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The silent treatment: siRNAs as small molecule drugs

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As soon as RNA interference (RNAi) was found to work in mammalian cells, research quickly focused on harnessing this powerful endogenous and specific mechanism of gene silencing for human therapy. RNAi uses small RNAs, less than 30 nucleotides in length, to suppress expression of genes with complementary sequences. Two strategies can introduce small RNAs into the cytoplasm of cells, where they are active – a drug approach where double-stranded RNAs are administered in complexes designed for intracellular delivery and a gene therapy approach to express precursor RNAs from viral vectors. Phase I clinical studies have already begun to test the therapeutic potential of small RNA drugs that silence disease-related genes by RNAi. This review will discuss progress in developing and testing small RNAi-based drugs and potential obstacles.
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Introduction

RNA interference (RNAi) is a ubiquitous mechanism in eukaryotic cells to suppress the expression of genes that determine fundamental cell fate decisions of differentiation and survival.[1,2] In plants and lower organisms RNAi also protects the genome from viruses and insertion of rogue genetic elements, like transposons.[3–6] In RNAi, small double-stranded RNAs processed from long double-stranded RNAs or from transcripts that form stem-loops, silence gene expression by several mechanisms – by targeting mRNA for degradation, by preventing mRNA translation or by establishing regions of silenced chromatin.[7,8] All of these mechanisms suppress expression of genes bearing complementary sequences of at least seven nucleotides.[9–13] mRNA degradation is probably the most powerful mechanism for silencing gene expression and is the most specific. It is this RNAi pathway that therapeutic strategies have been designed to activate.[8] mRNA degradation occurs when one strand (the antisense or guide strand) of the short interfering RNA (siRNA, ~22 nucleotides in length) directs the RNA-induced silencing complex (RISC) that contains the RNA endonuclease Ago2 to cleave its target mRNA bearing a complementary sequence.[14–16] Although some RNAi mechanisms, especially inhibition of translation, do not require extensive base-pairing over the length of the siRNA, efficient mRNA cleavage likely requires high complementarity, thereby providing specificity.[17]

As every cell contains the RNAi machinery and any gene can be targeted with a good deal of (but still imperfect, see below) specificity, the prospect of specifically suppressing the expression of disease-causing genes has generated a lot of enthusiasm for developing RNAi-based therapies. Because RNAi is an endogenous and ubiquitous pathway, the effectiveness of gene silencing achieved with RNAi surpasses what has been possible in the past using other small nucleic acids, such as antisense oligonucleotides or ribozymes.[7,8] In one head-to-head comparison, siRNAs knocked down gene expression about 100–1000 fold more efficiently than antisense oligonucleotides.[18] The high efficiency may be related to the catalytic nature of RNAi, where one siRNA can be used over and over to guide the cleavage of many mRNAs.[19] The high efficiency may also be due to protection of the active component of the siRNA (the antisense or guide strand) from digestion by endogenous RNases by its incorporation into the RISC, although this has not been explicitly demonstrated.

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Silencing specificity and potential toxicity from off-target effects

Specificity is such that by clever siRNA design, disease-linked alleles of genes that differ by a single nucleotide polymorphism from their wild-type allele can be targeted for silencing without suppressing the expression of the corresponding wild-type gene.[20–22] Although initial reports suggested that siRNAs would have previously unheard of specificity for their targets, several mechanisms have since been described that can lead to unintended off-target effects on gene expression and need to be seriously considered in developing RNAi-based drugs. One potential problem is induction of the interferon response, which causes global, nonspecific inhibition of protein translation. Although small double-stranded RNAs, less than 30 nucleotides in length, do not efficiently activate a full interferon response,[17] a subset of interferon genes can be activated by siRNAs, particularly in specialized highly sensitive cell lines and at high concentrations of siRNAs.[23–26] Fortunately, this problem

has not been observed in most animal studies, even when looked at with highly sensitive RT-PCR assays. siRNAs containing GU-rich sequences can also indirectly induce IFN production by binding to Toll-like receptors (TLR3, TLR7 and TLR8) that alarm immune activating cells to the presence of RNA viral pathogens.[27–30] Toxicity from the ensuing inflammatory response, which activates interferons and other proinflammatory cytokine genes, might also pose a therapeutic problem.[23–25] However, binding of double-stranded RNAs to TLRs appears to be sequence specific and may be abrogated by chemical modifications of the sugar backbone of the siRNAs without jeopardizing efficacy.[31] Also the inflammatory effect requires much higher RNA concentrations than those required for gene silencing.

Perhaps more serious are unanticipated off-target effects that occur by siRNA recognition of other mRNAs bearing only partial homology.[32,33] Although microarray studies have shown that differences in off-target mRNA levels are generally small (usually much less than twofold, except for a few genes), effects on protein expression, which is more difficult to survey, might become important if the microRNA pathway of translation inhibition is activated.[32–34] siRNAs can act like microRNAs to inhibit translation, while microRNAs with high homology can direct mRNA degradation.[35,36] The rules for identifying microRNA targets are still not defined well enough to reliably predict genes that might be inadvertently silenced by any particular siRNA. Because microRNA silencing may be activated by base-pairing of only seven consecutive nucleotides, careful comparison of candidate siRNA guide strand sequences with mRNAs in the human genome is a prudent step to diminish potential off-target effects. Although short stretches of homology are unavoidable, longer stretches, which are likely to have a more profound effect on gene expression, can be avoided. These nonspecific off-target effects are not limited to the guide strand, but can also be generated by the sense strand of the double-stranded siRNA if it becomes incorporated into RISC and binds to another set of mRNAs bearing partially complementary sequences.[33] Because RISC incorporation favors the strand whose 5′-end is least thermodynamically stable to unwinding, it is now possible to design siRNAs destabilized at the 5′-end of the guide strand to promote incorporation of only the guide strand and thus minimize this potential problem.[37–39] A recent comparison of the effects that siRNAs and delivery agents had on the nonspecific silencing of gene expression found that the majority of off-target effects were due to the lipid-based transfection reagent and not the siRNA.[40] This is consistent with findings that naked siRNAs produced no detectable interferon response upon injection into mice.[41] Whether off-target silencing of unintended genes will pose a real problem for clinical use remains to be seen. As the microRNA effect on gene expression is weak and is likely to require the concerted action of multiple microRNAs working on a single mRNA to block translation, it may not turn out to be a significant problem in practice.[34]

