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REVIEW ARTICLE

doi:10.1016/j.ymthe.2006.01.001

Progress Towards *in Vivo* Use of siRNAs

Mark A. Behlke*

Integrated DNA Technologies, Inc., Coralville, IA 52241, USA

**To whom correspondence and reprint requests should be addressed at Integrated DNA Technologies, Inc., 1710 Commercial Park, Coralville, IA 52241, USA. Fax: +1 319 626 8444. E-mail: mbehlke@idtdna.com.*

Available online 14 February 2006

RNA interference (RNAi) has become the method of choice to suppress gene expression *in vitro*. It is also emerging as a powerful tool for *in vivo* research with over 90 studies published using synthetic small interfering RNAs in mammals. These reports demonstrate the potential for use of synthetic small interfering RNAs (siRNAs) as therapeutic agents, especially in the areas of cancer and viral infection. The number of reports using siRNAs for functional genomics applications, for validation of targets for small-molecule drug development programs, and to address questions of basic biology will rapidly grow as methods and protocols for use in animals become more established. This review will first discuss aspects of RNAi biochemistry and biology that impact *in vivo* use, especially as relates to experimental design, and will then provide an overview of published work with a focus on methodology.

Key Words: antisense, RNA interference, siRNA, oligonucleotide, therapeutics, mammals drug delivery systems, liposomes, innate immunity

Contents

1525-0016/$30.00

INTRODUCTION

Compounds that can selectively alter expression levels of any gene have been sought by basic scientists and clinicians for many years. Nucleic acid hybridization is very specific, and theoretically the sequence of any desired gene could be uniquely targeted by an oligonucleotide of complementary sequence. The first "antisense" experiments were performed over 25 years ago and employed DNA oligonucleotides to block translation of Rous sarcoma virus RNA [1,2]. Experiments demonstrating the use of antisense RNA to inhibit gene expression soon followed [3,4]. Most antisense methods that use single-stranded DNA (ssDNA) or single-stranded RNA (ssRNA) oligonucleotides rely upon either RNase H-mediated degradation of the target mRNA or steric blockade of translation for their mechanism of action [5]. However, other pathways have been exploited with favorable results. For example, 2′,5′-oligoadenylate (2-5A) triggers action of RNase L; conjugation of 2-5L to an antisense oligonucleotide sequence enables sequence-specific targeting of this otherwise nonspecific ribonuclease [6].

Extensive medicinal chemistry has been done on antisense nucleic acids and hundreds of compounds have been tested in a search for modifications that improve nuclease stability, increase binding affinity (melting temperature, or T_m), and improve the *in vivo* pharmacodynamic properties of oligonucleotides as drugs [7,8]. Pharmaceutical companies have been working to develop antisense drugs for more than 15 years and extensive knowledge of the pharmacology and toxicology of synthetic nucleic acids *in vivo* has been compiled [9]. As of 2005, one antisense drug is on the market. Formivirsen (Vitravene), a product of Isis Pharmaceuticals and Novartis Ophthalmics, is a 21-mer phosphorothioate DNA oligonucleotide that is complementary to the immediate early region 2 of cytomegalovirus (CMV). This compound has been shown to be effective in the treatment of CMV retinitis and was approved by the FDA for use by direct intravitreal injection in 1998 [10,11]. Currently, over 20 antisense drugs are in various stages of clinical testing [12]. Although antisense methods have been successfully used for years to study gene function, antisense technology has failed to gain widespread acceptance as a gene knockdown tool, largely due to the extensive experimental testing that often needs to be done to find effective target sites within the gene of interest.

In 1998 Fire and Mello described a process in *Caenorhabditis elegans* in which long dsRNA (several hundred bases in length) caused suppression of complementary genes and called this process RNA interference (RNAi) [13]. Although the mechanism was not understood at the time, RNAi had actually been seen as early as 1990 in plants as the phenomenon of cosuppression [14]. In plants, it is suspected that the degradative pathways of RNAi evolved as an antiviral defense mechanism. In higher organisms, antiviral defense mechanisms are more complex and long dsRNAs induce interferon secretion, activating a cascade of interferon-stimulated genes (ISGs) as well as RNAi. Therefore, long dsRNAs should not be used as an experimental tool to trigger RNAi in mammalian cells. Fortunately, long dsRNAs do not mediate suppression directly. Instead, long dsRNAs are processed by the endoribonuclease Dicer into short 21-bp duplexes with 2-base 3′ overhangs called small interfering (si) RNAs, which are the actual effector species [15–17]. Synthetic siRNAs can be transfected into mammalian cells and specifically suppress expression of complementary genes [18], usually without triggering interferon responses. The siRNA enters a multimember protein complex called RISC (RNA induced silencing complex) by which one strand of the duplex (the "passenger strand") is cleaved and discarded [19,20]. The other strand is retained and serves as the "guide strand," which directs the sequence specificity of RISC and therefore of RNAi silencing. Some of the components of human RISC have been identified and have assigned functions. Argonaute 2 (Ago2) functions as "slicer," the RNA endonuclease that cleaves a target RNA species as directed by the siRNA guide strand [21–23]. Dicer and TRBP form a heterodimer, which processes long dsRNA into siRNAs [24,25], and together complex with the siRNA and Ago2 to form a functional silencing complex [26]. The biochemistry of degradative RNAi has been extensively studied and has been well summarized in recent reviews [27–31].

RNAi exploits a complex, natural pathway that regulates gene expression and includes machinery for sequence-specific mRNA degradation. Specific siRNA design rules have been developed from studies that compared effective vs ineffective duplexes and it is now possible to obtain potent RNAi reagents without the extensive testing needed when using traditional antisense approaches [32–37]. SiRNAs can be surprisingly potent. Transfections done using subnanomolar concentrations of RNA sometimes achieve >90% reduction in mRNA levels [38]. RNAi has rapidly been adopted by the research community and a keyword search for "RNAi or siRNA or RNA interference" finds that almost 9000 citations already exist in the PubMed database. RNAi has largely replaced antisense as the gene knockdown tool of choice for most applications *in vitro* and is showing similar promise for *in vivo* applications.

Two different general approaches can be used to trigger RNA interference *in vivo*. First, synthetic RNA duplexes can be introduced directly into animals; the RNAi effect will be transient. In this case, the dsRNA is usually chemically synthesized and the problems surrounding its use parallel those encountered in small-molecule or antisense drug development. Alternatively, dsRNA can be expressed inside cells using DNA templates that direct synthesis of RNA duplexes or short hairpin RNAs; in this case the RNAi effect can be sustained and could theoretically be permanent, depending upon the vector employed and whether

doi:10.1016/j.ymthe.2006.01.001

expression levels are adequately maintained long term. DNA-directed RNAi (ddRNAi) expression cassettes can be introduced into animals using plasmid or viral vectors. Problems surrounding this approach parallel those encountered with other forms of gene therapy. The present review is restricted to the use of chemically synthesized siRNA duplexes *in vivo* with a focus on methodology. Other recent reviews consider *in vivo* use of siRNA from different perspectives [39–48].

CONSIDERATIONS FOR *IN VIVO* USE OF siRNAs

A number of important issues need to be considered before embarking on *in vivo* studies using RNAi. These include site selection, compound design and chemistry, controls, route of administration, and use of a delivery vehicle.

Selection of Target Site and Duplex Design

Site selection and optimization do not appear to be as difficult for siRNA as would be expected from historical experience with antisense oligonucleotide site selection. Effective site selection algorithms are available from several commercial and academic sources and key elements of design criteria have been published [32–37]. Before undertaking an *in vivo* experiment, however, actual validation of duplexes *in vitro* should be performed; it is usually necessary to test only a small number of duplexes, possibly as few as three or four, to find suitable candidates. It is preferable to have more than one effective siRNA available for each target to help control for off-target effects (see below). For siRNA therapeutic applications, more extensive optimization will of course be expected.

Most *in vivo* siRNA experiments reported to date used 21-mer duplexes that have a 19-base central double-stranded domain with terminal 2-base 3′ overhangs. These reagents mimic the products naturally produced by Dicer processing *in vivo*. Other designs are available that may be worth consideration. Blunt 19-mer duplexes with extensive 2′-*O*-methyl modifications have been shown to have favorable properties [49]; blunt 25-mer duplexes, with sense-strand 2′-*O*-methyl modifications, have also been used with good results [50]. Asymmetric 27-mer duplexes that are substrates for Dicer have been shown to have increased potency *in vitro* compared with 21-mers [38,51] and may have similar benefit *in vivo*.

Chemical Modification

Although duplex RNA is more resistant to nuclease attack than single-stranded DNA, unmodified siRNAs are nevertheless rapidly degraded when administered intravenously in mammals; degradation can be delayed or avoided by chemical modification of the oligonucleotide and/or by complexation with a carrier/delivery particle [52]. A wide variety of chemical modifications that confer nuclease resistance have been successfully used in single-stranded antisense oligonucleotides and ribozymes; many of these modifications can be imported directly into siRNA use and will provide similar benefit.

Phosphorothioate or boranophosphate modification of the internucleoside linkage improves nuclease stability. Boranophosphate-modified RNAs have substantial nuclease resistance; unfortunately, boranophosphate-modified RNAs cannot simply be manufactured using standard chemical synthesis methods. Instead, borano-modified bases are incorporated in RNA made by enzymatic synthesis using *in vitro* transcription [53], which makes site-selective placement of this modification difficult. Phosphorothioate (PS) modifications can be easily placed in the RNA duplex at any desired position and will prolong the life of the duplex when exposed to serum or other nuclease sources. Limited PS modification preserves potency of the siRNA; however, extensively modified duplexes show reduced potency and/or toxicity [54–57]. Extensive PS modification can also lead to artifacts due to nonspecific protein binding [58], so it may be preferable to limit the use of this modification to terminal positions (or 3′ overhangs) where added nuclease resistance is probably most important. Other nuclease-resistant modifications that do not have these kinds of problems are available.

Modification of the 2′ position of the ribose can increase duplex stability (T_m) and confers varying degrees of nuclease resistance. 2′-*O*-methyl RNA is a naturally occurring base that is present in mammalian ribosomal RNA. A number of reports have described patterns of 2′-*O*-methyl incorporation that retain full potency of the siRNA compared with unmodified RNA and are stable in serum [49,55,57]. The precise pattern of incorporation is important and RNAs that are entirely substituted with 2′-*O*-methyl are inactive as triggers of RNAi. Alternating the 2′-*O*-methyl bases retains potency and confers substantial nuclease resistance [49]. The 2′-fluoro (2′-F) modification has also been used with good results [54–56]. This modification is usually incorporated selectively at pyrimidine bases due to reagent issues. Their use either alone or in combination with 2′-*O*-methyl purines will stabilize RNA duplexes. In fact, duplexes can be fully modified such that no unmodified RNA remains [59]. Locked nucleic acids (LNAs) are a different class of 2′ modification in which a methylene bridge connects the 2′-O with the 4′-C of the ribose. This modification "locks" the ribose in the 3′-*endo* conformation and improves both duplex stability and nuclease resistance. LNAs can be incorporated into siRNAs; however, placement to achieve best effect is more restricted than for 2′-*O*-methyl or 2′-F bases [56,60,61].

Several studies have already been conducted in mice that compared the functional performance of modified vs unmodified siRNA duplexes. Layzer and colleagues modified an anti-luciferase siRNA with 2′-F pyrimidines and compared it with unmodified siRNA *in vivo* using hydrodynamic tail vein injection. SiRNAs were co-injected with

a luciferase expression plasmid and luciferase activity in the liver was assessed by *in vivo* bioluminescence imaging 2–7 days after injection [62]. Despite improved nuclease stability, no functional difference in luciferase knockdown was observed between modified and unmodified siRNAs. Using hydrodynamic delivery, injected nucleic acids are rapidly taken up by hepatocytes, thus nuclease resistance may not be as important for siRNA administered using this approach than when using more traditional low-pressure intravenous administration. Unfortunately, only a single 10-μg siRNA dose was used, making it difficult to assess potency accurately without the additional data obtained from dose–response curves. Other groups have reported substantial benefit from the use of highly modified siRNA duplexes. Morrissey and colleagues compared activity of an unmodified siRNA with that of a highly modified duplex that included DNA bases, 2′-*O*-methyl purines, 2′-F pyrimidines, terminal inverted-dT bases, and PS linkage modification at select positions (leaving no unmodified RNA). A hepatitis B virus (HBV) expression plasmid was co-injected with modified or unmodified duplexes into the tail vein of mice using the same hydrodynamic delivery method, and production of HBV DNA and hepatitis B surface antigen was assayed 72 h postinjection [63]. A 2-log dose–response curve was performed and the modified duplexes were significantly more potent. Unmodified RNA may be suitable for use in some *in vivo* research applications, especially if a delivery vehicle that offers protection from serum nucleases is employed. However, there may be reasons beyond nuclease resistance to include modified bases in siRNAs used *in vivo* (see discussion of innate immunity below).

Various chemical groups can be placed at the ends of oligonucleotides that facilitate entry into cells or improve biodistribution. Experience with modifications in this category has been gained from work done to improve properties of antisense oligonucleotides and can be applied directly to siRNAs [8]. For example, conjugation of cholesterol to a siRNA can improve serum protein binding, improve pharmacokinetics, and increase delivery to hepatocytes [64,65]. Other lipid conjugates can be used, although synthesis is currently more complex [64]. A dinitrophenol end modification has been shown to improve transfection and increase intracellular stability in tissue culture [66]. Perhaps of greatest interest, peptide–oligonucleotide conjugates can have improved cell permeation properties as well as nuclear targeting, if desired [67]. Peptide delivery will be considered in greater detail below.

