDL-cholesterol (LDL-C). Retrieved from: <http://www.clinicaltrials.gov/show/NCT01437059>.
- ClinicalTrials.gov. (2012). NCT01559077: Trial to evaluate safety, tolerability, and pharmacokinetics of ALN-TTR02 in healthy volunteer subjects. Retrieved from: <http://www.clinicaltrials.gov/show/NCT01559077>.
- ClinicalTrials.gov. (2012). NCT01617967: Trial to evaluate safety and tolerability of ALN-TTR02 in transthyretin (TTR) amyloidosis. Retrieved from: <http://www.clinicaltrials.gov/show/NCT01617967>.
- ClinicalTrials.gov. (2013). NCT01960348: The study of an investigational drug, ALN-TTR02, for the treatment of transthyretin (TTR)-mediated amyloidosis. Retrieved from: <http://www.clinicaltrials.gov/show/NCT01960348>.
- ClinicalTrials.gov. (2013). NCT01872065: Safety and tolerability study of ARC-520 in healthy volunteers. Retrieved from: <http://www.clinicaltrials.gov/show/NCT01872065>.
- ClinicalTrials.gov. (2014). NCT02065336: Study of ARC-520 in patients with chronic hepatitis B virus.
- Coelho, T., Adams, D., Silva, A., Lozeron, P., Hawkins, P. N., Mant, T., et al. (2013). Safety and efficacy of RNAi therapy for transthyretin amyloidosis. *The New England Journal of Medicine*, 369(9), 819–829.
- Dahlman, J. E., Barnes, C., Khan, O., Thiriot, A., Jhunjunwala, S., Shaw, T. E., et al. (May 2014). *In vivo* endothelial siRNA delivery using polymeric nanoparticles with low molecular weight. *Nature Nanotechnology*, 19, 648–655.
- Davis, M. E., Zuckerman, J. E., Choi, C. H. J., Seligson, D., Tolcher, A., Alabi, C. a., et al. (2010). Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. *Nature*, 464(7291), 1067–1070.
- Deleavey, G. F., & Damha, M. J. (2012). Designing chemically modified oligonucleotides for targeted gene silencing. *Chemistry & Biology*, 19(8), 937–954.
- Dohmen, C., Fröhlich, T., Lächelt, U., Röhl, I., Vornlocher, H.-P., Hadwiger, P., et al. (2012). Defined folate-PEG-siRNA conjugates for receptor-specific gene silencing. *Molecular therapy. Nucleic acids*, 1, e7.
- Dong, Y., Love, K. T., Dorkin, J. R., Sirirungruang, S., Zhang, Y., Chen, D., et al. (2014). Lipopeptide nanoparticles for potent and selective siRNA delivery in rodents and nonhuman primates. *Proceedings of the National Academy of Sciences*, 111(11), 3955–3960.
- Drickamer, K. (1996). Selective sugar binding to the carbohydrate recognition domains of the rat hepatic and macrophage asialoglycoprotein receptors. *Journal of Biological Chemistry*, 271(12), 6686–6693.
- Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., & Mello, C. C. (February 1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*, 391, 806–811.
- Fitzgerald, K., Frank-Kamenetsky, M., Shulga-Morskaya, S., Liebow, A., Bettencourt, B. R., Sutherland, J. E., et al. (2014). Effect of an RNA interference drug on the synthesis of proprotein convertase subtilisin/kexin type 9 (PCSK9) and the concentration of serum LDL cholesterol in healthy volunteers: a randomised, single-blind, placebo-controlled, phase 1 trial. *Lancet*, 383(9911), 60–68.
- Fogal, V., Zhang, L., Krajewski, S., & Ruoslahti, E. (2008). Mitochondrial/cell-surface protein p32/gC1qR as a molecular target in tumor cells and tumor stroma. *Cancer Research*, 68(17), 7210–7218.