Another possible source of unintended toxicity might arise if the introduced RNAs compete with limited amounts of Dicer and RISC to interfere with endogenous RNAi pathways required to maintain the cell in its differentiated state. In fact, there is evidence that the RNAi machinery can be limiting.[42] One of the ways adenovirus circumvents a potential host RNAi antiviral effect is to express high amounts of a non-coding RNA stem-loop that interferes with nuclear-cytoplasmic transport by binding to exportin 5 and inhibits processing of host cell siRNA and microRNA precursors by binding to Dicer.[43] Although siRNAs silence at such low concentrations that this source of toxicity is unlikely in most cells, it may set a limit on how many different siRNAs can be used simultaneously in one target cell. In clinical situations, where escape from RNAi-based drugs is likely and where silencing multiple gene targets is therefore a good idea (such as in suppressing viral infection or treating tumors) competition for the RNAi machinery may need to be considered.

Optimizing siRNA design for silencing

Silencing can vary from less than twofold suppression by inefficient siRNAs to undetectable target mRNA even by RT-PCR by some 'super' siRNAs.[44,45] How much a gene is transcribed does not seem to be an important determinant of the extent of silencing as abundant mRNAs appear to be as well silenced as rare mRNAs. Situations where transcription is being activated *de novo* may be particularly fortuitous for therapeutic intervention. To use siRNAs or their small RNA precursors as drugs, they must be efficiently delivered into the cytoplasm of cells, the site of precursor RNA processing by the enzyme Dicer and uptake of the siRNA guide strand into RISC.[19] Because Dicer may direct endogenously processed siRNAs and microRNAs to the RISC, small double-stranded RNAs that are between 25 and 30 nucleotides in length and require Dicer processing appear to be more efficient at inducing RNAi than smaller siRNAs designed for direct incorporation into RISC.[46] Once within the RISC, the durability and efficiency of silencing depend on many factors, most of which are poorly understood, but may include sequence preferences for RISC binding along the siRNA, accessibility of the target site on the mRNA,[47,48] and thermodynamic considerations.[39] Although existing algorithms readily available on several websites (http://www.dharmacon.com; http://www.ambion.com/techlib/misc/siRNA_finder.html; http://molecula.com/new/siRNA_inquiry.html; http://www1.qiagen.com/siRNA) are fairly good at predicting sequences that will work, only experimental testing can identify the most efficient sequences. The most important factor that determines durability of silencing in cells appears to be the rate of cell division, which leads to dilution of the activated RISC as it is divided between daughter cells.[7,8] In rapidly dividing tumor cells, used for most of the early RNAi *in vitro* experiments, silencing peaks 2–3 days after transfecting siRNAs and begins to wane around day 5 and disappears by day 7.[49] However, in terminally differentiated non-dividing cells, such as macrophages, silencing lasts for as long as the cells can be cultured (several weeks).[44] There is also some suggestion that the persistence of the activated RISC for any siRNA may depend on the presence of the target mRNA.[44] For therapeutic purposes, the rate of division of the target cell will be an important determinant of the dosing interval.

Pharmacokinetic considerations

The half-life of unmodified siRNAs *in vivo* is short (seconds to minutes).[50] This is predominately due to their rapid elimination by kidney filtration because of their small size (~7 kDa). They also can be degraded by endogenous serum RNases with a serum half-life of ~5–60 min. The circulating siRNA half-life can be extended to days by complexing the siRNAs with other molecules or incorporating them into various types of particles (to bypass renal filtration)[50–52] and by chemically modifying the sugar backbone[51,53–58] and capping the ends of the siRNA[50,55,58–60] to make them resistant to RNase digestion. Experience with developing oligonucleotide and ribozyme therapies has been used to develop chemically modified siRNAs with improved resistance to endogenous nucleases.[50,51,54,55,58,61] The backbone ribose of some residues is generally modified at the 2′ position to 2′-deoxyribose or 2′-*O*-methyl or 2′-fluoro substitutions.[51,53–58] Although, the siRNA half-life in serum can be extended from minutes to days by chemical modifications, modifying siRNAs often comes at the cost of less-efficient silencing. The impact of the modification on silencing may depend on its position in the siRNA sequence, whether it occurs on the sense or antisense strand, and the particular residue that is altered. Similarly, chemical modifications can greatly increase the *in vivo* siRNA half-life. Because renal clearance is faster than the rate of siRNA degradation, the increase in the *in vivo* half-life is most pronounced when siRNAs are delivered in a way that bypasses glomerular filtration (e.g. by producing serum complexes when siRNAs conjugated to cholesterol[50] bind to serum albumin or by incorporating siRNAs into stable nucleic acid-lipid particles[62]). Much of the experimentation that has been done to design the optimal strategy for balancing the opposing considerations of half-life and potency is unpublished. Moreover, what modifications are optimal will likely depend on the clinical indication and the strategy used to deliver the siRNA, as incorporation into complexes and particles may variably protect the siRNA from exposure to endogenous nucleases. It is noteworthy that many of the *in vivo* studies that have shown disease protection used unmodified siRNAs that were not optimized for half-life.[63–67]