Adverse Effects and Controls

It seems unlikely that major adverse effects will be observed from the use of siRNAs *in vivo* that could not be predicted from prior experience with the toxicology of antisense oligonucleotides and ribozymes. Early results suggest that the biodistribution of siRNAs will be grossly similar to that seen using single-stranded DNA oligonucleotides [68]. Phase I clinical trials are already ongoing and more information will soon be available about the pharmacology of siRNAs in humans. Some specific problems have already been identified, which need to be considered in experimental design and inclusion of appropriate controls, including stimulation of the innate immune system (leading to interferon pathway cascade) and off-target effects (OTEs).

Interferon Induction and the Innate Immune System

Perhaps the most significant unexpected "side effect" encountered in the use of antisense DNA oligonucleotides was a sequence-specific ability to trigger the innate immune system. This property was traced to the recognition of motifs that contained nonmethylated dC bases in the context of a "CpG" dinucleotide by Toll-like receptor 9 (TLR9). Although originally viewed as a nuisance side effect, this observation opened an entirely new therapeutic use for DNA oligonucleotides as modulators of the immune system [69–72]. The innate immune system relies upon a series of receptor molecules that recognize "pathogen-associated molecular patterns" and that initiate a variety of cellular responses when these motifs are encountered [73,74]. One class of these receptors, the Toll-like receptor family, can recognize compounds that range from bacterial lipopolysaccharide (LPS) to DNA.

While long dsRNA is a well-known trigger of type-1 interferon (IFN) responses in mammals, it was originally hoped that short dsRNAs, like siRNAs, would be "safe" to use. However, siRNAs are also capable of triggering IFN responses. These effects can be both sequence specific and cell-type specific and therefore are not always encountered. Members of the Toll-like receptor family that recognize nucleic acids reside intracellularly within endosomal or lysosomal compartments (other TLRs reside on the cell surface). Of the 11 known TLRs, 3 that specifically interact with RNA have been identified, including TLR3, 7, and 8 [75–77]. Other molecules that recognize dsRNA and can trigger IFN pathway responses include dsRNA-dependent protein kinase (PKR), 2′,5′-oligoadenylate synthetase (OAS), and retinoic acid-inducible gene I (RIG-I) [78,79].

Sledz and colleagues reported that 21-mer siRNAs could cause interferon-mediated activation of the JAK–STAT pathway and a dose-dependent induction of ISGs in T98G cells that was attributed to PKR activation [80]. This effect appeared to be sequence independent as two different siRNAs that did not share any obvious sequence motifs triggered the same effect. In contrast, stimulation of IFN-α, IFN-β, or phospho-PKR was not detected after administration of either 21-mer or 27-mer dsRNAs in HEK293 cells [38]. Activation of 2′,5′-OAS by dsRNA requires a minimum RNA length of 30 bp and maximal activation is not achieved until length reaches 60–80 bp [81], so direct triggering of 2′,5′-OAS by siRNAs is unlikely

doi:10.1016/j.ymthe.2006.01.001

to be a problem. Requirements for RIG-I activation are under investigation, but it appears that it can recognize short siRNAs and may be a major pathway for IFN production and activation of ISGs, at least *in vitro* (J. T. Marques and B. R. G. Williams, personal communication). RNA synthesized by *in vitro* transcription retains a triphosphate group at the 5′ end, which can trigger a robust IFN response [82]; this is easily avoided by use of chemically synthesized RNA of known structure and purity.

Toll-like receptor 3 recognizes dsRNA [83] and certain siRNAs have been shown to trigger release of IFN-α, IFN-β, and interleukin 8 (IL-8) in HEK293 cells; this effect is dose dependent and is magnified by overexpression of TLR3 [84,85]. Toll-like receptors 7 and 8 recognize ssRNA [86] and can be triggered by siRNAs in a sequence-specific fashion. Single-stranded RNA is more effective than double-stranded RNA in triggering TLR7 and 8 responses [87]. The sequences UGUGU [88,89] and GUCCUUCAA [90] have been demonstrated to stimulate secretion of a variety of cytokines from TLR7- or 8-bearing cell types. TLR7 and 8 are expressed in a limited number of cell types including myeloid dendritic cells, plasmacytoid dendritic cells, neutrophils, B cells, and monocytes. However, these cells are found throughout the body; siRNAs can trigger IFN secretion from human peripheral blood mononuclear cells (PBMCs), so stimulation of the innate immune system must be considered when using any kind of systemic administration [89]. TLR3, 7, and 8 are localized intracellularly in endosomes and delivery to this compartment must take place for activation to occur. In cell culture, cationic lipid transfection agents deliver nucleic acids to endosomal compartments, while electroporation does not, and siRNA delivery done using electroporation is less likely to stimulate IFN secretion [87]. These same principles appear to apply *in vivo*. Intravenous administration to mice of unmodified siRNAs complexed in lipid particles is more likely to cause IFN pathway activation [52] than intravenous administration of unmodified "naked" siRNAs using high-volume hydrodynamic delivery [91]. Cationic DOTAP liposomes significantly enhance the ability of siRNA to trigger IFN responses in PBMCs and in mice [92,93]. More information about siRNA activation of innate immunity via TLR signaling can be found in a recent review by Marques and Williams [94].

As more and more sequences that are capable of activating IFN via TLR7/8 binding are being identified [87], a better strategy than simply avoiding "bad" motifs may be needed to generalize use of siRNA *in vivo,* where all cell types are present. Use of chemically modified RNAs may overcome many of these problems. A recent study by Kariko and colleagues compared the capacity of RNA from prokaryotic and eukaryotic sources for their ability to trigger IFN responses. They observed that prokaryotic RNAs were a much more potent trigger of signaling through TLR3, 7, and 8 pathways than eukaryotic RNAs. Weaker or absent receptor signaling from eukaryotic RNAs was not sequence dependent but rather was due to the base and/or sugar modifications that were present in these nucleic acids. Much like 5-methyl-dC, which can reduce or prevent activation of TLR9 in CpG motif DNA, the presence of 5-methyl-C, N^6-methyl-A, pseudouridine, or 2′-*O*-methyl bases prevents recognition of RNA by TLR3, 7, and 8 [95]. Importantly, it has been shown that extensive 2′ modification of a sequence that is otherwise strongly immunostimulatory as unmodified RNA can prevent an immune response when that same sequence is administered intravenously (iv) in mice using a lipid-based particle delivery system [52]. However, it might not be necessary to modify an RNA duplex this extensively to block immune responses. Judge and colleagues reported that substitution of as few as two 2′-*O*-methyl bases in one strand of a siRNA duplex can be sufficient to block TLR activation both *in vitro* (human PBMCs) and *in vivo* (iv delivery in mice using stabilized nucleic acid lipid particle (SNALP)-facilitated delivery). Modified U and G bases were most effective in achieving this affect [96]. Since selective incorporation of 2′-*O*-methyl bases is inexpensive and does not compromise siRNA functional activity, use of this modification in all duplexes intended for *in vivo* applications should be considered.

Just as certain sequences are active in stimulating innate immune responses via TLR pathways, some sequences can inhibit or block these responses. Barrat and colleagues described several sequence motifs that prevent TLR7 and/or 9 activation when present in ssDNA oligonucleotides [97]. It would also be interesting to see if endosomal inhibitors such as chloroquine might blunt innate immune responses to siRNAs.

Given the sequence-specific variation in the ability of a duplex to trigger innate immune responses, it may be impossible to ensure that any single "negative control duplex" is really a valid control. It might therefore be judicious to measure serum cytokine levels routinely to verify whether IFN induction has actually occurred to assist with interpretation of *in vivo* results, even if modified duplexes are employed.

Off-Target Effects

Nucleic acid base pairing is highly specific and mismatches at one or a small number of positions in a short sequence are often sufficient to prevent hybridization completely under physiological conditions. Based upon antisense oligonucleotide experience, it was originally hoped that performing a genome-wide homology search to eliminate sites with significant cross-hybridization potential would ensure specificity of siRNA gene targeting. Indeed, even a single mismatch can disrupt siRNA action against an intended target [57,98]. Systematic analysis of the functional impact caused by base changes at all positions in a target site has been reported and can

be used as a guide to assist with siRNA design [99]. Unfortunately, this kind of traditional homology and cross-hybridization analysis is insufficient and siRNAs can cause widespread changes in expression levels in seemingly unrelated genes [100–102].

RNAi is mediated by multimember protein complexes that mechanistically repress gene expression in different ways when using perfect vs imperfect duplexes. Degradative RNAi leads to sequence-specific cleavage of a target mRNA as directed by RISC using Ago2 [21,22,103] and initiation of this event requires perfect or near-perfect base pairing with the siRNA-derived guide strand. Conversely, the microRNA (miRNA) pathway results in translational repression and is directed by imperfect base pairing between target and guide strand [104,105]. This pathway is theorized to be mediated by a variant of RISC using Ago1. Functionally, the siRNA degradative pathway and miRNA translational repression pathway overlap. Exogenously supplied siRNA duplexes can function as miRNAs [106,107] and miRNAs can direct target cleavage events [108,109]. It seems likely that many of the OTEs attributed to siRNAs derive from miRNA-like target interactions, which can be triggered by surprisingly limited sequence homology. Target specificity for miRNAs is largely directed by a 7-nucleotide "seed region" at the 5′ end of the antisense strand of the miRNA [110]. Unfortunately, even this small amount of homology can give rise to OTEs. Lin and colleagues reported results from a large-scale siRNA library screen in which their top three "hits" all derived from off-target events and, further, that two of these events resulted from only a 7-bp complementation between the siRNA and the "off target" [111].

Given the expected frequency of finding 7-base matches between a siRNA and nontargeted genes within the entire transcriptome of a mammal, simple homology screening alone is insufficient to predict or eliminate off-target events. It is possible that some pattern of chemical modification will decrease the ability of a siRNA to participate in this class of OTE. When using siRNAs as research tools, a simple strategy to help ensure that results are not misinterpreted because of unsuspected OTEs is to require that two independent siRNAs against the same target produce the same biological results. For development of siRNAs as therapeutic agents, extensive testing may be needed to ensure that a potential drug candidate has an acceptable degree of OTEs. It is not clear what metrics should be employed to assess OTEs *in vivo.* While microarrays have been employed to document changes in gene expression profiles that result from siRNA treatment on a genome-wide scale *in vitro,* no studies have yet reported this kind of experiment *in vivo.* Further, up or down regulation of "unrelated" genes might or might not result in biologically significant adverse events *in vivo.* Traditional "adverse event" assessment, as is routinely performed in small-molecule drug development programs, may be the most relevant method to consider unwanted affects from siRNA drugs without the need for additional studies to catalog the presence or absence of "unintended gene regulation events."

The cellular machinery responsible for degradative RNA interference also participates in processing miRNAs. MicroRNAs are important in cellular gene regulation networks and anything that disrupts or alters miRNA processing could potentially have broad affects on gene expression in a cell. It is theoretically possible that high doses of exogenous siRNAs administered as therapeutics or investigational tools could "saturate" the RNAi machinery and thereby alter miRNA levels, further complicating the spectrum of OTEs. Hong and colleagues described that high doses of a siRNA can induce expression of some genes involved in siRNA degradation and processing (*meri-1* and *adar-1*) [112]. Stimulation of IFN pathways, OTEs, and desired gene knockdown are all dose dependent. The precise dose needed to trigger each response can be different and it may be possible to influence the relative magnitude of undesired side effects by careful attention to dosing. Dose–response profiles should be performed *in vivo* and the siRNA regents subsequently administered at the lowest levels that achieve the desired degree of target knockdown or therapeutic effect.

Route of Administration and Use of Delivery Vehicles

The route of siRNA administration will affect the total dose needed, the effective tissue distribution, potential side effects, and the need for chemical modification or use of an agent that facilitates delivery. Although iv administration is often considered for broadest tissue distribution, it is important to consider alternative injection sites such as intraperitoneal (ip) and subcutaneous (sc) routes. Local delivery can be used to achieve high concentration at the intended target site while using a low dose and minimizing risk of systemic side effects. Local delivery can be achieved by direct injection (such as into a tumor or joint or intraocularly) or by topical application to a surface (such as transepithelial absorption through oral, rectal, or vaginal mucosa). Direct administration into the central nervous system (CNS) can be achieved by intrathecal or intraventricular injection, bypassing issues with blood–brain barrier penetration. Inhaled, intranasal, or intratracheal administration can be employed for pulmonary delivery.

Although reports about the life span of unmodified siRNA in serum vary from minutes to days, it is obvious that chemically modified duplexes will survive longer in nuclease-rich environments than unmodified RNA [49,56,59]. Even when chemical modifications are employed to stabilize a duplex from degradation, plasma clearance may still be rapid and use of a delivery vehicle can improve pharmacokinetics. Morrissey and colleagues reported that the plasma half-life of an unmodified siRNA duplex in mice was 0.03 h, the half-life of a modified

doi:10.1016/j.ymthe.2006.01.001

duplex was 0.8 h, and the half-life of a modified duplex packaged in a SNALP was 6.5 h [52]. Similarly, Soutschek and colleagues reported studies using modified siRNAs in rats in which an unconjugated duplex had an elimination half-life of 0.1 h, while a cholesterol-conjugated duplex had a half-life of 1.5 h. While it may be reasonable to employ unmodified duplexes for basic research applications *in vivo,* a more sustained effect and potential therapeutic benefit will be achieved using chemically modified duplexes together with some kind of delivery system.