- Fridman, W. H., Pagès, F., Sautès-Fridman, C., & Galon, J. (2012). The immune contexture in human tumours: impact on clinical outcome. *Nature Reviews Cancer*, 12(4), 298–306.

- Gilleron, J., Querbes, W., Zeigerer, A., Borodovsky, A., Marsico, G., Schubert, U., et al. (2013). Image-based analysis of lipid nanoparticle-mediated siRNA delivery, intracellular trafficking and endosomal escape. *Nature Biotechnology*, 31(7), 638–646.
- Gonzalez, H., Hwang, S. J., & Davis, M. E. (1999). New class of polymers for the delivery of macromolecular therapeutics. *Bioconjugate Chemistry*, 10(6), 1068–1074.
- Gray, J. (2010). Cancer: genomics of metastasis. *Nature*, 464, 989–990.
- Hagberg, C. E., Falkevall, A., Wang, X., Larsson, E., Huusko, J., Nilsson, I., et al. (2010). Vascular endothelial growth factor B controls endothelial fatty acid uptake. *Nature*, 464(7290), 917–921.
- Hagberg, C. E., Mehlem, A., Falkevall, A., Muhl, L., Fam, B. C., Ortsäter, H., et al. (2012). Targeting VEGF-B as a novel treatment for insulin resistance and type 2 diabetes. *Nature*, 490(7420), 426–430.
- Heidel, J. D., Liu, J. Y. -C., Yen, Y., Zhou, B., Heale, B. S. E., Rossi, J. J., et al. (2007). Potent siRNA inhibitors of ribonucleotide reductase subunit RRM2 reduce cell proliferation in vitro and in vivo. *Clinical cancer research*, 13(7), 2207–2215.
- Heidel, J. D., Yu, Z., Liu, J. Y. -C., Rele, S. M., Liang, Y., Zeidan, R. K., et al. (2007). Administration in non-human primates of escalating intravenous doses of targeted nanoparticles containing ribonucleotide reductase subunit M2 siRNA. *Proceedings of the National Academy of Sciences of the United States of America*, 104(14), 5715–5721.
- Herbert, S. P., & Stainier, D. Y. R. (2011). Molecular control of endothelial cell behaviour during blood vessel morphogenesis. *Nature Reviews. Molecular Cell Biology*, 12(9), 551–564.
- Hermanson, G. T. (1996). *Bioconjugate techniques* (1st ed.). Academic Press.
- Heyes, J., Palmer, L., Bremner, K., & MacLachlan, I. (2005). Cationic lipid saturation influences intracellular delivery of encapsulated nucleic acids. *Journal of Controlled Release*, 107(2), 276–287.
- Hilgenbrink, A. R., & Low, P. S. (2005). Folate receptor-mediated drug targeting: from therapeutics to diagnostics. *Journal of Pharmaceutical Sciences*, 94(10), 2135–2146.
- Jain, K., Kesharwani, P., Gupta, U., & Jain, N. K. (2012). A review of glycosylated carriers for drug delivery. *Biomaterials*, 33(16), 4166–4186.
- Jain, R. K. (1994). Barriers to drug delivery in solid tumors. *Scientific American*, 271, 58–65.
- Jain, R. K. (2005). Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. *Science*, 307(5706), 58–62.
- Jayaraman, M., Ansell, S. M., Mui, B. L., Tam, Y. K., Chen, J., Du, X., et al. (2012). Maximizing the potency of siRNA lipid nanoparticles for hepatic gene silencing in vivo. *Angewandte Chemie (International ed. in English)*, 51(34), 8529–8533.
- Jeck, W. R., & Sharpless, N. E. (2014). Detecting and characterizing circular RNAs. *Nature Biotechnology*, 32(5), 453–461.
- Kanasty, R., Dorkin, J. R., Vegas, A., & Anderson, D. (2013). Delivery materials for siRNA therapeutics. *Nature Materials*, 12(11), 967–977.