Local versus systemic delivery

Although siRNAs are readily taken up into worm and fly cells,[68,69] most mammalian cells do not efficiently internalize these small molecules. This is even true of cells, such as dendritic cells and macrophages, that are actively sampling their environment.[44,70] Therefore, the major obstacle for using small RNAs as drugs is to deliver them into the cytoplasm of cells. An exception may be mucosal tissues. In the lung and vagina, siRNA uptake is extremely efficient and occurs even in the absence of transfection reagents.[42] For clinical indications where siRNAs only need to be delivered to a localized region, such as the eye,[71,72] pulmonary[73] or vaginal mucosa,[74] or superficial tumors,[75–78] efficient siRNA delivery and silencing can be achieved by mixing siRNAs with cationic lipid transfection reagents used for *in vitro* transfection and directly injecting the siRNA-lipid complexes into the relevant tissue or instilling it into the body cavity. A similar approach is certain to apply to the skin. Mixing siRNAs with other molecules known to carry nucleic acids into cells, such as certain cationic peptides, might also be used effectively for local delivery.[79,80] However, some cell types, such as lymphocytes, dendritic cells and hematopoietic stem cells, are refractory to transfection using cationic lipids. Therefore, even when these targets might be localized (e.g. in a joint inflamed by an autoimmune process), alternate delivery strategies may be needed.

The first demonstrations of the therapeutic potential of siRNAs for silencing disease-related genes delivered siRNAs systemically by rapid high-pressure intravenous injection ('hydrodynamic delivery'). This method leads to transient right-sided heart failure, where elevated venous pressures somehow enable siRNAs to get into cells in highly vascularized organs like the liver, pancreas and lungs.[64] Nonetheless, this strategy is too risky for human use. It is however possible to deliver siRNAs into an organ, such as the kidney, by rapid retrograde injection via catheter into the draining vein.[81] It may be possible to use hydrodynamic injection into a peripheral vein to treat skeletal muscle by blocking venous outflow using a tourniquet.[82] Elevated venous pressures are generated only in the targeted tissue without inducing potentially fatal heart failure. However, a minimally invasive method for delivering siRNAs requires alternate approaches. A variety of strategies that involve complexing siRNAs to cationic polymers or peptides or incorporating siRNAs into nanoparticles or liposomes have been proposed.[62,72,76,77,79,80,83] Alternately, siRNAs can be covalently or noncovalently linked to antibody fragments or ligands to cell surface receptors to limit the delivery of the siRNAs to cells that bear the specific receptor. These strategies probably deliver siRNAs via receptor-mediated endocytosis, although the trafficking of siRNAs into and within cells has not been well studied. The directed delivery of siRNAs into specific cells will decrease the amount of siRNAs needed for the efficient silencing of gene expression in the target organ or tissue and will reduce potential toxicity by preventing targeting of unintended cells and tissues. The optimal delivery strategy may differ between different therapeutic indications and will depend on efficiency and duration of delivery and silencing, lack of systemic toxicity, and lack of immunoreactivity, which would interfere with repetitive treatments. The first examples of effective systemic delivery have only recently been described.[50,62,84]

Choice of genes and diseases to target

As all cells have the RNAi machinery and any gene is a potential target, any disease caused by or greatly exacerbated by the expression of a dominant gene can in principle be treated by RNAi. This means that the list of potential indications is long. Diseases that are intractable or poorly responsive to current therapy are high on everyone's list, especially cancer, neurodegenerative disease, viral infection, and macular degeneration, and these are the disease models that have been most studied so far.[8,85–88] Viral infections are particularly attractive as RNAi constitutes an important primitive

antiviral response in plants and lower organisms. However, for viral infection and cancer, the potential for genetic mutation to escape from RNAi needs to be taken into account. Targeting multiple genes at once is one approach. Another is to target essential genes or highly conserved sequences whose mutation would come at a high cost for viral fitness or tumor-cell survival. For viruses, targeting host genes required for viral replication in addition to viral genes is another approach to minimizing the chances of escape.

Although the focus of this review is on using RNAi as a small molecule drug, an increasingly important application of RNAi – to pinpoint important disease-related genes that would be useful targets for other small molecule drugs – deserves mention. By knocking down a gene *in vitro* or in transgenic mice, it is possible to determine how important the gene might be in the pathogenesis of a particular disease. 'Knockdown' mice, bearing a transgene encoding for a short hairpin RNA (shRNA) precursor of an siRNA that can be expressed constitutively or conditionally in certain tissues or at specific times, provide a relatively quick way to assess the role of that gene *in vivo*.[89,90] Moreover, libraries of shRNAs designed to silence a subset of mouse or human genes can be used either *in vitro* (or potentially *in vivo* in mice) to screen for potential drug targets, which if inhibited might ameliorate disease course.[91–93]

The remainder of this review will discuss *in vivo* studies that have locally or systemically introduced siRNAs to silence disease-related genes in small animals. As the obstacles for systemic delivery are greater, local and systemic studies are discussed separately.