A wide variety of methods have been used to facilitate delivery of nucleic acids. Nucleic acids have a high negative charge density and cationic polymers are usually employed to bind the nucleic acid, neutralize charge, and assist with transport across the cell membrane. Cationic lipids are routinely used with great success as transfection agents in tissue culture; however, their *in vivo* use by iv administration presents significant problems as these reagents can be quite toxic. Despite problems with iv use, cationic lipids can sometimes be employed for ip injection [113–115], for CNS injection [116,117], or in topical epithelial surface application [118,119]. Toxicity varies with the precise chemical composition of the lipids employed, dose, and a variety of other factors. Variations in chemical composition can have a large impact on the functional properties of cationic lipid mixtures [120] and lipoplex/liposomal preparations have been devised with decreased toxicity that are more compatible with iv administration. Liposomal formulations have been FDA approved for small-molecule drug delivery since the late 1990s [121]. Liposomes can be modified with ligands such as folate or small peptides, which assist with delivery and help target specific cell types or tissues [122,123]. Through the use of neutral polyethylene glycol-substituted surfaces and other approaches, liposomes can be stabilized and made more "stealthy," showing reduced clearance and improved pharmacokinetics [124,125]. These kinds of lipid nanoparticles have been successfully used to deliver antisense oligonucleotides and siRNAs *in vivo* [52,126–128].

Polyethylenimine (PEI) has been used for many years to facilitate nucleic acid delivery [129]; however, due to toxicity and variable performance it has not found generalized acceptance as a delivery tool for either antisense oligonucleotides or siRNAs. Nevertheless, PEI can be used as a "base" for formulation of more complex particles with improved properties. For example, a PEGylated PEI particle with a vasculature-targeting RDG peptide has been shown to deliver siRNAs successfully in a mouse xenograft tumor model [130]. Deacylation of PEI lowers toxicity and improves pulmonary delivery of siRNAs in mice [131] and folate-modified PEGylated PEI is being tested for siRNA delivery in tumor cells [132].

Cyclodextrin-modified PEI has favorable properties for nucleic acid delivery. Compared with unmodified PEI, the cyclodextrin polymer (CDP) has relatively low toxicity and with the addition of adamantine-PEG, will spontaneously form stable particles with the nucleic acid cargo under physiological conditions. PEI-based CDPs have been shown to function for *in vivo* delivery of nucleic acids [133]. CDPs have been synthesized using other polycations (than PEI) and optionally can include terminal imidazole groups to improve endosomal trafficking [183]. Conjugation of transferrin to a linear cyclodextrin-containing polymer/adamantine/PEG complex improves tumor targeting *in vivo* and this kind of polyplex has been shown to effectively deliver siRNAs in mice [134]. As a general strategy, transferrin-conjugated polycations were shown to facilitate transfection of DNA 15 years ago [135]. A variety of different conjugates that facilitate delivery of nucleic acids have been characterized [8] and novel small-molecule libraries are under development that assist targeting of different cell types and may eventually offer a "menu" that enables selective delivery of therapeutic compounds wherever desired [136].

Other polycations can be complexed with siRNAs to facilitate delivery. Protamine has been employed as a carrier for nucleic acids and can be conjugated to a variety of molecules that improve its performance, including lipids [137]. Protamine can be conjugated to antibody Fab fragments to target delivery selectively to specific cell types and this approach has been successfully used to deliver siRNAs in mice [138]. Cationic peptides have also been used to facilitate nucleic acid entry into cells by exploiting the capability of certain sequences to enter cells directly (cell-penetrating peptides) or to enter specific cell types selectively (cell-targeting peptides) [67,139]. The peptides MPG, penetratin, and transportin have all been shown to promote delivery of siRNAs in tissue culture [140–142] and may have promise for *in vivo* use. General peptide carrier effects can also be exploited, and atelocollagen, a purified pepsin digest of collagen, has been shown to function as a delivery vehicle for siRNAs in mice [143]. The DNA-packaging motor of bacteriophage ϕ29 has been conjugated to siRNAs and shown to facilitate cell entry [144].

Modified virus envelopes can be adapted to deliver nucleic acids to cells. Fusogenic influenza virus envelopes were used to deliver fluorescent dye-labeled siRNAs by ip injection into mice [145], hemagglutinating virus of Japan envelopes were used to deliver siRNAs via direct intratumor injection in mice [146], and SV40 pseudovirions are being tested as a delivery vehicle [147]. The potential antigenicity of these delivery tools will need to be addressed for consideration in therapeutic applications.

Physical and mechanical delivery methods should also be considered. In fact, the single most commonly employed strategy for *in vivo* siRNA delivery to date has

been the "hydrodynamic delivery" (HD) method [62,63,91,112,148–165]. In this approach, a large volume of a nucleic acid solution is rapidly injected into the tail vein of a mouse, which transiently disrupts vascular and tissue integrity and achieves delivery [166]. Morbidity can be significant and success using this method is technique dependent. Nevertheless, HD is useful as a research tool and is especially effective in delivering nucleic acids to the liver. Recent reports have also demonstrated success using electroporation [167–172], ultrasound [173], and particle-based "gene gun" [174] delivery systems.

Delivery-Related Toxicity

Although a large number of studies have reported successful *in vivo* delivery of nucleic acids using liposome or polyplex reagents, issues of toxicity remain and must be considered. Chien and colleagues compared the toxicity of iv injection of cationic DOTAP liposomes directly with the cardiolipin analogue CCLA:DOPE liposomes in mice as part of an evaluation of the use of this delivery system with siRNAs [128]. Liposomes were administered by tail vein injection and mice were followed for 2 weeks. Two of three mice given a single dose of 100 mg/kg DOTAP liposomes died compared with 0/3 mice given CCLA liposomes. The CCLA liposomes were also studied with a multiple dose regimen and 1/3 mice that received 100 mg/kg/day for 3 consecutive days died. Animals may survive yet still suffer delivery-related morbidity, which can affect experimental results and must be considered.

Much of the toxicity of cationic lipids relates to electrostatic effects, and precise complexation ratios of the positively charged lipid with the negatively charged cargo nucleic acid are important. Interaction with negatively charged serum proteins can inactivate the complex [175] and particle aggregation with clumping in capillary beds can be problematic; interaction with complement proteins can lead to inflammation. When loaded with a nucleic acid cargo, cationic lipids can trigger the release of a variety of cytokines (see discussion of TLR activation above). Lymphopenia, thrombocytopenia, and hepatic necrosis can occur with higher doses of lipid [176]. Newer classes of lipid/nucleic acids delivery systems fully encapsulate (not just complex with) the nucleic acid cargo and present a stabilized, more neutral surface to the intravascular environment that lowers toxicity, improves stability, and prolongs serum circulation time [177]. Recent reviews by Dass [178], Audouy [179], and Simberg [180] provide greater detail on cationic liposome delivery methods and *in vivo* toxicity.

Polyplex delivery systems have similar toxicity problems. The LD_{50} of linear PEI is around 4 mg/kg in mice [181], significantly limiting *in vivo* utility. Cyclodextrin polymers are much less toxic, having an LD_{50} of ~200 mg/kg in mice [182]. Grafting of β-cyclodextrin onto PEI reduces toxicity but also can decrease efficiency of nucleic acid transfection, so careful optimization is needed. The addition of adamantine–PEG forms inclusion complexes that coat the complex in a way that further stabilizes the polymer/nucleic acid particle under physiological salt conditions without increasing size and helps further lower toxicity [133]. Transferrin or other ligands can be added to the polymer to help with cell-type targeting. For an overview of issues involved with polyplex design, see the recent review by Davis and colleagues [183].

It is also possible to perform *in vivo* gene knockdown experiments using *ex vivo* delivery methods, which avoids potential complications and/or toxicity associated with each of the various *in vivo* delivery methods. If a cell line can be transfected with siRNAs in tissue culture using standard methods, it can subsequently be implanted into recipient animals in which changes in phenotype and behavior *in vivo* can be studied, avoiding the challenges associated with traditional *in vivo* delivery. For example, in one study that employed *ex vivo* methods, human melanoma tumor cells were transfected in culture using electroporation with either control siRNAs or siRNAs specific for a mutant ^{V599E}*B-Raf* gene. In this study, "stealth" siRNAs were employed, which are chemically modified blunt 25-mer duplexes with selective incorporation of *2′-O*-methyl RNA in the sense strand. Transfected cells were implanted in nude mice. Cells transfected with control siRNAs took ~5 days to develop 10-mm tumor foci, whereas cells transfected with anti-^{V599E}*B-Raf* siRNAs took 14–16 days to develop 10 mm tumor foci [184].

Overview of Studies Using siRNAs in Mammals

Over 90 reports that involve *in vivo* administration of siRNAs in mammals have been published. The following survey of these studies will focus on the methodologies employed, such as route of administration, use of delivery vehicles, use of chemically modified duplexes, and controls (if any) for OTE or IFN induction. Table 1 includes a listing of studies organized by "system" studied. Selected studies are examined in greater detail below, organized by route of administration.

Intravenous Administration Using Hydrodynamic Delivery

The first published reports of the *in vivo* use of siRNAs in mammals appeared in mid-2002. McCaffrey and colleagues used HD delivery to introduce 2 μg of a luciferase expression plasmid with 40 μg of unmodified control or anti-*luciferase* siRNAs into mice [148]. A human α-1 antitrypsin (hAAT) expression plasmid was co-injected and

doi:10.1016/j.ymthe.2006.01.001

TABLE 1: Reports using synthetic siRNAs in mammals

Target(s)	System	Route/delivery	IFN studied	Modified siRNAs	Reference and year
Luc	Mouse—reporter	IV–hydrodynamic	No	No	[148] 2002
Luc, EGFP	Mouse—reporter	IV–hydrodynamic	No	No	[149] 2002
EGFP	Mouse—reporter	DIT–cationic lipid	No	No	[222] 2002
Luc	Mouse—reporter	IV–hydrodynamic	No	Yes	[62] 2004
Luc, Fas	Mouse—reporter	IV–hydrodynamic	Yes	No	[91] 2004
Luc	Mouse, rat, primate—reporter	IV–hydrodynamic	No	No	[155] 2004
EGFP, RFP	Rat—reporter	IV–hydrodynamic	No	No	[164] 2005
Luc	Mouse—reporter	IV–hydrodynamic; DIT–electroporation	No	No	[163] 2005
EGFP, Tnf	Mouse—reporter	IV, IP–DOTAP liposomes	Yes	No	[92] 2003
Luc, EGFP, Gapdh	Mouse—reporter	IM–electroporation	No	No	[167] 2004
EGFP	Mouse—reporter	IM–electroporation	No	No	[171] 2005
EGFP	Mouse—reporter	Cardiac sonoporation	No	No	[173] 2005
EGFP	Mouse—reporter	IP–virosomes	No	No	[145] 2006
Abcb1a (Mdr1a/1b)	Mouse—liver	IV–hydrodynamic	No	No	[162] 2005
Rho	Mouse—liver plasmid codelivery	IV–hydrodynamic	No	No	[161] 2005
Gapdh	Mouse—lung	IN–InfaSurf	No	No	[215] 2004
Tgfb1, EGFP, Luc	Rat—glomerulonephritis	Renal artery electroporation	No	No	[168] 2005
Fas	Mouse—hepatitis	IV–hydrodynamic	No	No	[150] 2003
Casp8	Mouse—hepatitis	IV–hydrodynamic	No	No	[152] 2003
Fas	Mouse—ischemia–reperfusion injury	IV–hydrodynamic	No	No	[156] 2004
Casp8, Casp3	Mouse—ischemia–reperfusion injury	IV–hydrodynamic	No	No	[154] 2004
Hmox1	Mouse—ischemia–reperfusion injury	IN–naked	No	No	[223] 2004
Hsf1	Mouse—ischemia–reperfusion injury	IP–cationic lipid	No	No	[115] 2005
P4ha2	Mouse—ischemia–reperfusion injury	IP–cationic lipid	No	Yes	[224] 2006
Tlr4, Bcl2, Irak3	Mouse—immune stimulation	IV–DOTAP liposomes	Yes	No	[93] 2005
Mif	Mouse—transplant	TOP–cationic lipid	No	No	[225] 2005
Tbx21	Mouse—multiple sclerosis	IV–naked	Yes	No	[226] 2004
Casp8, Fas, EGFP	Mouse—CLP sepsis and reporter	IV–hydrodynamic	No	No	[160] 2005
Casp8, Fas	Mouse—CLP sepsis	IN–naked intratracheal	Yes	No	[227] 2005
Cxcl1 (KC), Cxcl2 (MIP2)	Mouse—CLP sepsis	IN–naked intratracheal	Yes	No	[228] 2005
Il12b (IL-12p40)	Mouse—LPS/sepsis	IP–cationic lipid	Yes	No	[114] 2004
Tnf, EGFP	Mouse—LPS/sepsis	IV, IP–DOTAP liposomes	Yes	No	[198] 2003
Tnf, Luc, EGFP	Mouse—arthritis	IA–electroporation	No	No	[170] 2005
Tnf	Rat—arthritis	IA–electroporation + cationic lipid	No	No	[172] 2005
Apob	Mouse—metabolic	IV–Chol conjugated	No	Yes	[65] 2004
Apob	Mouse—metabolic	IV–SNALP	Yes	Yes	[96] 2006
Avpr2	Mouse—metabolic	IV–DOTAP liposomes	No	No	[229] 2005
Cav1	Mouse—metabolic	IV–DDAB liposomes	No	No	[230] 2006
Cyba	Rat—metabolic	IV–HD, cationic lipid	Yes	No	[165] 2006
RRM2	Mouse—tumor	IV–naked	No	No	[188] 2004
CEACAM6	Mouse—tumor	IV–naked	No	No	[189] 2004
CXCR4	Mouse—tumor	IV–naked	No	No	[190] 2005
BCL2	Mouse—tumor	IV, SC–LIC-101 liposomes	No	No	[231] 2004
BCL2	Mouse—tumor	IP–naked	No	No	[232] 2005