- Kanasty, R. L., Whitehead, K. A., Vegas, A. J., & Anderson, D. G. (2012). Action and reaction: the biological response to siRNA and its delivery vehicles. *Molecular Therapy*, 20(3), 513–524.
- Kaufmann, J., Ahrens, K., & Santel, A. (2010). RNA interference for therapy in the vascular endothelium. *Microvascular Research*, 80(2), 286–293.
- Kim, E.-M., Jeong, H.-J., Park, I.-K., Cho, C.-S., Moon, H.-B., Yu, D.-Y., et al. (2005). Asialoglycoprotein receptor targeted gene delivery using galactosylated polyethyleneimine-graft-poly(ethylene glycol): in vitro and in vivo studies. *Journal of Controlled Release*, 108(2–3), 557–567.
- Kumar, V., Abbas, A., Fausto, N., & Aster, J. (2009). *Pathologic basis of disease* (8th ed.). Robbins and Cotran.

- Lee, H., Lytton-Jean, A. K. R., Chen, Y., Love, K. T., Park, A. I., Karagiannis, E. D., et al. (June 2012). Molecularly self-assembled nucleic acid nanoparticles for targeted *in vivo* siRNA delivery. *Nature Nanotechnology*, 7, 389–393.
- Leung, A. K. K., Hafez, I. M., Baoukina, S., Belliveau, N. M., Zhigaltsev, I. V., Afshinmanesh, E., et al. (2012). Lipid nanoparticles containing siRNA synthesized by microfluidic mixing exhibit an electron-dense nanostructured core. *The Journal of Physical Chemistry C*, 116, 18440–18450.
- Love, K. T., Mahon, K. P., Levins, C. G., Whitehead, K. a, Querbes, W., Dorkin, J. R., et al. (2010). Lipid-like materials for low-dose, *in vivo* gene silencing. *Proceedings of the National Academy of Sciences of the United States of America*, 107(5), 1864–1869.
- Lu, J., Li, Z., Zink, J. I., & Tamaroi, F. (2012). *In vivo* tumor suppression efficacy of mesoporous silica nanoparticles-based drug-delivery system: enhanced efficacy by folate modification. *Nanomedicine: Nanotechnology, Biology, and Medicine*, 8(2), 212–220.
- Maier, M. A., Jayaraman, M., Matsuda, S., Liu, J., Barros, S., Querbes, W., et al. (2013). Biodegradable lipids enabling rapidly eliminated lipid nanoparticles for systemic delivery of RNAi therapeutics. *Molecular therapy: The Journal of the American Society of Gene Therapy*, 21(8), 1570–1578.
- Mamidyala, S. K., Dutta, S., Chrunyk, B. a, Préville, C., Wang, H., Withka, J. M., et al. (2012). Glycomimetic ligands for the human asialoglycoprotein receptor. *Journal of the American Chemical Society*, 134(4), 1978–1981.
- Meier, M., Bider, M. D., Malashkevich, V. N., Spiess, M., & Burkhard, P. (2000). Crystal structure of the carbohydrate recognition domain of the H1 subunit of the asialoglycoprotein receptor. *Journal of Molecular Biology*, 300(4), 857–865.
- Meylan, E., Dooley, A. L., Feldser, D. M., Shen, L., Turk, E., & Ouyang, C. (2009). Requirement for NF- κ B signaling in a mouse model of lung cancer. *Nature*, 462(7269), 104–107.
- Monopoli, M. P., Aberg, C., Salvati, A., & Dawson, K. a (2012). Biomolecular coronas provide the biological identity of nanosized materials. *Nature Nanotechnology*, 7(12), 779–786.
- Nguyen, D. X., & Massagué, J. (2007). Genetic determinants of cancer metastasis. *Nature Reviews Genetics*, 8(5), 341–352.
- Nolan, C. J., Damm, P., & Prentki, M. (2011). Type 2 diabetes across generations: from pathophysiology to prevention and management. *Lancet*, 378(9786), 169–181.