Local siRNA therapy

Clinical situations where siRNAs would need to silence disease-related gene expression locally in easily accessible tissues provide some of the most readily testable opportunities for exploring RNAi-based drug therapy. Local siRNA administration has shown benefit in small animal models involving the lung, vagina, subcutaneous tissue, muscle, eye and central nervous system.

Lung

Successful silencing in the lung has been achieved by intranasal or intratracheal administration of siRNAs. Remarkably, pulmonary epithelial cells have even been transduced *in vivo* by siRNAs given without transfection reagents or other delivery molecules.[42,73] This suggests that the lung (and possibly other tissues) may have a means for siRNA uptake, not generally present in most mammalian cells. The transformed cell lines and primary hematopoietic cells commonly used for *in vitro* RNAi experiments require a transfection reagent or other mechanism for siRNA uptake. The first therapeutic benefit in the lung was demonstrated for influenza A infection. siRNAs directed against conserved influenza sequences when complexed with the polycation polyethyleneimine (PEI) could be delivered to the lung by intravenous low pressure injection and reduce viral titers by 1–2 logs when given either before or after infection.[83] A concurrent study that combined hydrodynamic injection of the same siRNAs and intranasal instillation of siRNA complexed to oligofectamine was able to protect mice from lethal challenge with several highly pathogenic stains of influenza A.[94] Neither study found any evidence of interferon induction induced by siRNA treatment *in vivo*. These experiments established the proof of principle for treating pulmonary infections with siRNAs, although the hydrodynamic injection method is unlikely to be suitable for clinical use and the PEI carrier may also have unacceptable toxicity. The therapeutic possibility of siRNA treatment in the lung was advanced significantly by the recent study of Bitko *et al*.,[42] who were able to prevent and treat respiratory syncytial virus and parainfluenza virus, two significant pathogens in neonates that cause croup, pneumonia and bronchiolitis, by siRNAs directed against essential viral genes given intranasally with or without the transfection reagent Trans-IT TKO. Even in the absence of a transfection reagent, mice were protected from infection. Both viruses could be targeted simultaneously; however, if higher doses of one siRNA were used, protection against the second virus was not as efficient, suggesting possible competition for the RNAi machinery. In addition to lowering viral titers, clinical signs of infection including weight loss and increased respiratory rate were dramatically improved, as were laboratory indicators of disease severity such as leukotriene levels in bronchoalveolar lavage fluid and lung pathology. In another clinical setting, following traumatic hemorrhagic shock, the intratracheal instillation of siRNAs targeting proinflammatory chemokines, involved in the pathogenesis of acute lung injury, reduced chemokine production and consequent neutrophil infiltration in the lung.[95] This group also showed that intratracheal instillation only reduced gene expression in the lung, but not in more distal tissues, such as the liver. These recent studies suggest that intranasal delivery of chemically unmodified siRNAs to the lung should be an effective strategy for clinical use in the near future.

Vagina

Another mucosal surface, the vagina, has also recently been the target for RNAi-mediated silencing.[74] siRNAs mixed with Oligofectamine were efficiently taken up by epithelial and lamina propria cells throughout the vagina and ectocervix, but not in distal organs. Endogenous GFP expression in a fluorescent mouse in which every cell expresses GFP was silenced deep into the tissue. Moreover, silencing lasted for at least 9 days. By silencing viral genes, mice were protected from lethal herpes simplex virus 2 (HSV-2) infection when challenged intravaginally. Protection was even possible when siRNAs were not administered until 3 h after viral exposure. No toxicity from the siRNA-Oligofectamine complexes was found by histological examination for cell death or inflammatory infiltrates or by assaying for induction of interferon or interferon response genes by quantitative RT-PCR.

Subcutaneous tumor

Multiple studies have been able to inhibit the growth of subcutaneous tumors by injection of siRNAs targeting oncogenes or angiogenic genes when complexed with a variety of agents. In one study, *VEGF* siRNA mixed with proteolytically cleaved collagen (atelocollagen) was able to suppress the growth of the PC-3 prostate cancer cell

line in nude mice.[76] In another study, siRNAs directed against fibroblast growth factor were able to inhibit germ-cell tumor growth when mixed with either atelocollagen or constituted into liposomes.[77] Similarly intratumoral injection of liposomes containing *bcl-2* siRNA inhibited the outgrowth of subcutaneous PC-3 tumors.[96] Intraperitoneal administration of siRNAs directed against the growth factor receptor Her2/ErbB2 complexed with PEI efficiently targeted and inhibited a subcutaneous Her2+ ovarian tumor in nude mice.[97] More recently, intratumoral injection of a cocktail of siRNAs directed against *c-myc*, *MDM-2* and *VEGF* when mixed with an antibody fragment-protamine fusion protein was able to specifically target and inhibit subcutaneous B16 melanoma cells by directing the siRNAs only into the tumor cells via antibody binding to a cell surface receptor on the tumor.[84] These studies suggest the possible application of locally injected siRNAs as adjuvant therapy before or after surgical resection of tumors that have not yet metastasized. siRNAs might also be used to shrink a non-operable tumor to make it amenable to surgical removal. Topical application of siRNAs might also be highly effective for both benign and pre-maligant skin conditions, as well as malignant pigmented and non-pigmented skin cancers.