TABLE 1 (*continued*)

Target(s)	System	Route/delivery	IFN studied	Modified siRNAs	Reference and year
RAF1	Mouse—tumor	IV–CCLA liposomes	No	No	[128] 2005
RAF1	Mouse—tumor	IV–CCLA liposomes	No	No	[127] 2005
RAF1	Mouse—tumor	DIT–peptide carrier	No	No	[211] 2005
EPHA2	Mouse—tumor	IV–DOPC liposomes	No	No	[192] 2005
EWS-FLI1	Mouse—tumor	IV–cyclodextrin polyplex	Yes	No	[134] 2005
Vegfa, Mdm2, Myc, EGFP, gag	Mouse—tumor	IV–Fab-protamine	Yes	No	[138] 2005
EZH2, PIK3CA, Luc	Mouse—tumor	IV–atelocollagen	Yes	No	[143] 2005
CTNNB1 (β-catenin)	Mouse—tumor	IP–cationic lipid	No	No	[113] 2003
ERBB2 (HER-2)	Mouse—tumor	IP–PEI	No	No	[199] 2005
Csf1, Csf1r	Mouse—tumor	DIT–naked	No	No	[210] 2004
RAD51	Mouse—tumor	DIT–HVJ viral envelope	No	No	[146] 2005
FGF4	Mouse—tumor	DIT–atelocollagen	No	No	[233] 2004
VEGF	Mouse—tumor	DIT–atelocollagen	No	No	[212] 2004
VEGFR2 (Kdr)	Mouse—tumor	IV–RPP-polyplex	No	No	[130] 2004
Vegfa, Luc	Mouse—tumor	IV, IP, SC, DIT–naked	No	No	[187] 2003
Vegfa, Sst	Mouse—tumor	DIT–jetSI	No	No	[234] 2005
Edg1 ($S1P_1$)	Mouse—tumor	DIT–DOTAP liposomes	No	No	[235] 2004
Rhoa, Rhoc	Mouse—tumor	DIT–cationic lipid	No	No	[236] 2005
Plk1	Mouse—tumor	TOP–cationic liposomes, bladder	No	No	[237] 2005
VEGF, EGFP	Mouse—ocular neovascularization	IO–cationic lipid	No	No	[200] 2003
Vegfa, Flt1 (VEGFR1), Kdr (VEGFR2)	Mouse—ocular neovascularization	IO–naked; IV–polyplex	No	No	[202] 2004
VEGFR1	Mouse—ocular neovascularization	IO–naked	No	Yes	[201] 2006
TGFBR2	Mouse—ocular fibrosis	IO–cationic lipid	No	No	[238] 2004
Bax, Apaf1, Jun	Rat—ocular axotomy apoptosis	IO–naked	No	No	[239] 2005
App	Rat—amyloid, CNS	IO–cationic lipid	No	No	[240] 2006
Bax, Bak	Mouse—apoptosis and immunization	Dermal–gene gun	No	No	[174] 2005
GJB2	Mouse—auditory	TOP–DOTAP liposomes	No	No	[118] 2005
Agrp	Mouse—CNS metabolic	CNS–naked	No	No	[241] 2002
Slc6a3	Mouse—CNS behavioral	CNS–naked	No	Yes	[206] 2004
Slc6a4	Mouse—CNS behavioral	CNS–naked	No	Yes	[207] 2005
Luc	Mouse—CNS reporter	CNS–PEI, DOPE	No	No	[116] 2005
Drd1a	Rat—CNS behavioral	CNS–naked	No	No	[208] 2003
P2rx3	Rat—CNS behavioral	CNS–naked	No	Yes	[205] 2004
Adra2a	Rat—CNS behavioral	CNS–naked	No	No	[242] 2004
Oprd1	Rat—CNS behavioral	CNS–cationic lipid	No	No	[117] 2005
Mc4r	Rat—CNS behavioral	CNS naked, cationic lipid, DOTAP liposome	No	No	[209] 2005
Gria2, Ptgs1	Rat—CNS physiology	CNS electroporation	No	No	[169] 2005
Hepatitis B	Mouse—virus	IV–hydrodynamic	No	No	[151] 2003
Hepatitis B	Mouse—virus	IV–hydrodynamic	No	No	[153] 2003
Hepatitis B, meri-1	Mouse—virus	IV–hydrodynamic	No	No	[112] 2005
Hepatitis B	Mouse—virus	IV–hydrodynamic	No	Yes	[63] 2005
Hepatitis B	Mouse—virus	IV–SNALP	Yes	Yes	[52] 2005
Influenza	Mouse—virus	IV–PEI	Yes	No	[197] 2004
Influenza	Mouse—virus	IV–deacyl-PEI	No	No	[131] 2005
Influenza	Mouse—virus	IV–hydrodynamic; IN–cationic lipid	No	No	[157] 2004
CVB3	Mouse—virus	IV–hydrodynamic	No	No	[159] 2005
West Nile virus	Mouse—virus	IV–hydrodynamic	No	No	[158] 2005

(continued on next page)

doi:10.1016/j.ymthe.2006.01.001

TABLE 1 (*continued*)

Target(s)	System	Route/delivery	IFN studied	Modified siRNAs	Reference and year
HSV2	Mouse—virus	TOP–cationic lipid	Yes	No	[119] 2006
RSV, PIV	Mouse—virus	IN–naked	No	No	[216] 2005
SARS	Primate—virus	IN–naked	No	No	[217] 2005
Berghepain 1 and 2	Mouse—malaria	IV–naked	No	No	[186] 2003

IFN, interferon or other cytokine assay performed to assess innate immune activation; Naked, low-pressure administration without delivery assistance; CNS, direct injection into the central nervous system (intrathecal, intraventricular, or stereotactic); DIT, direct intratumor injection; IA, intraarticular injection (joint); IM, intramuscular injection; IN, intranasal/respiratory infusion; IO, intraocular administration (subretinal, subconjunctival, intravitreous injections, etc.); IP, intraperitoneal injection; IV, intravenous injection; SC, subcutaneous injection; TOP, topical application to epithelial surface.

serum levels of hAAT were used for normalization between animals. Luciferase activity in the liver was assessed by *in vivo* bioluminescence imaging 3 days after injection and the anti-*luciferase* siRNA was found to specifically reduce light emission by 81%. IFN levels (or other cytokines) were not measured; however, use of the hAAT marker served as a control for nonspecific effects. Similar results using HD delivery to suppress luciferase in mice were reported by Lewis and colleagues [149]. In this case RNAi activity was demonstrated to occur in multiple tissue types, including liver, kidney, spleen, lung, and pancreas. Duration of silencing was investigated using an expression plasmid encoding secreted human placental alkaline phosphatase (SEAP). Ten micrograms of the pSEAP plasmid and 5 μg of an anti-*SEAP* siRNA were administered by HD injection. SEAP levels were suppressed by 83% on day 1 postinjection and by 32% on day 4 and were at control levels on day 14 (possibly sooner, but intermediate time points were not reported).

Song and colleagues reported using siRNAs to protect mice from liver damage in two model systems involving Fas-mediated apoptosis [150]. Anti-*Fas* siRNAs or control anti-*EGFP* siRNAs (50 μg in 1 ml PBS) were administered using HD delivery for three doses. Both *Fas* mRNA and Fas protein levels were shown to be specifically reduced about 10-fold (Fas/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) ratio) in recipient mouse hepatocytes relative to controls. Suppression lasted ~10 days. Mice treated with the anti-*Fas* siRNA did not develop fulminant hepatitis or hepatic fibrosis after treatment with concanavalin A (which induces hepatitis). Treatment with the anti-*Fas* siRNA was also protective against treatment with an agonist, an anti-Fas antibody (Jo2), which is normally fatal within 2–3 days of injection. Similar beneficial effects were reported using the same anti-*Fas* siRNA in protecting mice from renal ischemia–reperfusion injury [156]. SiRNAs were administered by HD delivery via the tail vein (50 μg in 1 ml PBS) or by low-pressure injection directly into the renal vein. In one series of experiments, renal ischemia was induced by clamping one renal pedicle for 35 min, and the other kidney was removed. Survival was significantly higher (8/10) for mice treated with anti-*Fas* siRNA than with control siRNA or saline (2/10).

Caspase 8 is a critical enzyme in the Fas apoptosis pathway. Zender and colleagues reported use of siRNAs targeting *caspase 8* to block hepatic necrosis following challenge with the agonist anti-Fas antibody (Jo2) or recombinant adenovirus encoding Fas ligand [152]. SiRNAs were administered using HD tail vein injection at a dose of 0.6 nmol/g (8 mg/kg, or 200 μg per dose per 25-g mouse) or a 0.45 nmol infusion directly into the portal vein. The *caspase 8* mRNA levels were reduced about fourfold (*caspase 8/GAPDH* ratio) following siRNA treatment in mouse hepatocytes. Following administration of the Jo2 Fas-agonist antibody, 5/11 mice treated with the anti-*caspase 8* siRNA survived, while 0/17 control animals survived. Using a strategy similar to that discussed previously for protection of Fas-induced renal ischemia–reperfusion injury, Contreras and colleagues reported success in using anti-*caspase 8* and anti-*caspase 3* siRNAs in protecting mice from hepatic ischemia–reperfusion injury [154]. A variant of HD delivery was employed in which low dose 0.45 nmol (6 μg) siRNA was administered in 1 ml volume directly into the portal vein. SiRNAs were administered 1 h before injury, hepatic blood supply was clamped for 90 min, and hepatic caspase 3 and 8 levels were assayed 1 h after reperfusion. Both caspase enzymes are strongly induced by ischemia; however, the relative level of induction was reduced approximately twofold by siRNA treatment. In one arm of the study, mice were observed for 30 days after a 75-min ischemic injury to the liver, with or without siRNA pretreatment. Mice treated with anti-*caspase 3* showed 50% survival (5/10), mice treated with anti-*caspase 8* showed 30% survival (3/10), while control mice showed 10% survival (1/10, control siRNA) or no survival (0/10, vehicle).

Hydrodynamic delivery has also been used to study the antiviral potential of siRNAs *in vivo*. Giladi and colleagues demonstrated that a plasmid encoding the hepatitis B virus (HBV) genome can be codelivered with siRNAs to the liver of mice using the HD method to study the effects of anti-HBV siRNAs on viral replication [151]. Five HBV siRNAs were tested, which, not surprisingly, showed different functional potency. The most potent reagent was capable of reducing HBV titers produced in transfected livers by 10^5 on day 2 and 10^2 on day 3 at the

dose studied (15 μg HBV plasmid with 1 nmol, or 13 μg siRNA). Morrissey and colleagues employed a similar HBV plasmid model system to study the use of anti-HBV siRNAs in mice, also using HD delivery [63], and compared performance of modified vs unmodified RNAs. The modified duplexes (called "small interfering nucleic acids, or siNAs) performed better, showing a 1.5-log greater reduction in HBV DNA and surface antigen levels 72 h postinjection than when the same sequence was administered as an unmodified siRNA. Importantly, only the modified duplexes suppressed HBV when treatment was started 3 days after "infection" (via delivery of the HBV plasmid by HD injection); however, a significantly higher dose was needed in this setting (30 mg/kg).

Although HD has largely been viewed as a research tool, Hagstrom and colleagues reported success using this method to deliver siRNAs and other nucleic acids to skeletal muscle in rats, dogs, and rhesus macaques [155]. In this variant of the HD method, a limb is functionally isolated by application of a blood pressure cuff or tourniquet and a large-volume bolus of nucleic acid solution is administered intravenously. Therapeutic delivery of plasmids or siRNAs in humans using this approach was proposed; if care is taken in technique, only moderate, reversible tissue damage was observed [185].