- Parmar, R. G., Busuek, M., Walsh, E. S., Leander, K. R., Howell, B. J., Sepp-Lorenzino, L., et al. (2013). Endosomolytic bio-reducible poly(amido amine disulfide) polymer conjugates for the *in vivo* systemic delivery of siRNA therapeutics. *Bioconjugate Chemistry*, 24(4), 640–647.
- Pober, J. S., & Sessa, W. C. (2007). Evolving functions of endothelial cells in inflammation. *Nature Reviews Immunology*, 7(10), 803–815.
- Rader, D. J., Cohen, J., & Hobbs, H. H. (2003). Monogenic hypercholesterolemia: new insights in pathogenesis and treatment. *The Journal of Clinical Investigation*, 111(12), 1795–1803.
- Rana, T. M. (2007). Illuminating the silence: understanding the structure and function of small RNAs. *Nature Reviews Molecular Cell Biology*, 8(1), 23–36.
- Ren, Y., Cheung, H. W., von Maltzhan, G., Agrawal, A., Cowley, G. S., Weir, B. a, et al. (2012). Targeted tumor-penetrating siRNA nanocomplexes for credentialing the ovarian cancer oncogene ID4. *Science Translational Medicine*, 4(147), 1–12.
- Rensen, P. C., Sliedregt, L. A., Ferns, M., Kieviet, E., van Rossenberg, S. M., van Leeuwen, S. H., et al. (2001). Determination of the upper size limit for uptake and processing of ligands by the asialoglycoprotein receptor on hepatocytes *in vitro* and *in vivo*. *The Journal of Biological Chemistry*, 276(40), 37577–37584.
- Rinn, J. L., & Chang, H. Y. (2012). Genome regulation by long noncoding RNAs. *Annual Review of Biochemistry*, 81, 145–166.

- Rozema, D. B., Lewis, D. L., Wakefield, D. H., Wong, S. C., Klein, J. J., Roesch, P. L., et al. (2007). Dynamic PolyConjugates for targeted *in vivo* delivery of siRNA to hepatocytes. *Proceedings of the National Academy of Sciences of the United States of America*, 104(32), 12982–12987.
- Sabin, L. R., Delás, M. J., & Hannon, G. J. (2013). Dogma derailed: the many influences of RNA on the genome. *Molecular Cell*, 49(5), 783–794.
- Sander, J. D., & Joung, J. K. (2014). CRISPR–Cas systems for editing, regulating and targeting genomes. *Nature Biotechnology*, 32(4), 347–355.
- Santel, A., Aleku, M., Keil, O., Endruschat, J., Esche, V., Durieux, B., et al. (2006). RNA interference in the mouse vascular endothelium by systemic administration of siRNA–lipoplexes for cancer therapy. *Gene Therapy*, 13(18), 1360–1370.
- Santel, A., Aleku, M., Keil, O., Endruschat, J., Esche, V., Fisch, G., et al. (2006). A novel siRNA–lipoplex technology for RNA interference in the mouse vascular endothelium. *Gene Therapy*, 13(16), 1222–1234.
- Schroeder, A., Heller, D. A., Winslow, M. M., Dahlman, J. E., Pratt, G. W., Langer, R., et al. (2012). Treating metastatic cancer with nanotechnology. *Nature Reviews Cancer*, 12(1), 39–50.
- Seiple, S. C., Akinc, A., Chen, J., Sandhu, A. P., Mui, B. L., Cho, C. K., et al. (2010). Rational design of cationic lipids for siRNA delivery. *Nature Biotechnology*, 28(2), 172–176.
- Soutschek, J., Akinc, A., Bramlage, B., Charisse, K., Constien, R., Donoghue, M., et al. (2004). Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature*, 432(7014), 173–178.