Muscle

Although multiple studies have used shRNA-encoding viral vectors to transduce muscle cells *in vivo*, only a few studies have delivered synthetic siRNAs into skeletal muscles either using electrical stimulation[98,99] or localized hydrodynamic injection;[82] however, none of these studies have used siRNAs to investigate siRNA therapy in a disease model. Electrical stimulation was used to deliver previously injected siRNAs into muscle cells to silence reporter genes, such as luciferase and eGFP or an endogenous gene (GAPD). This delivery method resembles electroporation, commonly used for *in vitro* transfection. It is not clear how far along a muscle, genes could be silenced; in one study images show silencing for at least 1 cm.[99] Silencing persisted without diminution for 11 days with some silencing still evident 23 days after treatment. Hydrodynamic injection of siRNAs into a peripheral vein of a mouse, rat or monkey limb that had been transiently isolated by reducing blood flow to and from the limb via a tourniquet led to the efficient silencing of a coinjected luciferase reporter plasmid.[82]

Eye

The eye was an early target for animal studies of siRNA-based therapy and is the target site of the first pilot clinical studies of siRNA-based therapy. Subretinal injection of siRNAs directed against VEGF complexed with a transfection lipid blocks the signals that induce neovascularization in the retina in response to laser photocoagulation damage, a model for age-related macular degeneration and other diseases, such as diabetic retinopathy, where blindness arises from hemorrhage of leaky new blood vessels.[72] Neovascularization in siRNA-treated eyes was only 25% of that in control eyes subjected to laser damage. Similarly, ocular neovascularization induced by proinflammatory CpG oligonucleotides or herpes simplex virus (HSV) infection was inhibited by injecting a cocktail of siRNAs targeting VEGF-A and two VEGF receptors without any delivery agent.[100] These same siRNAs could be delivered systemically in nanoparticles constructed with a polymer designed with PEI at one end, polyethylene glycol in the middle and an RGD peptide at the other end to direct binding to integrins on activated endothelial cells. In a disease model of post-surgical inflammation and fibrosis, subconjunctival injection of siRNAs targeting a TGF-β receptor complexed with lipids reduced inflammatory infiltrates and fibrosis. Such an approach might be useful to reduce postoperative scarring, although in practice, this therapy would have to balance the need for wound healing with the dangers of scarring. In yet another indication, siRNAs targeting c-jun and Apaf-1 were used to prevent degeneration of retinal ganglion cells following optic nerve transection.[101] Naked siRNAs directly injected into the optic stump after axotomy were taken up by some of the damaged neurons, allowing recovery from mechanical trauma. Whether such an approach could be developed to prevent permanent neuronal loss in other traumatic settings (such as spinal cord injury) remains to be seen.

Nervous system

Viral vectors encoding for shRNAs targeting mutated genes implicated in otherwise untreatable neurodegenerative diseases (SCA1 for spinocerebellar ataxia, SOD1 for amyotrophic lateral sclerosis) have shown therapeutic benefit in mice.[102–104] A few studies have also been able to demonstrate effective delivery and therapeutic effect of siRNAs injected into the central nervous system. Continuous intrathecal infusion by osmotic minipump in rats of stabilized siRNAs (0.4 mg/day) targeting a cation channel involved in sensing pain reduced the expression of the pain channel by 40% in dorsal root ganglia neurons and raised the threshold for pain responses in a model for chronic neuropathic pain induced by partial ligation of the sciatic nerve.[56] There was no overt inflammation from the treatment and siRNAs were more potent than antisense oligonucleotides targeting the same gene at alleviating pain in this model. Interestingly, the transduction of neuronal cells *in vivo* did not require a transfection reagent. Similarly, in mice stereotactic insertion of a cannula into the third ventricle for continuous infusion of stabilized siRNAs by minipump over 1–2 weeks was able to silence expression of a reporter EGFP gene as well as of endogenous dopamine and serotonin transporter genes at distal sites in the brain, again without any transfection reagent. Silencing was widespread, but variable in different regions of the brain and increased with the duration of the infusion. Changes in motor and behavioral activity were observed that were similar to those obtained by infusing specific inhibitors of the respective transporters. In another approach, localized transduction of neurons was achieved by electroporation in rats.[105] After siRNAs were stereotactically injected into the brain, the application of a mild electric current that did not on its own cause cellular apoptosis was able to reduce endogenous gene expression only in the area of the brain between the two electrodes. In addition, lipid complexes and immunoliposomes that have been used to deliver plasmid DNA encoding shRNAs might also be adapted to deliver siRNAs into brain cells.[106,107]

Systemic siRNA therapy

Although localized delivery of siRNAs to specific organs or tissues has been successful in an increasing number of experimental small animal models, this approach is limited to sites that are readily accessible. Delivery to deep-seated tissues and organs or to disseminated targets (i.e. lymphocytes or metastatic tumor cells) requires other strategies. One possible exception is treatment of parasitic infection. Parasites like other primitive organisms readily take up siRNAs in the absence of any transfection reagent.[108–110] Parasitic infections might therefore be readily treated by conventional intravenous administration of siRNAs silencing genes essential for the parasite to survive or replicate. Because siRNA uptake into mammalian cells is likely to be extremely inefficient and parasite genes are so distinct, toxicity is also likely to be minimal.