Intravenous Administration without Delivery Assistance

Despite the relative susceptibility of unmodified siRNA duplexes to degradation in serum, several groups have reported positive results using unmodified siRNAs administered by low-volume intravenous injection in mice without the use of any facilitated delivery system [186–190]. Filleur and colleagues administered unmodified siRNAs targeting vascular endothelial growth factor (*VEGF*) to nude mice bearing subcutaneous implants of rat fibrosarcoma cJ4 cells that express luciferase [187]. SiRNAs were injected iv, ip, or sc at a dose of 3 μg per injection (125 μg/kg/day) in PBS. Animals given a single anti-*VEGF* siRNA by all three of these routes showed 40–50% suppression of luciferase activity in the tumor implants at day 3 postinjection compared with animals receiving a control siRNA. Other animals were given daily ip injections of siRNAs in PBS and after 16 days those receiving anti-*VEGF* treatment showed a 66% reduction in tumor volume and a 70% reduction in tumor-associated VEGF levels compared with controls.

Duxbury and colleagues studied the effects that changes in levels of ribonucleotide reductase (RR) had on the efficacy of gemcitabine treatment of pancreatic tumor cells implants in nude mice [188]. A high level of RR is believed to be one cause of resistance to gemcitabine chemotherapy. RR levels can be reduced using siRNAs targeting the M2 subunit of RR (*RRM2*) and this treatment increases tumor cell susceptibility to gemcitabine *in vitro*. MIAPaCa2 pancreatic tumor cells were implanted in nude mice and were given control treatments, an anti-*RRM2* siRNA (150 μg/kg iv), and/or gemcitabine (150 mg ip) twice weekly for 6 weeks. Mice treated with the anti-*RRM2* siRNA showed reduced RRM2 levels in tumor extracts by Western blot compared with control siRNA-treated mice. Treatment with gemcitabine or the anti-*RRM2* siRNA alone resulted in a modest reduction in tumor volume, while treatment with the combination of gemcitabine with anti-*RRM2* siRNA showed a synergistic effect with 87% reduction in tumor size. Mice receiving combination treatment had no detectable liver metastasis, whereas 80% of control and 40% of gemcitabine or siRNA monotherapy animals had significant tumor burden in their livers. This group continued to investigate using siRNAs as a therapy for pancreatic adenocarcinoma using a different target, CEACAM6, a cell-surface adhesion molecule thought to play a role in tumor progression and metastasis. As before, unmodified siRNAs were administered at a dose of 150 μg/kg in PBS iv via the tail vein twice weekly. BxPC3 cells were implanted in the pancreas of nude mice and animals were given PBS alone, a control siRNA, or an anti-*CEACAM6* siRNA for 6 weeks. Mice treated with target-specific siRNAs had smaller tumors, 46% lower CEACAM6 levels, reduced evidence of angiogenesis ($CD34^+$ structures using immunohistochemical staining), and reduced hepatic metastasis compared to control mice. Survival after 6 weeks of treatment was 0/10 for PBS-treated mice, 0/10 for control siRNA-treated mice, and 9/10 for anti-*CEACAM6*-treated mice [189].

Intravenous Administration with Delivery Assistance

A variety of delivery systems have been successfully used to improve siRNA uptake following iv injection, including liposomes, polymer-based nanoparticles, peptides, antibodies, and small molecule ligands. Soutschek and colleagues described the use of cholesterol-conjugated siRNAs to reduce expression of apolipoprotein B (apoB) in mice and assessed the impact that this had on serum cholesterol and lipoprotein particle levels [65]. Duplexes were chemically modified by selective incorporation of 2′-*O*-methyl RNA bases and phosphorothioate linkages. Cholesterol modification increased serum half-life 15-fold compared with unconjugated siRNAs. Following iv injection, siRNA levels present in various tissues were measured using a sensitive RNase protection assay (RPA) and only the modified, conjugated RNA duplexes were detectable; these siRNAs were seen in liver, heart, kidney, adipose, and lung tissue. Extensive sequence optimization was performed; 84 siRNAs were screened *in vitro* and the 2 best candidates were used *in vivo*. Mice received the anti-*apoB* siRNAs or a control siRNA at a dose of 50 mg/kg iv daily for 3 days and cholesterol levels were measured 24 h after the last dose. ApoB-100 protein levels were reduced by 68% (siRNA apoB-1) and by 31% (siRNA apoB-2); the more potent siRNA showed a 37% reduction in total serum cholesterol. Although IFN or other cytokines

doi:10.1016/j.ymthe.2006.01.001

were not measured directly to assess activation of the innate immune system, mRNA levels for three unrelated control genes were found to be unaffected by siRNA treatment. Mechanism of action was demonstrated *in vivo* using a 5′-RACE procedure. ApoB species were identified that had a cleavage point exactly at the site predicted using the apoB-1 siRNA (10 bp from the 5′ end of the antisense strand of the siRNA).

It is informative to compare what dose is needed to achieve the same physiological effects if the same siRNA sequence was delivered using a liposomal or polymer-based delivery system. Judge and colleagues administered a siRNA having the same sequence as apoB-1 discussed above in mice intravenously using a lipid-based delivery vehicle (SNALP). The siRNA was not cholesterol conjugated and had a different modification pattern (compared to that employed by Soutschek) and was limited to five 2′-*O*-methyl-U residue substitutions in the sense strand [96]. A 50% reduction in serum cholesterol was achieved using a dose of 5 mg/kg/day for 3 days, 1/10 the dose used previously for the cholesterol-modified anti-apoB siRNA. Using a traditional antisense approach, Crooke and colleagues reported that ip administration of a 2′-*O*-methoxyethyl/DNA "gapmer" phosphorothioate oligonucleotide specific for apoB reduced total cholesterol to a similar degree when mice were given a dose of 25 mg/kg twice weekly for 8 weeks and by 55% when dose was increased to 50 mg/kg [191].

A synthetic cardiolipin analogue (CCLA) has been used to make a new family of cationic liposomes that can be used for iv administration of siRNAs. Chien and colleagues described the use of the CCLA liposomes to deliver siRNAs by tail vein injection in mice [128]. Delivery efficiency was estimated to be approximately sevenfold better than with traditional DOTAP liposomes and had lower toxicity. The Ras/Raf/MAPK signaling pathway is thought to be important in around 30% of human cancers. An unmodified siRNA specific for *c-raf* was administered via the tail vein at a dose of 7.5 mg/kg twice a day for 5 days to SCID mice bearing sc implants of the breast cancer tumor MDA-MB-231. Tumors in mice treated with naked siRNAs grew at the same rate as controls, while tumors in mice treated with liposomal–siRNAs were 73% smaller at day 8, suggesting that the delivery vehicle had a significant impact on the therapeutic efficacy of the anti-*c-Raf* siRNA treatment. A similar series of experiments studied the effect of an anti-*Raf-1* siRNA with or without the chemotherapy agent Taxotere in suppressing the growth of sc implants of the prostate cancer cell line PC-3 in SCID mice [127]. Mice were administered unmodified siRNAs at a dose of 7.5 mg/kg twice a day for 5 days, either naked or complexed in CCLA liposomes, with or without the coadministration of Taxotere (10 mg/kg on day 2 and 5 mg/kg on day 5). Tumors were excised and Western blots were performed to examine Raf-1 protein levels. Relative to the control siRNA, administration of free *Raf-1* siRNAs reduced Raf-1 protein levels by 37% (Raf-1/GAPDH ratio), liposomal *Raf-1* siRNAs reduced Raf-1 levels by 89%, and the combination of liposomal siRNAs + Taxotere reduced Raf-1 levels by 98%. Naked siRNAs had no effect on tumor growth, while the liposomal anti-*Raf-1* siRNA inhibited tumor growth by 49%, and, when combined with Taxotere, inhibited tumor growth by 89%.

Use of neutral liposomes made of 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) to deliver siRNAs iv in mice was described by Landen and colleagues [192]. Dye-labeled RNA duplexes were injected into mice iv and the use of naked, DOTAP liposome, and DOPC liposome delivery was compared using fluorescence microscopy. The DOPC liposomes gave an estimated 10-fold improved delivery compared with DOTAP liposomes and 30-fold improved delivery compared with naked RNA; the greatest uptake of siRNA was seen in liver, kidney, and lung. EphA2 is a tyrosine kinase receptor that is overexpressed in some human cancers and is associated with poor outcome in ovarian cancer. Nude mice with ip implants of the ovarian cancer cell line HeyA8 or SKOV3ip1 were given control or anti-*EphA2* siRNA (150 μg/kg iv twice weekly in DOPC liposomes) for 4 weeks, with or without the chemotherapeutic agent Paclitaxel (100 μg ip weekly). Compared with mice receiving the control siRNA, average tumor size was reduced by 35–50% using the anti-*EphA2* siRNA, 45–68% using Paclitaxel, and 86–91% with combination therapy. Several studies have reported similar observations in which the combination of siRNA with a cytotoxic chemotherapeutic agent worked additively or synergistically in reducing tumor size. It is also worth noting the very low dose of siRNA employed in this study, presumably due to improved delivery using the neutral liposome carrier.

A different type of lipid particle was employed by Morrissey and colleagues to deliver anti-HBV siRNAs in a mouse hepatitis B model system [52]. The particle, called a SNALP, contained cationic, neutral, and fusogenic lipids that fully encapsulate the nucleic acid cargo; an earlier version of this particle was reported to improve delivery of phosphorothioate antisense oligonucleotides in mice [193]. Both unmodified and highly modified siRNAs (including 2′-*O*-methyl, 2′-F, DNA, and PS bonds) were tested by Morrissey. Unmodified siRNAs administered using this delivery method strongly stimulated innate immune responses while the highly modified duplexes did not. Delivery of a fluorescent siRNA was examined and use of SNALPs resulted in highest levels of RNA in liver and spleen but showed little accumulation in lung. Plasma half-life of modified siNAs in SNALPs was 6.5 h compared with 2 min for unmodified naked siRNAs. Hepatitis "infection" was initiated with HD delivery of 0.3 μg of pWTD HBV plasmid and siRNA therapy was started 6 days later. Mice received control or anti-HBV siRNAs in SNALPs at doses of 0.3 or 3 mg/kg/day for 3 days. Serum

HBV DNA levels dropped by 1.5 $\log_{10}$ for the higher dose, and reduction in HBV DNA levels persisted for 6 weeks after the last siNA dose. Effective reduction in HBV DNA was achieved using 3 mg/kg dosing with SNALPs compared with 30 mg/kg for the same sequences administered using HD delivery [63].

Nonliposomal compounds are being developed to deliver nucleic acids *in vivo* that form a "nanoparticle drug delivery complex." Typically, this kind of compound includes a cationic polymer that binds the DNA or RNA cargo (such as PEI), a neutral "stealth" coating (such as PEG), and a targeting ligand (such as transferrin). Schiffelers and colleagues described the use of a "sterically stabilized nanoparticle" to deliver unmodified siRNAs to neuroblastoma N2A tumors in nude mice [130]. The particles contained PEI ("P" nanoparticles), PEI + PEG ("PP" nanoparticles) or PEI + PEG plus an Arg-Gly-Asp (RGD) peptide that promotes vascular targeting ("RPP" nanoparticles). Complex formation of siRNAs in the particles (nanoplexes) protected unmodified siRNAs from degradation in serum and altered *in vivo* delivery patterns. A fluorescence-labeled naked siRNA did not accumulate in tumors, lung, or liver and was mostly degraded and/or excreted in urine. The fluorescence-labeled P nanoplex accumulated more in lung than in liver or tumor, while the labeled RPP nanoplex improved uptake more selectively in the N2A tumor masses (distribution of PP nanoplexes was not reported). SiRNA duplexes specific for the murine vascular endothelial growth factor receptor-2 (*VEGFR2*) were administered to mice bearing N2A tumors at a dose of 40 μg iv every 3 days. After three cycles of treatment, mice receiving the anti-*VEGFR2* siRNA as an RPP nanoplex showed >90% inhibition of tumor growth compared with vehicle-only or control siRNA-treated mice. Newer formulations of this particle that may have even greater potential in siRNA drug development have been devised [194].

Another novel particle employs a linear cyclodextrin-containing polymer stabilized with adamantine-PEG and has been shown to deliver both small molecule drugs as well as nucleic acids *in vivo* [183]. This cyclodextrin-polycation ("polyplex") was conjugated to transferrin to improve uptake into tumor cells and used to deliver unmodified siRNAs in a mouse model of Ewing sarcoma [134]. Many Ewing sarcoma tumors contain a translocation, t(11;22), that results in production of an aberrant transcript, *EWS-FL11,* which is thought to function as an oncogenic transcription factor. An anti-*EWS-F11* siRNA (siEFB2) and a control siRNA were administered to SCID mice bearing tumors formed from TC71 cells that stably express luciferase (enabling bioluminescence imaging of tumor implants). Administration of siEFB2 for 3 days using the transferrin polyplex at a dose of 50 μg (2.5 mg/kg/day) resulted in transient suppression of the luciferase signal and 60% reduction in *EWS-F11* mRNA levels (normalized to β-actin) in established tumors. In another series of experiments, siRNA administration was started concurrent with injection of the TC71-Luc tumor cells. Mice were followed for up to 8 weeks with twice weekly dosing (2.5 mg/kg) of siRNAs. Mice receiving control siRNAs (in any form) and those receiving naked siEFB2 had rapid tumor development. Mice receiving siEFB2 with nontargeting polyplexes had delayed onset of tumors and those receiving siEGB2 delivered with transferrin polyplexes had minimal to no tumor formation. Ogris and colleagues described the use of transferrin and epidermal growth factor as ligands to improve targeting of DNA polyplexes (PEGylated PEI) to tumor cells in mice [195]. Employing some kind of specific cell-surface marker or receptor targeting technique seems generally to improve *in vivo* delivery of nucleic acid polyplexes.

