Knocking down disease: a progress report on siRNA therapeutics

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Abstract | Small interfering RNAs (siRNAs), which downregulate gene expression guided by sequence complementarity, can be used therapeutically to block the synthesis of disease-causing proteins. The main obstacle to siRNA drugs — their delivery into the target cell cytosol — has been overcome to allow suppression of liver gene expression. Here, we review the results of recent clinical trials of siRNA therapeutics, which show efficient and durable gene knockdown in the liver, with signs of promising clinical outcomes and little toxicity. We also discuss the barriers to more widespread applications that target tissues besides the liver and the most promising avenues to overcome them.

RNA interference
(RNAi). An endogenous gene silencing mechanism, present in virtually all eukaryotic cells, by which short double-stranded RNA molecules induce translational inhibition and/or degradation of mRNAs containing partially complementary sequences.

Gene knockdown
An experimental technique used to reduce gene expression using sequence-specific oligonucleotides, typically by RNA interference (RNAi) or antisense mechanisms.

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Since the discovery in 1978 that a 13-mer DNA oligonucleotide could inhibit Rous sarcoma virus translation and proliferation in a sequence-specific manner[1,2], substantial efforts have been devoted to harnessing antisense oligonucleotides (ASOs) for therapy[3]. ASOs that bind by complementary base pairing to intracellular mRNAs were initially designed to inhibit translation or to modify splicing. The challenges in turning ASOs into drugs were their degradation within body fluids full of nucleases, the potential to trigger innate immune nucleic acid sensors and, the most difficult, their delivery across the cell membrane into the cytosol (and also into the nucleus for some applications). Alterations in the chemistry of the basic nucleotide building blocks led to the development of nucleic acid analogues that are more stable, bind to their target with higher specificity and have improved cell penetration (reviewed in REFS 3–5), culminating in the first ASO-approved drug (fomivirsen) to treat cytomegalovirus retinitis in 1998 (at that time, a declining complication of HIV infection). More recently, mipomersen targeting *APOB*, a gene encoding the apolipoprotein B-100 in low-density lipoprotein (LDL)-cholesterol particles, was approved for the treatment of familial hypercholesterolaemia[6].

The discovery of RNA interference (RNAi)[7] approximately 20 years ago opened up a new mechanism for ASO therapeutics: gene silencing (FIG. 1). Transfection of short double-stranded RNAs — namely, small interfering RNAs (siRNAs), which are 21–23 nucleotides in length and contain an mRNA sequence (sense strand) and its complement (antisense active strand) —harnesses this ubiquitous pathway to degrade target gene mRNA and suppress its expression with high specificity[8].

The potential of siRNA therapeutics was first demonstrated just 12 years ago when injection of *Fas* siRNAs protected mice from autoimmune hepatitis[9]. Drug development since then has been rapid. The obstacles to turning siRNAs into drugs are similar to those faced with ASO drugs (see BOX 1 for a summary of the major strategies currently being evaluated to exploit the therapeutic potential of oligonucleotides). Although intracellular delivery of double-stranded RNAs is more challenging than that of mostly single-stranded ASOs, some of the ASO strategies could be adapted to siRNA therapeutics, thus accelerating siRNA preclinical drug development and clinical evaluation. Simple chemical modifications of the 2′-position of the ribose and substitution of phosphorothioate linkages protect siRNAs from nuclease digestion and thus prolong half-life in serum[10,11] and other body fluids. 2′-modifications can also prevent recognition by innate immune receptors[12,13] and limit off-target effects owing to suppression of partially complementary sequences[14]. Clinical Phase I and II studies of siRNA therapeutics in the past 2 years have demonstrated potent (as high as 98%) and durable (lasting for weeks) gene knockdown in the liver, with some signs of clinical improvement and without unacceptable toxicity. Two Phase III studies are in progress to treat familial neurodegenerative and cardiac syndromes caused by mutations in transthyretin (*TTR*).

In this Review, we discuss the advances in the development of siRNA-based therapy, recent clinical studies that apply this technology to target gene knockdown in the liver and promising approaches for developing siRNA drugs that knock down gene expression in target tissues beyond the liver.

This Review will not cover other oligonucleotide therapeutics, such as antisense and exon-skipping oligonucleotides, which are reviewed elsewhere[3,4].