Hydrodynamic delivery

The first attempts at systemic delivery of siRNAs followed hydrodynamic transfection protocols previously used to deliver plasmid DNA into cells.[111,112] These involve the rapid intravenous injection ($\sim$5 s) of DNA or RNA in a large volume (typically 20–40% of the mouse circulating blood volume). This leads to uptake into liver, kidney, spleen, heart and lung cells. Hydrodynamic injection functions by creating a transient inversion of blood flow resulting from elevated venous pressures.[113] This purportedly promotes massive endocytosis. Most studies that used hydrodynamic injection focused on the liver because it is very efficiently targeted. Gene expression throughout the organ can be reduced by $\sim$80%. In an early study, co-injection of a luciferase expression plasmid with an siRNA-targeting luciferase led to efficient suppression of luciferase expression in the liver and other central organs.[64,65,114] No long-term negative effects have been demonstrated after hydrodynamic injection, even after multiple injections, provided the animal survives the acute heart failure.[113] However, hydrodynamic injection requires a high level of technical skill and causes transient liver damage, characterized by cell swelling, some necrosis and modestly elevated serum liver transaminases. Moreover, it is difficult, if not impossible, to scale up for larger animals and is unlikely to be safe enough for human use. Therefore, the hydrodynamic injection studies of siRNA application to disease models should be interpreted as establishing proof of principle for human siRNA drug therapy, rather than as a potential mode of administration.

The susceptibility of the liver to a variety of agents that can induce acute or chronic liver injury including viral infection, autoimmune hepatitis, toxins, and liver transplantation, make it a good candidate for testing the therapeutic potential of siRNAs.[115–117] Many of these insults cause hepatic injury via engagement of the proapoptotic cell surface receptor Fas expressed on hepatocytes and upregulated in response to hepatic inflammation.[115,117] Fas-deficient (lpr) mice are protected from insults that would induce fulminant hepatitis in normal mice.[118,119] Hydrodynamic injection of labeled siRNAs showed that nearly 90% of hepatocytes took up the siRNAs, compared to $\sim$40% of cells that could take up DNA vectors, making this approach extremely effective for siRNA delivery.[8,63] Injection of siRNAs targeting Fas led to degradation of Fas mRNA without affecting the expression of Fas-related genes, with the levels of Fas mRNA and protein being stably reduced by $>$80% for 10 days post transfection.[63] Importantly, this reduction of Fas had physiological consequences, preventing liver cell necrosis and inflammatory infiltration in concanavalin A-treated mice, a model for autoimmune hepatitis (Figure 1). In addition, Fas siRNA greatly increased survival in a fulminant hepatitis model induced by intraperitoneal injection of an agonistic Fas antibody. While all the control siRNA-treated mice died within three days, greater than 80% of the Fas siRNA-treated mice survived. Because Fas-mediated apoptosis plays a critical role in a wide variety of liver diseases, silencing of Fas by siRNA treatment may be of therapeutic value for preventing and treating acute and chronic liver injury induced by a range of insults. In fact, silencing caspase 8 (activated downstream after fas receptor engagement) has shown protection from both autoimmune and adenoviral hepatitis, and hydrodynamic injection of either Fas or caspase 8 siRNAs protected mice from sepsis in a bowel puncture model.[67,120]

Infection with hepatitis C virus (HCV) and hepatitis B virus (HBV) represents a major global health problem. These viruses infect $\sim$270 and $\sim$350 million people worldwide, respectively.[121–123] Although they are completely unrelated viruses, they both cause chronic hepatitis in a subset of infected individuals, which is associated with progression to cirrhosis and increased

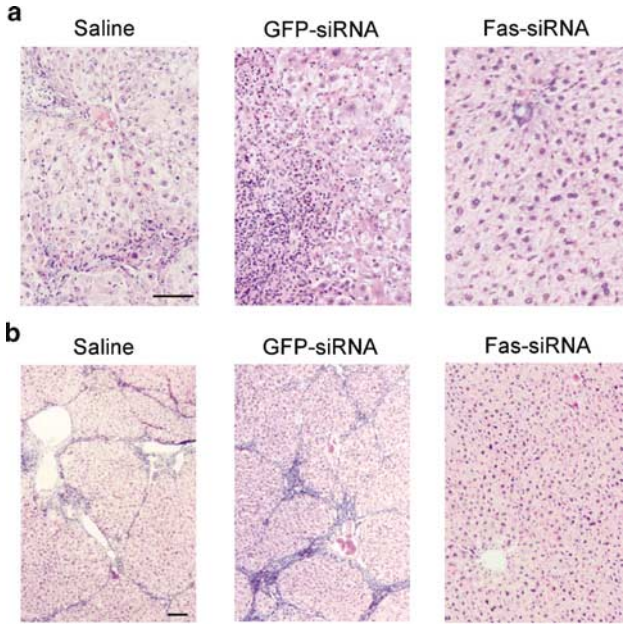

Figure 1 Fas gene silencing protects mice from fulminant hepatitis. (**a**) Concanavalin A was injected into mice as a model of acute autoimmune hepatitis. Mice were pretreated by hydrodynamic injection with saline or siRNAs directed against GFP or Fas. Representative liver histology is shown. This was the first demonstration of disease protection using siRNAs in an animal model. (Figure reprinted from Song *et al*.[63] with permission.) (**b**) In a chronic hepatitis model mice were injected every week with concanavalin A for 6 weeks and siRNA administration was deferred until after the second and fourth injections. Mice that received Fas siRNAs had reduced hepatic fibrosis at 7 weeks.