Antibodies have high specificity and a variety of well-characterized reagents are available "off the shelf" that recognize many different mouse or human cell surface markers. Song and colleagues described a method in which protamine was conjugated to the C-terminus of an antibody Fab fragment and used as a cell-marker-specific reagent to deliver unmodified siRNAs in mice [138]. In one series of experiments, a Fab fragment (F105) specific for the HIV envelope glycoprotein gp160 was coupled to protamine (F105-P). Six siRNAs formed a delivery complex with one F105-P carrier. This complex was effective in delivering siRNAs to all cell types tested that expressed the HIV *env* gene, even T cells (which are notoriously difficult to transfect). Administration of siRNAs using the F105-P carrier did not elicit an immune response in B16 melanoma cells *in vitro* (β-*IFN, STAT1,* and *OAS1* mRNA levels were assayed using qRT-PCR). While B16 cells are competent for some TLR responses and express TLR3 [196], it may be worthwhile to determine if this method of delivery triggered any innate immune response in myeloid or dendritic cell lines or *in vivo*. Murine B16 cells were implanted in C57BL/6 mice and different siRNAs were administered iv with or without the F105-P carrier. A fluorescent dye-labeled control siRNA was used to assess specificity of delivery. Naked siRNA did not enter tumor cells. Using the F105-P carrier, gp160-B16 tumors showed high uptake of the dye-labeled siRNA, whereas native B16 tumors did not, demonstrating the specificity of this delivery method. Tumor gp160-B16 cells were injected into the flanks of recipient mice and a cocktail of three siRNAs specific for *c-myc, MDM2,* and *VEGF* was administered either iv or by direct tumor injection with or without the F105-P carrier on days 0, 1, and 3 at a dose of 80 μg (~4 mg/kg); tumors were removed at day 9. Both intratumor and iv administration of the siRNA cocktail with F105-P delivery significantly reduced tumor size, whereas naked siRNAs or control treatments had no effect.

Although complicated polyplex delivery systems can offer significant advantages in administration of siRNAs *in vivo,* some very simple cationic polymers have also

doi:10.1016/j.ymthe.2006.01.001

been used with success. Ge and colleagues reported the use of PEI to deliver siRNAs targeting influenza virus via retroorbital iv injection in mice [197]. Unmodified siRNAs specific for genes encoding nucleocapsid protein (*NP*) or viral transcriptase (*PA* and *PB1*) were administered at doses of 30, 60, or 120 μg iv complexed to PEI in 200 μl PBS (using a nitrogen to phosphorus ratio of 5). This treatment did not trigger an obvious innate immune response and IFN-α was not detected in the lungs of mice using either influenza or control siRNAs with PEI. Administration of either of the influenza-specific siRNAs reduced viral titers in infected mouse lungs and a combination of anti-*NP* + anti-*PA* siRNAs was most effective. A reduction of viral titers ranging from 1 to 3 $\log_{10}$ was seen and siRNA treatment was effective even when given 5–24 h after infection. Thomas and colleagues reported that deacylated PEI (PEI187) improved delivery of plasmid DNA in mice by iv administration 10^4-fold and dramatically improved pulmonary targeting [131]. The deacylated PEI187 was used to deliver anti-*NP* siRNAs in mice by retroorbital iv injection using the same *in vivo* influenza model system described by Ge. A 94% reduction in viral titers was achieved with administration of a single 120-μg dose of siRNA. However, these results are similar to those reported previously by Ge using similar siRNA doses with unaltered PEI.

Atelocollagen is a peptic digest of collagen with low immunogenicity. Takeshita and colleagues report the use of atelocollagen to assist in iv delivery of siRNAs in a bone tumor metastasis model system in mice [143]. Luciferase-expressing PC-3M-luc-C6 cells were injected into nude mice. After 4 weeks, unmodified siRNAs were administered at a dose of 50 μg (2.5 mg/kg) by iv injection either with or without 0.05% atelocollagen, and luciferase expression was measured by *in vivo* bioluminescence 24 h later. The control siRNA or the naked anti-*Luc* siRNA had no effect. The anti-*Luc* siRNA administered with atelocollagen showed a 90% reduction in luciferase light emission. Tissue distribution of siRNAs was assessed using a RPA. Atelocollagen was seen to facilitate delivery into liver, lung, spleen, and kidney. Innate immune stimulation was tested (2 h postinjection) and siRNAs delivered by this approach did not result in a detectable increase in IL-12 or IFN-α levels; however, the single time point examined may have been inadequate to support this conclusion fully. Two genes thought to be involved in metastasis were targeted, enhancer of zeste homolog 2 (*EZH2*) and 3′-hydroxykinase p110-α-subunit (*p110*-α). Anti-*EZH2* or anti-*p110*-α siRNAs were administered iv at a dose of 50 μg on days 3, 6, and 9 after injection of tumor cells, and animals were imaged for the presence of bone implants. Use of control or naked siRNAs had no effect on tumor development; however, both anti-*EZH2* and anti-*p110*-α siRNAs gave dramatic reductions in the number of bone lesions when administered with atelocollagen.

Although some researchers have reported success in the use of unmodified siRNAs with naked iv administration, a number of studies report that improved uptake and efficacy are seen when siRNAs are administered as a complex with some kind of polymer that facilitates delivery and/or tissue targeting.

Intraperitoneal Administration

Injection of bacterial LPS into mice triggers secretion of tumor necrosis factor (TNF-α) and other cytokines by macrophages, resulting in a septic shock-like syndrome and death from cardiovascular collapse. Sorenson and colleagues describe the use of an unmodified anti-*TNF* siRNA to rescue mice from what would otherwise be a fatal dose of LPS [198]. Mice were given the anti-*TNF* siRNA in cationic DOTAP liposomes by ip injection (dose was not stated) 18 h before administration of 350 μg of bacterial LPS. Only 1/8 mice given a mismatch control siRNA "survived" this treatment, while 12/16 mice given the anti-*TNF* siRNA "survived." The precise meaning of "survival" in this experiment, however, is not entirely clear since the mice were euthanized based on a subjective evaluation of morbidity. Cytokine levels were measured in peritoneal lavage fluid and TNF-α was reduced by ~75% in the anti-*TNF*-treated animals, while IL-1α levels were unchanged.

Yin and colleagues used ip delivery of siRNAs with cationic lipid to study the role that heat-shock proteins (HSPs) have on thermotolerance and cardiac ischemia–reperfusion injury in mice [115]. Unmodified siRNAs targeting heat shock factor 1 (HSF1) were given at a dose of 25 μg (1.2 mg/kg) by ip injection with cationic lipid; 72 h after siRNA injection, mice were subjected to whole-body hyperthermia (WBH), elevating rectal temperature to 42°C for 15 min. Cardiac ischemia–reperfusion injury was performed 48 h after WBH treatment. HSF1 is usually induced to fourfold control levels by WBH. Treatment with the anti-*HSF1* siRNA blocked elevation of *HSF1* mRNA (*HSF1/GAPDH* ratio) relative to an amine-alone control (not a control siRNA). Further, treatment with the anti-*HSF1* siRNA resulted in increased size of myocardial infarction following ischemic injury and increased mortality from WBH, as would be expected if the protective effects of HSPs were removed by siRNA treatment.

Urban-Klein and colleagues used linear low-molecular-weight PEI as a carrier to deliver unmodified siRNAs by ip administration in nude mice with sc implants of SKOV-3 tumors [199]. The ability of PEI to protect unmodified siRNAs from degradation in serum was examined and naked siRNAs were degraded within 15 min of exposure to serum, while ~2/3 of the siRNAs complexed with PEI were intact after 6 h. Intraperitoneal administration of siRNA/PEI led to highest accumulation in muscle and tumor and to a lesser extent in liver. SKOV-3 tumors were implanted into the flanks of nude mice and a siRNA

specific for the *HER2* oncogene was administered three times a week at a dose of 0.6 nmol (~8 μg) starting when tumors reached a size of 10 mm^2. Control or naked siRNAs did not influence tumor growth but the anti-*HER2* siRNA administered with PEI led to a marked reduction in tumor size as well as an average of 50% reduction in *HER2* mRNA in excised tumors.

Direct Injection, Intraocular

Direct injection of siRNAs into a target tissue can allow for lower dosing relative to systemic administration, reduce the risk of systemic toxicity, and permit access to otherwise protected sites such as the CNS or the interior of the eye. As mentioned previously, the only FDA-approved antisense drug (Vitravene) is given by intraocular injection to treat CMV retinitis. Intraocular injection is therefore an obvious approach for *in vivo* use of siRNAs for both research applications and pharmaceutical development. Reich and colleagues described the use of unmodified siRNAs administered by intraocular injection to inhibit neovascularization following laser photocoagulation [200]. To demonstrate the feasibility of the method, recombinant adenovirus expressing enhanced green fluorescent protein (EGFP) were injected subretinally in the eyes of mice with or without 20 pmol (~0.27 μg) of an anti-*EGFP* siRNA, and retina were examined at 48 h. The anti-*EGFP* siRNA almost totally eliminated EGFP fluorescence, even using this very low dose. Administration of a similar low dose of an anti-*VEGF* siRNA reduced the area of choroidal neovascularization after laser photocoagulation injury by ~75%.

Shen and colleagues also reported the use of a siRNA that disrupts the VEGF pathway to reduced neovascularization following laser photocoagulation in mice [201]. The compound studied, Sirna-027, is a chemically modified siRNA that targets VEGFR1 and is under development as a therapeutic for treatment of age-related macular degeneration (see below). SiRNA was administered as a direct intravitreal injection at doses of 1.5 or 0.5 μg in 1 μl PBS on days 1 and 7 following laser-induced rupture in Bruch's membrane. On day 14, choroidal neovascularization was visualized using fluorescein-labeled dextran and fluorescence microscopy. Neovascularization was reduced by 56% using the 1.5-μg dose compared with injection of a control siRNA (in the other eye) and by 66% using the 0.5-μg dose compared with injection of PBS alone. A dose of 7.5 μg given by periocular injection (a less traumatic method of administration) on days 1, 4, 7, 10, and 13 resulted in a 45% reduction in neovascularization following laser treatment. Treatment with Sirna-027 was also protective against oxygen-induced ischemic retinopathy. Both VEGFR1 mRNA and protein were demonstrated to be specifically reduced by injection of Sirna-027. Distribution of a BrdU-labeled siRNA after intravitreous injection was studied. After 6 h, labeled siRNA was detected in ganglion cells and by 12–24 h the siRNA was present in ganglion cells and the inner nuclear layer. By 48 h, staining was absent in ganglion cells but was increased in photoreceptor cell bodies. Injected siRNA was no longer detectable at 5 days postinjection.

Ocular infection with herpes simplex virus (HSV) can induce stromal keratitis and neovascularization. Kim and colleagues used unmodified siRNAs targeting three genes in the VEGF pathway (*VEGFA, VEGFR1,* and *VEGFR2*) to block angiogenesis from HSV infection [202]. A cocktail of all three siRNAs was administered either by subconjunctival injection (10 μg) or by tail vein iv injection (40 μg) complexed with a RPP polymer (see above). Using the anti-*VEGF* cocktail, both methods of treatment significantly improved the clinical angiogenic score relative to the control siRNA. Subconjunctival administration reduced *VEGF* mRNA in infected cornea approximately sixfold, while system administration achieved only approximately twofold reduction (using a fourfold higher dose of siRNA).

Direct Injection, CNS

Antisense oligonucleotides have a long history of successful use in neuroscience research [203]. Although charged nucleic acids do not readily cross the blood–brain barrier, oligonucleotides can be directly injected into the CNS. Compared with plasma and many tissues, the CNS is a relatively "nuclease-poor" environment and antisense oligonucleotides with minimal modification have been employed with good results [204]. It is therefore not surprising that direct injection of unmodified siRNAs into the CNS has met with success. Dorn and colleagues reported the use of minimally modified siRNAs administered using an implanted intrathecal pump in rat CNS to modulate chronic neuropathic pain [205]. RNA duplexes had 2′-*O*-(2-methoxyethyl)-modified residues (MOE-modified) with phosphorothioate linkages comprising the 2-base 3′ overhangs on each strand and targeted the pain-related cation-channel $P2X_3$. SiRNAs were administered by continuous infusion at a dose of 400 μg/day (20 mg/kg) for 6–7 days. Dorsal root ganglia showed ~40% reduction in $P2X_3$ mRNA and protein. Rats exhibited an attenuated pain response in several models of neuropathic pain, including tactile allodynia and agonist-induced mechanical hyperalgesia.

Thakker and colleagues reported knockdown of two targets in mouse brain using direct CNS administration of 3′-overhang MOE-modified siRNAs. Alzet osmotic minipumps delivered 400 μg/day of siRNA (20 mg/kg) into the dorsal third ventricle. In the first series of studies, anti-*EGFP* siRNAs were infused into EGFP-expressing mice. Following 2 weeks of infusion, the effect on EGFP fluorescence ranged from no suppression (in the locus coeruleus and the spinal trigeminal nucleus) to 50% suppression (in the dentate gyrus and hippocampus) [206]. SiRNAs targeting the dopamine transporter (*DAT*)

doi:10.1016/j.ymthe.2006.01.001

gene were also infused and were found to reduce *DAT* mRNA levels and produce a temporal hyperlocomotor response similar to that seen using a small-molecule antagonist of DAT [206]. Infusion of siRNAs targeting the serotonin transporter (*SERT*) reduced *SERT* mRNA levels and produced an antidepressant-like response similar to that observed from administration of Citalopram, a small-molecule serotonin-reuptake inhibitor [207].