Delivery and uptake of siRNA therapeutics

Efficient cellular delivery and uptake of large, negatively charged oligonucleotides are the main obstacles to widespread application of RNA therapeutics (BOX 2). The roadblocks involve getting across the cell membrane into endosomes and then across the endosomal membrane into the cytosol. Once inside a cell, one strand of an siRNA binds to the endogenous cytoplasmic RNA-induced silencing complex (RISC), which captures an mRNA bearing a complementary sequence, and then the RISC Argonaute RNase cuts the target mRNA to initiate

Figure 1 | Mechanism of gene knockdown by siRNAs.
Exogenously administered small interfering RNAs (siRNAs; double-stranded ~22-nucleotide-long RNA molecules with 2-nucleotide overhangs at the 3′ ends) exploit the endogenous RNA interference (RNAi) machinery. In the endogenous pathway, Dicer generates microRNAs (miRNAs) from RNA hairpins encoded by miRNA genes. The Dicer machinery also processes exogenous double-stranded RNA (dsRNA) sequences containing loops called short hairpin RNAs (shRNAs). Synthetic siRNAs bypass Dicer processing and can directly associate with the RNA-induced silencing complex (RISC) to mediate recognition of target mRNAs through base-pair complementarity. Argonaute 2 in the RISC complex then enzymatically cleaves the target mRNA, leading to target gene knockdown.

its degradation (FIG. 1). The active (antisense) strand of the siRNA is stable within the RISC for weeks, but it is diluted with every cell division[15]. Thus, the same siRNA molecule can target multiple transcripts and knock down gene expression in slowly dividing or non-dividing cells for weeks. Because of the highly catalytic nature of RNAi, only a few hundred cytosolic siRNAs per cell are needed for efficient and sustained gene knockdown[16,17]. This low number makes overcoming the delivery obstacle less formidable, and the number is probably lower than what is required using other antisense mechanisms.

Intracellular delivery of siRNAs is foiled by their large size (~12 kDa) and negative charge. Although they are too large to cross cell membranes, siRNAs are small enough to be filtered by the kidney. As a consequence, unless they are conjugated to other molecules or incorporated into complexes, intravenously injected siRNAs are rapidly excreted. Many different siRNA delivery approaches have been proposed (reviewed in REF. 18), and the clinically most advanced strategies (all liver-targeted) are described below and summarized in BOX 3.

Non-targeted delivery. Early strategies for solving the dual problems of intracellular delivery and rapid excretion involved incorporating siRNAs into lipid nanoparticles (LNPs) — smaller, more homogeneous analogues of lipoplexes used for laboratory transfection[19–22]. However, these complexes (and other nanoparticle strategies for siRNA delivery) accumulate in the liver and other filtering organs, which limits their effectiveness in penetrating other tissues. Neutral LNPs directly interact with apolipoprotein E3 in the circulation, which targets these particles for hepatocyte uptake[23], whereas cationic LNPs also accumulate in the liver, independently of apolipoprotein E3 (REF. 22). As a consequence, most of the clinical focus of siRNA drug development has been on hepatic gene targets. Because the liver synthesizes many blood proteins and is a key metabolic hub, gene knockdown in the liver offers a broad array of therapeutic targets. So far, however, efforts have concentrated on treating rare genetic diseases. Accelerated drug approval for orphan diseases makes this an attractive drug development strategy. Readily accessible tissues, such as the skin, eye or mucosa, in which topical drug delivery is possible, are also attractive sites for siRNA drug development. LNPs and nanoparticles can also penetrate leaky or more permeable vasculature[24,25]. It remains uncertain whether the enhanced permeability and retention of tumour vasculature can lead to effective gene knockdown in solid cancers using nanoparticles, especially as poor tumour vascularization (and its consequence, hypoxia) are common.