risk of hepatocellular carcinoma, with severely affected individuals requiring liver transplants. The similarity of symptoms is largely due to the liver damage associated with the immune response to these viruses, as neither virus is cytopathic. These viruses do not infect mouse or rat hepatocytes; as a consequence, no good small-animal model for these infections exists. Several groups have used hydrodynamic injection both to transduce mouse hepatocytes with viral replicons (cDNA versions of the viral genome) that then replicate the virus (but do not cause disease in mice)[124,125] and to deliver siRNAs designed to silence HCV and HBV gene expression and viral replication.[64–66] In the first demonstration of gene silencing in the liver, hydrodynamic injection of a DNA vector expressing an HCV NS5B protein fused to luciferase with an siRNA against NS5B led to a substantial inhibition of luciferase activity compared to a control siRNA.[64] Unmodified siRNAs targeting HBV were then shown to reduce viral replication in the mouse HBV replicon model.[66,126] More recently, hydrodynamic injection of certain chemically modified siRNAs at high concentrations ($\sim$50 mg/kg) was shown to have a more profound effect at inhibiting HBV replication than injection of unmodified siRNAs.[58] It was even possible to reduce HBV replication somewhat ($\sim$0.9 log) by repeated low pressure intravenous injections of 10–30 mg/kg modified siRNA directed against HBV, but six injections over 2 days were needed to achieve that modest effect. This approach was improved by incorporating the chemically modified, HBV-specific siRNA into a novel liposome complex forming a stable nucleic acid-lipid particle (SNALP).[62] The passive, intravenous administration of these particles efficiently reduced the level of serum HBV DNA in mice that had previously been injected with an HBV replicon. Incorporating siRNAs into these particles resulted in more sustained silencing and required lower and less-frequent doses of siRNAs than the hydrodynamic injection of chemically stabilized siRNAs.[62]

It is likely that other serious acute or chronic viral diseases involving internal organs can be controlled by administering siRNAs. Recently, hydrodynamic injection of siRNAs targeting Coxsackie virus B3, which causes acute and chronic viral myocarditis, was able to reduce viral titers in the heart and lung by at least 6 logs, and modestly prolonged survival in a highly susceptible mouse strain.[127]

Although systemic hydrodynamic injection is not practical for human use, it can potentially be used to inject the vein draining an organ, such as the kidney, to create localized elevated venous pressures that effectively deliver siRNAs. This was used to deliver Fas siRNAs into the kidneys to protect mice from acute tubular necrosis and death from renal ischemia-reperfusion injury.[81] Local instillation of siRNAs into the renal vein in a small clinically acceptable volume was as effective in this disease model as systemic hydrodynamic injection. However, approaches such as this require catheterization and therefore would be more costly and less practical for most indications than finding a method for peripheral intravenous administration.

Receptor-mediated systemic delivery

To use siRNAs as a systemic drug, more efficient delivery strategies are necessary to improve *in vivo* half-life, intracellular uptake and ideally target-specific cell-types. Several recent studies have begun to develop novel methods to enhance the potential drug-like properties of siRNAs and suggest that the siRNA delivery obstacle can be overcome. To this end, Soutschek *et al*.[50] conjugated cholesterol to the 3′-end of the target (antisense) strand of a chemically modified siRNA. Conjugation of siRNAs with cholesterol (Chol-siRNA) enabled the siRNAs to be taken up into cells via the ubiquitous LDL receptor. Intravenous injection of radiolabeled Chol-siRNA into rats showed an elimination half-life of 95 min compared to a 6-min elimination half-life for an unconjugated siRNA. This increased retention rate may be due to the propensity of cholesterol to bind serum albumin and be retained in the circulatory system. Injection of Chol-siRNAs targeting apoliprotein B (ApoB), an essential protein for the formation of low-density lipoproteins (LDL), led to a significant reduction in *apo*B mRNA in the liver and jejunum, the primary sites of ApoB expression. This had a substantial effect on overall lipid metabolism: it decreased plasma ApoB by $\sim$68% and decreased plasma LDL and cholesterol by $\sim$40%. Although the conjugation of the siRNA with cholesterol promoted siRNA retention, the Chol-siRNAs did not discriminate into which tissues the siRNA would be delivered with labeled siRNAs being present in liver, heart, kidney, adipose and lung tissue, because the LDL receptor is ubiquitously expressed. However, the unconjugated control modified siRNAs were not detected in any of these organs. To obtain this impressive beneficial effect on serum cholesterol required a lot of siRNA (injections of 50 mg/kg on three consecutive days) and the effects were measured just 1 day later. There is no indication about how long the effect lasted. Nonetheless, this study led the way towards establishing a practical method for systemic intravenous injection of siRNA-based drugs.

Recently, a novel method for *in vivo* delivery of siRNAs to specific cell types was developed that takes advantage of the nucleic-acid binding properties of protamine, which nucleates DNA in sperm, and the specificity of fragment antibodies (Fab).[84] A protamine-antibody fusion protein (F105-P) was developed containing the protamine coding sequence linked to the C-terminus of the heavy-chain Fab fragment against the HIV-1 envelope protein. When mixed with siRNAs, F105-P delivered siRNAs and induced silencing only in cells expressing the HIV envelope. Moreover, the silencing was highly efficient and as effective as transfection *in vitro*. Using this system, siRNAs targeting the HIV-1 capsid protein inhibited HIV replication in hard-to-transfect HIV-infected primary T cells. *In vivo*, intratumoral or intravenous injection of mice with F105-P-complexed siRNAs specifically delivered fluorescently labeled siRNAs into HIV envelope-expressing B16 melanoma cells, but not into normal tissue or envelope-negative B16 cells (Figure 2). The antibody-mediated delivery of a cocktail of siRNAs (totaling $\sim$3 mg/kg) targeting several genes involved in tumorigenesis by intratumoral or intravenous injection inhibited envelope-expressing tumor growth but not the growth of melanoma cells not expressing HIV envelope. In this study, the siRNAs were not chemically modified and were not optimized for improved pharmacokinetics. Cell-specific delivery and silencing were also obtained