Use of a cationic lipid delivery agent may enable lower doses of siRNAs to be used for these kinds of studies and can be safe for use in the CNS. Luo and colleagues delivered an unmodified control siRNA or a siRNA specific for the delta opioid receptor (DOR) via an implanted intrathecal catheter in the lumbar spine of rats [117]. Dose ranged from 0.5 to 4.0 μg/day given as a 2 μg/10 μl solution in the cationic lipid i-Fect (very low dose of 2.5–20 μg/kg). Fluorescence signal was readily visualized in the spinal cord and dorsal root ganglia using a dye-labeled siRNA with cationic lipid but not with unassisted infusion. DOR protein levels were markedly reduced when examined by immunohistochemistry 72 h after the last dose of siRNAs in anti-*DOR*-treated animals but not mismatch control-treated animals. Levels of the related mu opioid receptor were unchanged, demonstrating specificity. Functionally, treatment with the anti-*DOR* siRNA specifically blocked the antinociceptive effect of a DOR agonist. Although eight groups have published data showing successful use of siRNAs in CNS studies, two groups have reported negative results [208,209], both using siRNAs that had been validated *in vitro*.

Direct Injection, Tumor

A number of groups have reported success using direct injection into or adjacent to tumor masses using naked and/or complexed siRNAs. Aharinejad and colleagues reported direct injection of unmodified siRNAs into subcutaneous masses of the human mammary tumor cell line MCF-7 in nude mice [210]. Injections were started after tumors were established (beginning 24 days after implantation) at a dose of 10 μg once every 3 days for 2 weeks. Two different siRNAs specific for murine colony-stimulating factor (CSF)-1 and the CSF-1 receptor (*c-fms* proto-oncogene) were employed. CSF-1 signaling recruits macrophage infiltration of tumors and several macrophage-derived factors are believed to facilitate metastasis and tumor growth (matrix metalloproteases and VEGF). SiRNAs were tested *in vitro* and the most active sequences were employed for subsequent *in vivo* experiments. Both CSF-1 and CSF-1R mRNA levels were reduced in tumor tissue. Consistent with the original hypothesis, both metalloprotease levels and VEGF levels were also reduced in tumors by this treatment and tumor growth was reduced by ~50%.

Leng and Mixon used cationic peptides to facilitate delivery of unmodified siRNAs by direct injection into human breast cancer MDA-MB-435 implants in nude mice [211]. The siRNA employed was specific for Raf-1, a member of the Raf kinase family involved in Ras/MEK signaling. A series of structurally different histidine–lysine peptides were synthesized and compared with DOTAP liposomes for their ability to deliver siRNAs in mice. Injections contained 12 μg peptide complexed with 4 μg siRNA and were administered every 5 days for two or three doses. The H^3K4b peptide/anti-*Raf-1* siRNA complex was most effective and reduced tumor size by 60% relative to treatment with the control siRNA/peptide and was superior to the liposomal preparation.

Atelocollagen has also been used to assist with direct intratumor injections. Takei and colleagues described injection of an unmodified anti-*VEGF* siRNA in 50 μl of a 1.75% solution of atelocollagen in PBS into established human prostate cancer PC-3 cell implants in nude mice [212]. Dose–response curves were performed using 1, 5, and 10 μM concentrations of the siRNA (0.67–6.7 μg); injections were done once every 10 days and tumor response was assessed at 40 days. Treatment with the anti-*VEGF* siRNA reduced tumor volume in a dose-dependent fashion and, using the highest dose, tumors were ~80% smaller than those in control siRNA-treated mice. An 80% reduction in VEGF protein was observed in day 17 tumors treated with 10 μM anti-*VEGF* siRNA. Properties of atelocollagen as a delivery agent were also described. Tumors injected with a fluorescent dye-labeled siRNA with atelocollagen had significantly more labeled cells (also "brighter") than tumors injected with naked siRNAs. Further, intact ^{32}P-labeled siRNA could be detected in tumors 7 days after injection with atelocollagen, whereas naked siRNAs were mostly undetectable only 1 day postinjection.

Topical Administration

Nucleic acids can be absorbed through epithelial or mucosal surfaces. Maeda and colleagues described the use of this delivery route to administer unmodified siRNAs in a murine deafness model system [118]. The *GJB2* gene (gap junction protein, β-2) encodes a transmembrane protein that is involved in potassium recycling during auditory signaling. Allelic variants of this gene are implicated in some severe forms of hereditary deafness. A dominant-negative mutant of this gene ($GJB2_{R75W}$) was fused to EGFP and was used to create a model of human deafness in mice. Anti-$GJB2_{R75W}$ siRNAs were delivered to the cochleae of mice in a mixture of DOTAP/cholesterol liposomes (GeneSHUTTLE) using 0.5 μg siRNA in 2 μl soaked in a piece of gel foam, which was surgically placed against the round window membrane. Administration of the anti-$GJB2_{R75W}$ siRNA reduced expression of $GJB2_{R75W}$ mRNA by 70% without affecting levels of normal murine *Gjb2* and reversed the auditory impairment caused by expression of the mutant human gene in mouse cochleae.

Palliser and colleagues used unmodified siRNAs in a cationic lipid carrier as a "microbicide" applied intravaginally to block HSV2 infection in mice [119]. Mice were vaginally infected with 2×10^4 pfu (~2× LD_{50} dose) of HSV2 and were given control or anti-HSV2 siRNAs at "−2 h and 4 h" time points or at "+3 and +6 h" time points. Duplexes targeting several viral genes were compared and one targeting the viral *UL29* was most potent in suppressing virus production *in vitro*. SiRNAs were administered intravaginally as described at a dose of 0.5 nmol (~7 μg) in 12-μl volume complexed with Oligofectamine. Mice receiving the anti-*UL29* siRNA had ~80% survival at 15 days, while mice receiving the control or no siRNA had 20–25% survival. Mice receiving a siRNA targeting a different viral gene, *UL27,* had ~60% survival. Importantly, no evidence for activation of the innate immune system was detected following siRNA treatment in the absence of viral infection (*IFN-β, STAT1,* and *OAS1* mRNA levels were tested by qRT-PCR). Thus, two independent anti-HSV2 siRNAs showed similar biological effects with no evidence of immune stimulation, so the antiviral effect could be attributed directly to RNAi-mediated suppression. Delivery of siRNAs in liposomal or cationic lipid "paint" might similarly be useful for other epithelial surfaces, such as oral or rectal mucosa.

Intranasal and Pulmonary Delivery

Antisense oligonucleotides have been administered by inhalation to treat asthma and other pulmonary diseases [213,214]. Similarly, several groups have delivered siRNAs by intranasal or intratracheal infusion in mice. Massaro and colleagues describe intranasal administration of both siRNA and small-molecule compounds to mice in InfaSurf (replacement pulmonary surfactant, more commonly used to treat neonatal severe respiratory distress syndrome) [215]. An unmodified siRNA targeting GAPDH was administered intranasally in lightly anesthetized mice at a dose of 10 μg in 10- to 20-μl volume. At 24 h postinfusion, GAPDH protein levels were reduced by 50% or more in lung, heart, and kidney, but not in liver or brain. At 7 days, GAPDH was further suppressed to 67% control levels in lung but other tissues showed limited or no effect.

A number of groups are exploring the use of siRNAs to treat respiratory viruses using direct pulmonary delivery in mammals. Tompkins and colleagues used unmodified siRNAs specific to viral NP and PA proteins to protect mice from lethal infection with influenza virus [157]. SiRNAs in PBS were administered iv by HD tail vein injection 16–24 h prior to infection (single dose of 50 μg, or ~2.5 mg/kg). Influenza virus was then coadministered with siRNAs complexed with the cationic lipid Oligofectamine by direct intranasal infusion in mice at a dose of 1.5 nmol (20 μg, or ~1 mg/kg). Viral titers in lung tissue were reduced as much as 63-fold using anti-influenza siRNAs compared with the control siRNA and survival was significantly improved, especially in mice receiving a combination of anti-*NP* + anti-*PA* siRNAs. Bitko and colleagues described the use of unmodified siRNAs to treat pulmonary infection with respiratory syncytial virus (RSV) and parainfluenza virus in mice by targeting the viral *P* gene for both viruses [216]. SiRNAs in PBS were administered either naked or complexed with the cationic lipid TransIT-TKO by direct intranasal infusion in mice at a dose of 35 μg in each nostril (70 μg total dose, or ~3.5 mg/kg). Treatment with naked siRNAs was ~70% as effective as with cationic lipid. Mice receiving antiviral siRNAs showed decreased lung pathology and overall distress compared with mice receiving control siRNAs. Although the greatest improvement was seen when treatment was started at the time of infection, some benefit was seen when siRNA treatment was given 1, 2, and even 3 days postinfection.

The severe acute respiratory syndrome coronavirus (SCV) has been the subject of intense investigation in recent years and development of any kind of therapeutic method to treat this virus other than supportive care is a high priority. Li and colleagues describe the use of unmodified siRNAs delivered intranasally in D5W in rhesus macaques (*Macaca mulatta*) [217]. Delivery of siRNAs targeting a reporter construct were first tested using D5W or InfaSurf via intranasal delivery in mice and the D5W carrier gave superior results and was therefore employed in the primate studies. A mixture of two siRNAs specific for the Spike protein-coding and ORF1b regions was administered 4 h prior to infection (single dose), at the time of infection (and at 1, 3, and 5 days postinfection), or 4 h postinfection (and at 1 and 3 days postinfection). Each dose was 30 mg in 3 ml D5W and total cumulative dosing was 30 to 120 mg siRNA (10–40 mg/kg). The severity of symptoms was improved in all treatment groups. Animals were tested at 4 days postinfection for the presence of SCV RNA using a qRT-PCR assay (from oropharyngeal swab samples); all control animals were positive for SCV viral RNA, while 75% of anti-SCV siRNA-treated animals had no detectable viral RNA.

Electroporation

Electroporation is a useful tool to transfect siRNAs *in vitro* into cell lines that are refractory to cationic lipid-mediated transfection. Interestingly, it is also being employed to assist with siRNA delivery *in vivo*. Golzio and colleagues described electroporation-assisted delivery of an EGFP expression plasmid with or without an unmodified anti-*EGFP* siRNA following direct injection into mouse muscles in PBS [171]. EGFP fluorescence was specifically reduced by the anti-*EGFP* but not the control siRNA; targeting of an endogenous target was not described. Schiffelers and colleagues used electroporation to assist with delivery of unmodified siRNAs targeting

doi:10.1016/j.ymthe.2006.01.001

TNF-α in mice with collagen-induced arthritis [170]. Injection of an EGFP expression plasmid into the knee joint of mice showed local expression of EGFP only if electroporation was employed. Coadministration (injection + electroporation) of 2 μg of a luciferase expression plasmid with 10 μg of either the anti-*EGFP* (control) or the anti-*Luc* siRNAs showed 90% suppression of luciferase activity when the anti-*Luc* siRNA was used. Collagen-induced arthritis was induced by injection of mice with type II collagen and inflammation was triggered with ip injection of LPS 3 days after the second dose of collagen. Anti-*TNF*-α siRNA treatment was initiated 3 days after LPS treatment at a dose of 10 μg (direct injection with electroporation into the knee joint) and was administered weekly for three doses. Animals treated with the anti-*TNF*-α siRNA showed a marked improvement in inflammation and reduced arthritis score relative to control animals. Similarly, Inoue and colleagues used electroporation to assist with delivery of an unmodified siRNA targeting TNF-α in rats with collagen-induced arthritis [172]. In this case, 0.8 nmol (~11 μg) of the anti-*TNF*-α siRNA or a control siRNA was injected into the knee joint in rats in cationic lipid (siPORT Amine) followed by electroporation. TNF-α mRNA levels were reduced in synovial tissue and inflammation was markedly reduced as assessed by both paw swelling and histological examination in anti-*TNF*-α-treated animals relative to controls.

Other Delivery Approaches

In addition to chemical and electrical methods to promote delivery of siRNAs *in vivo* discussed above, Kim and colleagues described a mechanical method using a gold nanoparticle gene gun in the context of improving the immune response in mice to DNA vaccines [174]. Infection with human papillomavirus type 16 (HPV-16) is associated with development of cervical carcinoma. Attempts to immunize mice against HPV-16 through the use of a DNA vaccine (a plasmid expressing the *E7* gene of HPV-16) resulted in an incomplete response, partially due to the transient nature of antigen presentation by resident antigen-presenting cells (APCs). The authors tested if coadministration of the HPV-16 E7 plasmid with siRNAs targeting the proapoptotic genes *BAX* and *BAK* would prolong survival of the APCs and improve vaccination. Nucleic acids were coated onto gold microcarriers and delivered subdermally using the Helios gene gun at a dose of 2 μg plasmid coadministered with 0.2 μg unmodified anti-*BAK* + anti-*BAX* siRNAs. Vaccine delivered with the *BAK* + *BAX* siRNAs increased the number of antigen-specific IFN-γ^+ $CD8^+$ T cell precursors. Further, mice receiving the combined vaccine (E7 with *BAX* + *BAK* siRNAs) showed a marked reduction in tumor burden when injected with TC-1 cells expressing the E7 antigen compared with mice that received the E7 vaccine with the control siRNA.