- Spies, M. (1990). The asialoglycoprotein receptor: a model for endocytic transport receptors. *Biochemistry*, 29(43).
- Tenzer, S., Docter, D., Kuharev, J., Musyanovych, A., Fetz, V., Hecht, R., et al. (2013). Rapid formation of plasma protein corona critically affects nanoparticle pathophysiology. *Nature Nanotechnology*, 8(10), 772–781.
- Tortorella, S., & Karagiannis, T. C. (2014). Transferrin receptor-mediated endocytosis: a useful target for cancer therapy. *The Journal of Membrane Biology*, 247(4), 291–307.
- Ulitsky, I., & Bartel, D. P. (2013). lincRNAs: genomics, evolution, and mechanisms. *Cell*, 154(1), 26–46.
- Wang, J., Loberg, R., & Taichman, R. S. (2006). The pivotal role of CXCL12 (SDF-1)/CXCR4 axis in bone metastasis. *Cancer Metastasis Reviews*, 25(4), 573–587.
- Wang, M., Alberti, K., Varone, A., Pouli, D., Georgakoudi, I., & Xu, Q. (2014). Enhanced intracellular siRNA delivery using bioreducible lipid-like nanoparticles. *Advanced Healthcare Materials*, 3(9), 1398–1403.
- Weis, W. I. (1996). Structural basis of galactose recognition by C-type animal lectins. *Journal of Biological Chemistry*, 271(12), 6679–6685.
- Whitehead, K. A., Dahlman, J. E., Langer, R. S., & Anderson, D. G. (2011). Silencing or stimulation? siRNA delivery and the immune system. *Annual Review of Chemical and Biomolecular Engineering*, 2(1), 77–96.
- Whitehead, K. A., Dorkin, J. R., Vegas, A. J., Chang, P. H., Veiseh, O., Matthews, J., et al. (2014). Degradable lipid nanoparticles with predictable *in vivo* siRNA delivery activity. *Nature Communications*, 5, 4277.
- Whitehead, K. A., Langer, R., & Anderson, D. G. (2009). Knocking down barriers: advances in siRNA delivery. *Nature Reviews Drug Discovery*, 8(2), 129–138.
- Whitehead, K. a, Matthews, J., Chang, P. H., Nirouy, F., Dorkin, J. R., Severgnini, M., et al. (2012). *In vitro-in vivo* translation of lipid nanoparticles for hepatocellular siRNA delivery. *ACS Nano*, 6, 6922–6929.
- Wisse, E., Jacobs, F., Topal, B., Frederik, P., & De Geest, B. (2008). The size of endothelial fenestrae in human liver sinusoids: implications for hepatocyte-directed gene transfer. *Gene therapy*, 15(17), 1193–1199.

- Wong, S. C., Klein, J. J., Hamilton, H. L., Chu, Q., Frey, C. L., Trubetsky, V. S., et al. (2012). Co-injection of a targeted, reversibly masked endosomolytic polymer dramatically improves the efficacy of cholesterol-conjugated small interfering RNAs *in vivo*. *Nucleic Acid Therapeutics*, 22(6), 380–390.
- Wooddell, C. I., Rozema, D. B., Hossbach, M., John, M., Hamilton, H. L., Chu, Q., et al. (2013). Hepatocyte-targeted RNAi therapeutics for the treatment of chronic hepatitis B virus infection. *Molecular Therapy*, 21(5), 973–985.
- Xue, W., Dahlman, J. E., Tammela, T., Khan, O. F., et al. (2014). Small RNA combination therapy for lung cancer. *Proceedings of the National Academy of Sciences of the United States of America*, 111(34), E3553–E3561.
- Yamada, T., Peng, C. G., Matsuda, S., Addepalli, H., Jayaprakash, K. N., Alam, M. R., et al. (2011). Versatile site-specific conjugation of small molecules to siRNA using click chemistry. *The Journal of Organic Chemistry*, 76(5), 1198–1211.