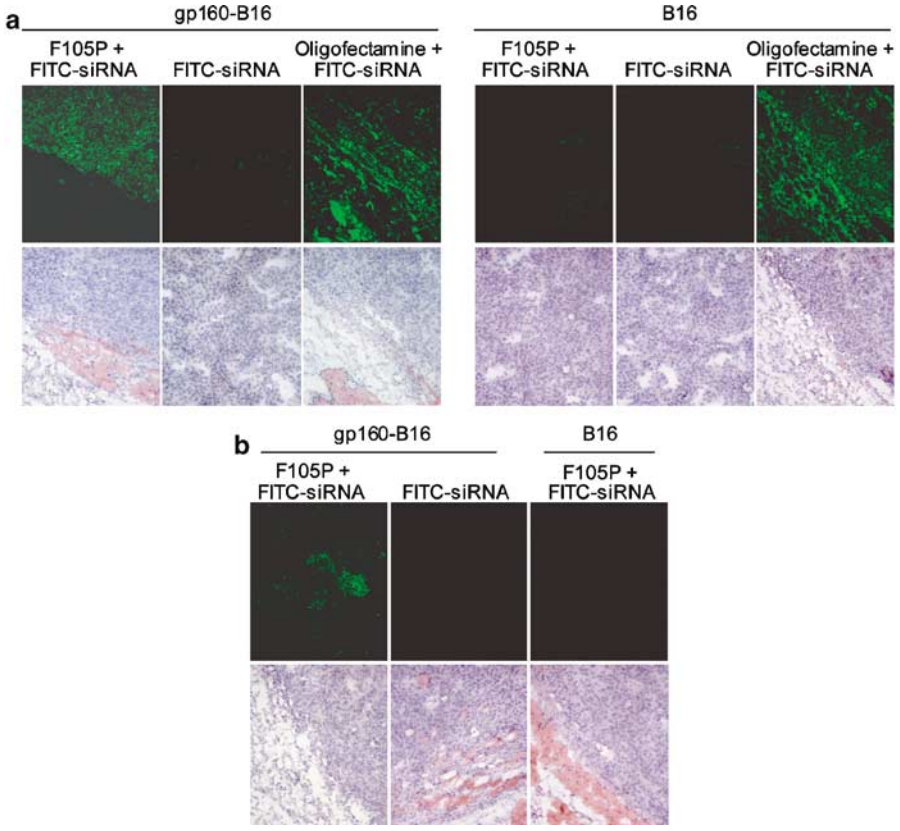

Figure 2 Intratumoral or intravenous injection of siRNAs complexed with an antibody fragment-protamine fusion protein delivers siRNAs only into mouse melanoma tumors expressing the cell surface receptor recognized by the antibody. (**a**) Fluorescent siRNAs, either naked or complexed with an anti-env-protamine fusion protein (F105-P) or oligofectamine, were injected into subcutaneous B16 melanomas expressing HIV env (left) or not (right). (**b**) Alternatively, F105-P loaded with fluorescent siRNA was injected intravenously. The tumors were harvested 12 h later for fluorescence microscopy (upper row) and hematoxylin and eosin staining (lower row). *In vivo* F105-P specifically delivers FITC-siRNA only into gp160-B16 tumors, but not into surrounding normal tissue or B16 tumors lacking *env*, while oligofectamine delivers FITC-siRNA into both tumor and neighboring tissues. Naked siRNAs do not efficiently get into any cells. Intratumoral injection is more efficient than intravenous injection. (Figure reprinted from Song *et al*.[84] with permission.)

with a single-chain antibody fusion protein that targeted ErbB2+breast cancer cells, demonstrating that this approach can be generalized to target other cell surface receptors. Because the siRNAs were not covalently linked to the antibody-protamine fusion protein, different siRNAs could be delivered with the same reagent. These results demonstrated that siRNA can be delivered systemically and target only cells expressing a specific cell-surface protein. Cell-specific targeting may reduce both the amounts of siRNA needed for therapeutic benefit as well as potential drug toxicity.

These examples are probably only the first of many strategies that are likely to be developed for systemic RNAi using siRNA-based drugs. Other methods used to deliver DNA plasmids for gene therapy, such as liposomes and immunoliposomes, which have been adapted to deliver plasmids encoding for shRNA precursors of siRNAs, could also deliver siRNAs or small siRNA precursors. In one study of mouse liver metastatic lung cancer, repeated injections of liposomes containing siRNAs directed against bcl-2 (10 mg/kg given 10 times) were able to inhibit tumor growth.[96] Similarly, nanoparticles containing cell targeting molecules on their outside and siRNAs within are also being developed.[52,100,128]

Conclusion

Just 3 years after RNAi was shown to work in mammalian cells, the first Phase I clinical studies targeting the VEGF angiogenic pathway in age-related macular degeneration have already begun. The interim analysis of one such study conducted by Sirna Therapeutics recently showed no evidence of clinical toxicity or disease progression in a small cohort. In the next few years it should become clear whether the promise of siRNA drug therapy to silence disease-causing genes with specificity and without undue toxicity will be realized. It should be an exciting time for researchers seeking to harness this powerful endogenous pathway to treat human disease.

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