Kinoshita and Hynynen described the use of microbubbles from focused ultrasound (sonoporation) to deliver siRNAs to cells *in vitro* [218]. Tsunoda and colleagues used sonoporation to deliver an EGFP expression plasmid with or without unmodified anti-*EGFP* siRNA to cardiac tissue in mice [173]. The EGFP expression plasmid was injected into the left ventricle of anesthetized mice (500 μg in 400 μl PBS with 100 μl BR14 microbubbles) and transthoracic ultrasound stimulation was applied. EGFP fluorescence was detected in the cardiac ventricular wall and in the coronary arteries. Co-injection of 40 μg of the anti-*EGFP* siRNA suppressed EGFP fluorescence.

DEVELOPMENT OF siRNA THERAPEUTICS

Over 30 pharmaceutical and biotechnology companies have declared an interest in or have an active drug development program already under way in RNAi-based therapeutics. A number of these companies and their drug development programs are outlined in Table 2. In addition, many large pharmaceutical companies that are traditionally associated with small-molecule drug development also have active internal or collaborative RNAi-related drug development projects.

Sirna Therapeutics reported interim results of a Phase I study of Sirna-027 at the American Society of Gene Therapy conference in June 2005 [219]. Sirna-027 is a chemically modified siRNA drug that targets VEGFR1 to treat age-related macular degeneration (AMD). The study involved administration of a single escalating dose of the drug by direct intravitreal injection. Doses from 100 to 800 μg were administered and no significant toxicity was observed. During the brief observation period, visual acuity stabilized and even improved in a dose-dependent manner. Preclinical studies of Sirna-027 in a mouse model system have also been reported (with Johns Hopkins University) [201].

Acuity Pharmaceuticals reported results of a Phase I clinical trial of Cand5, a siRNA drug targeting VEGF to drug to treat AMD, at the October 2005 meeting of the American Academy of Ophthalmology. Fifteen patients were administered Cand5 at five escalating dose levels up to 3.0 mg per eye by direct intravitreal injection. No adverse effects that related to the actual siRNA drug were reported; however, the expected side effects such as subconjunctival hemorrhage, etc., were observed (normal for this route of drug administration). No systemic delivery was detected. Patients are currently being enrolled in a Phase II clinical trial.

Alnylam Pharmaceuticals has drug development programs ongoing for multiple indications and has specifically partnered with Novartis, Merck, and Medtronic for certain projects. The most advanced drug in their pipeline targets RSV and an IND was filed on November 1, 2005, with the FDA for their lead compound, ALN-RSV01. Treatments for pandemic influenza are next in the

development pipeline and Alnylam plans to file an IND in this field later in 2006.

Many companies have reported preclinical data validating their siRNA drug development projects *in vivo* in mammalian model systems. Nastech Pharmaceuticals (working with Mayo Clinic) presented data on the use of an anti-*TNF*-α siRNA delivered using their peptide carrier system to treat rheumatoid arthritis at the November 2005 American College of Rheumatology meeting. Alnylam Pharmaceuticals has published preclinical work relating to treatment of hypercholesterolemia using anti-ApoB siRNAs [65] and Sirna Therapeutics has published preclinical work relating to treatment of hepatitis B infection (with Protiva Biotherapeutics, who provided the SNALP delivery system) [52,63]. Calando Pharmaceuticals published results of a collaborative study relating to siRNA treatment of Ewing sarcoma (with Children's Hospital of Los Angeles) [134] and Galenea has described the use of siRNAs to treat influenza in mice (work done at MIT) [197]. Intradigm has published a number of collaborative studies done relating to siRNA treatment of cancer, arthritis, ocular diseases, and viral diseases [130,170,194,202,217]. Abbott described the use of siRNAs in a mouse cancer model system [220] and Novartis has reported the use of siRNAs in CNS studies [205–207]. TransGenex Nanobiotech's chitosan delivery system was employed in a report using ddRNAi to treat RSV (University of South Florida) [221] and NeoPharm has published the use of their liposomes to deliver siRNAs in two mouse cancer model systems (one with Georgetown University Medical Center) [127,128]. Mirus has published the use of their hydrodynamic delivery system in mice [149] and larger mammals (with the University of Wisconsin, Madison, WI, USA) [155].

Considering the relative youth of the RNAi field, a remarkable number of *in vivo* studies have already been published that relate to preclinical development of siRNA drugs or the use of siRNA methods to validate targets for small-molecule drug development projects.

CONCLUSIONS

RNA interference has proven to be an extremely potent and versatile tool to specifically reduce expression of targeted genes. Use of this technology has rapidly moved from *in vitro* cell culture studies to *in vivo* administration in mammals, both to address questions of basic biology and in drug development programs. RNAi-based drugs are already in clinical trials and it is hopeful that a siRNA therapeutic will receive FDA approval in the not too distant future.

As understanding of the basic biochemistry and biology of RNAi advances, it is becoming clear that extra controls should be employed when using siRNAs *in vivo* to avoid the risk of misinterpreting results due to unintended stimulation of the innate immune system or off-target effects. Few published studies actually measured cytokine levels. Most simply employed "control" siRNAs to ensure that immune stimulation or other unintended events did not occur. Given the sequence-specific nature of innate immune responses, confidence in these kinds of controls may be misplaced, even if modified siRNAs that have a lower incidence of immune stimulation are employed. Measurement of serum IFN-α, TNF-α, and possibly IL-12 levels at ~4–6 h posttreatment should be considered, especially when systemic administration methods are employed. If local administration is employed, assays should include extracts prepared from the injected site/organ as well as from serum.

Off-target effects can be difficult to identify and are also sequence specific. Microarrays have been used to assess the extent of OTEs at the gene expression level on a genome-wide scale in studies done in cell culture; however, this kind of experiment has not been reported yet *in vivo* and these kinds of data might be difficult to interpret. One approach to control for OTEs would be to require the use of two (or more) different siRNAs against the same target with the expectation that both result in suppression of the targeted gene and produce the same biological response (phenotype). Few of the *in vivo* studies reported to date confirmed results using two independent siRNA sequences. Further, few of the studies discussed here performed any kind of dose–response optimization of the siRNA reagent *in vivo* and instead simply reported results using a single dosing regimen. Even though these studies are expensive to perform in mammals, it is important to characterize the system fully and this kind of information may uncover dose-dependent side effects. Actual OTE-related events will probably show variation between individuals, especially in outbred populations, making these controls even more important to include in this setting.

Although some labs have reported success using *in vivo* administration of naked siRNAs, a greater number of investigators reported that using some kind of delivery system improved results. Route of administration and choice of which delivery tool to use will be crucial to success. Other than hydrodynamic delivery, too few *in vivo* studies have been published using any other single method to accumulate the amount of information needed to assess functional performance adequately (with different target genes and different target tissues) or permit a general endorsement of that method, although several approaches do seem quite promising. Unfortunately, many of the most advanced delivery systems employed in the studies discussed above are not available to most researchers. Widespread adoption of RNAi using synthetic siRNAs as an *in vivo* research tool will accelerate as more effective mammalian delivery tools become commercially available. If the target tissue or system of study permits the use of a local delivery approach, this method may be easier to perform using

TABLE 2: Pharmaceutical and biotechnology companies with active siRNA drug development programs

Company	Notes	Indications	Clinical pipeline
Acuity Pharmaceuticals http://www.acuitypharma.com/	Cand5, siRNA targeting VEGF	Age-related macular degeneration (AMD); diabetic retinopathy (DR)	AMD–siRNA IND filed Aug 10, 2004; Phase II started Oct 14, 2005
AGY Therapeutics http://www.agyinc.com/	RNAi in neurons and glial cells	CNS	
Alnylam Pharmaceuticals http://www.alnylam.com/	Modified siRNAs, cholesterol conjugated; partnered with Novartis, Merck, and Medtronic; ''InterfeRx'' ip licensure program	ALN-RSV01–RSV; influenza; cystic fibrosis; Parkinson disease	RSV–siRNA IND filed Nov 1, 2005
Atugen http://www.atugen.com/	2′-*O*-Me-modified siRNAs; Aventis and Sankyo collaborations	Metabolic diseases; epithelial cancers	Preclinical stage
Benitec http://www.benitec.com/	Both siRNA and ddRNAi approaches; collaboration with Calando for delivery; InterfeRx partner with Alnylam	Hepatitis C (cocktail of three siRNAs); HIV	Phase I ddRNAi clinical trial for AIDS lymphoma with City of Hope, 2006
Calando Pharmaceuticals http://www.calandopharma.com/	Cyclodextrin polymer delivery system; collaboration with Benitec	Hepatitis C; cancer	Preclinical stage
CombiMatrix http://www.combimatrix.com/	Parallel chip-based target site optimization	Hepatitis C; HIV	Preclinical stage
CytRx http://www.cytrxlabs.com/	Small-molecule drug program more advanced than RNAi program	Amyotrophic lateral sclerosis; obesity; type II diabetes; CMV	
deVGen http://www.devgen.com/	Collaboration with Genentech; target validation using *C. elegans RNAi*	Diabetes/obesity	
Galapagos http://www.galapagosgenomics.com/	Adenoviral shRNA/ddRNAi vector approach	Arthritis; osteoporosis; Alzheimer disease; asthma	
Galenea http://www.galenea.com/	Small-molecule CNS drug development with Otsuka Pharmaceuticals; separate RNAi respiratory disease program	Influenza	Preclinical stage
GeneCare http://www.we-care-gene.com/	InterfeRx licensee with Alnylam for two helicase targets	Cancer	
Genesis R&D http://www.genesis.co.nz/	IgE and IgE receptor targeting	Asthma; atopic dermatitis	Preclinical stage
Genomica http://www.genomica.es/	Diagnostics company, recently added RNAi to its R&D program	Undeclared	
Genta http://www.genta.com/	Both antisense and RNAi	Oncology	
IC-Vec http://www.icvec.com/	Lipid nanoparticle delivery system	Hepatitis B and C; cancer	
International Therapeutics http://www.internationaltherapeutics.com/	Collaboration with Rossi lab, City of Hope for HIV	HIV; viral diseases	
Intradigm http://www.intradigm.com/	Synthetic nanoparticle delivery system; many collaborations, including Nucleonics	Cancer; SARS	Preclinical stage

doi:10.1016/j.ymthe.2006.01.001

doi:10.1016/j.ymthe.2006.01.001

Mirus http://www.mirusbio.com/	Hydrodynamic and polymer-based particle delivery systems	Undeclared	
Nastech Pharmaceuticals http://www.nastech.com/	Peptide based delivery systems; InterfeRx licensee with Alnylam for TNF-α target	Rheumatoid arthritis; metabolic diseases; cancer; others	Preclinical stage
NeoPharm http://www.neophrm.com/	Liposomal delivery platform	Undeclared	
Novosom http://www.novosom.com/	Liposomal delivery platform; collaborations with Nucleonics, Isis, and Santaris	Undeclared	
Nucleonics http://www.nucleonicsinc.com/	ddRNAi, expressed RNAs using a variety of configurations; collaboration with Intradigm	Hepatitis B and C	Preclinical stage
Phytovation http://www.phytovation.com/	Vaccines and other antiviral therapies, including RNAi.	Hepatitis C; influenza; HIV	Preclinical stage
Protiva Biotherapeutics http://www.protivabio.com/	Lipid particle delivery system for both small molecules and siRNA; Sirna collaboration	Viral diseases; cancer; metabolic diseases	Preclinical stage
Quark Biotech http://www.quarkbiotech.com/	Small-molecule, monoclonal antibody, and siRNA programs; using modified siRNAs	AMD, DR; acute renal failure	Preclinical stage
Santaris Pharma http://www.santaris.com/	LNA-based technologies; programs in both antisense and RNAi	Cancer	
Sirna Therapeutics http://www.sirna.com/	Experience with heavily modified RNAs from ribozyme work; collaborations with Eli Lilly and Archemix; AMD with Allergan	AMD–Sirna-027; hepatitis C; asthma; diabetes; Huntington disease; permanent hair removal	Phase I for AMD started Nov 2004, Sirna-027
siRNAsense http://www.sirnasense.com/	Commercialization of siRNA-related compounds developed in Norwegian institutions	Cancer; tissue factor; aquaporins	
ToleroTech http://www.tolerotech.com/	Variety of technologies relating to organ transplant	Transplantation	
TransDerm http://www.pachyonychia.org/	Founded through the PC project group	Pachyonychia congenital (PC)	
TransGenex Nanobiotech http://www.transgenex.com/	Nanochitosan polymer delivery system	Pulmonary diseases; cancer	

doi:10.1016/j.ymthe.2006.01.001

"off the shelf" tools and offer greater assurance of success than systemic iv administration.

ACKNOWLEDGMENTS

The author thanks Drs. John Rossi, Bryan Williams, Edouard Cantin, and Scott Rose for helpful discussions and for reading the manuscript.

RECEIVED FOR PUBLICATION DECEMBER 5, 2005; REVISED JANUARY 11 2006; ACCEPTED JANUARY 11, 2006.

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