Targeted delivery. siRNAs can also be targeted for uptake in selected tissues or cell types by taking advantage of high-affinity antibody or antibody fragments[26–28], aptamers (nucleic acids selected for high-affinity binding)[29–31], or receptor ligands[32–36] to bind to cell surface receptors and to mediate cell-specific uptake. Targeted uptake has the advantages of being effective at a lower dose and reduced toxicity from knockdown in unintended tissues.
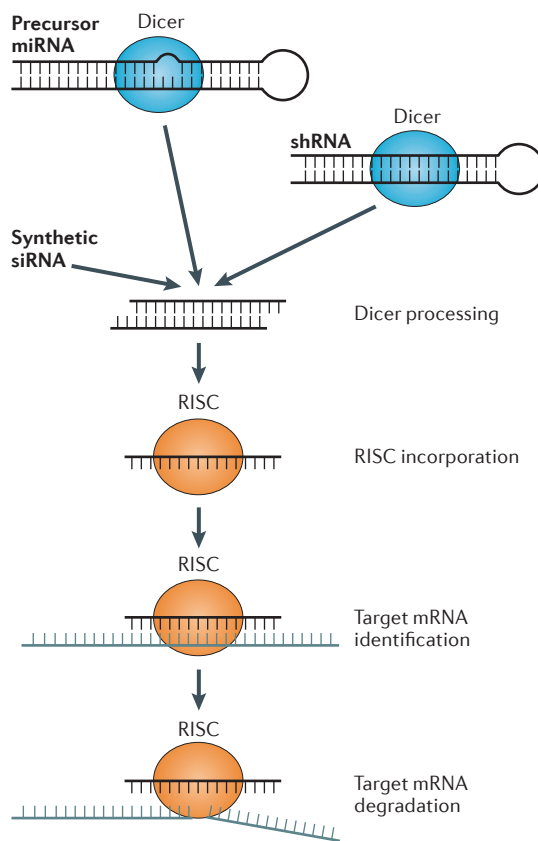
> **Box 1 | Classes of antisense oligonucleotide therapeutics and their mechanisms of action**
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> **RNase H-dependent ASOs**
> RNase H-dependent antisense oligonucleotides (ASOs) are single-stranded, chemically modified oligonucleotides that bind to complementary sequences in target mRNAs and reduce gene expression both by RNase H-mediated cleavage of the target RNA and by inhibition of translation by steric blockade of ribosomes. The most clinically advanced ASOs are 'gapmer' ASOs that incorporate a 5-nucleotide-long or longer central DNA stretch between chemically modified RNA flanks[4]. RNase H cleaves within the central DNA stretch. Once cleaved, the target mRNA is rapidly degraded.
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> **Exon-skipping ASOs**
> Exon-skipping ASOs are single-stranded, chemically modified ASOs that target intron–exon junctions (splice sites) or splicing-regulatory elements. Binding to the target site inhibits splicing at this site and forces the choice of an alternative splice site. Changing splice site leads to the translation of an alternative protein isoform that can restore stability or function to a mutated gene product[88]. Both 2′-O-methyl phosphorothioate and morpholino oligonucleotides have been evaluated clinically.
>
> **siRNAs**
> Small interfering RNAs (siRNAs) are 21–23-nucleotide-long, generally chemically modified, double-stranded RNAs with an antisense active strand that is exactly complementary to a sequence anywhere in the target mRNA. siRNAs are taken up by the cytosolic RNA-induced silencing complex (RISC), which ejects one strand, leaving the antisense strand to bind to the target mRNA and mediate its sequence-specific cleavage by the RISC RNase Argonaute 2 (REF. 8). Once cleaved, the target mRNA is rapidly degraded.
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> **Anti-miRs and miRNA mimics**
> Oligonucleotides can be used to antagonize (in which case they are known as anti-miRs) or mimic the function of endogenous microRNAs (miRNAs). Native miRNAs are taken up by the RISC, which suppresses gene expression of RNAs containing partially complementary sequences by blocking their translation or accelerating their degradation. They suppress the expression of hundreds of transcripts, but less efficiently than siRNAs. Single-stranded, chemically modified (primarily with phosphorothioates or with morpholino or locked nucleic acid backbones) ASOs can bind to miRNAs to block their activity. Because miR-122 stabilizes the hepatitis C virus (HCV) genomic RNA, anti-miRs targeting miR-122 in the liver have been tested in early-phase trials as a therapy for HCV, with promising results[89,90]. miRNAs are globally depleted in many cancers with poor prognosis[91]. Therefore, their replacement could be used as a form of cancer therapy. Ionizable liposomes encapsulating a mimic of a tumour suppressor miRNA (miR-34a)[92] are being evaluated in patients with primary and metastatic liver cancer.

These targeting moieties can be directly conjugated to siRNAs, bound non-covalently, or incorporated into LNPs or other nanoparticles. In particular, subcutaneous administration of siRNAs conjugated to trivalent *N*-acetylgalactosamine (GalNAc)[33,37–39], which mediates hepatocyte uptake through the hepatocyte-restricted asialoglycoprotein receptor (ASGPR) causes whole-liver, durable gene knockdown without limiting toxicity[40]. These GalNAc–siRNA conjugates are currently being evaluated in a Phase III study for the treatment of a rare genetic neurodegenerative disease. It is likely that they will eventually replace most therapeutic LNP efforts for liver targets, as conjugated siRNAs seem to be better tolerated than LNPs, are simpler and cheaper to manufacture, and can be administered subcutaneously. The GalNAc–ASGPR ligand–receptor pair appears to be more effective than other siRNA conjugates that have been tried thus far, which are generally taken up into cells but do not result in efficient knockdown, possibly because of inefficient endocytosis or endocytic release.

Endocytosis and endosomal escape. Most methods of siRNA delivery involve endocytosis. To function, siRNAs need to escape to the cytosol, where the RISC works. Thus, release from the endosome is an important barrier. Understanding the mechanism (or mechanisms) that promotes and limits endosomal release, which is not currently well understood, should help to optimize this limiting step. We recently developed a high-resolution imaging technique that enabled us
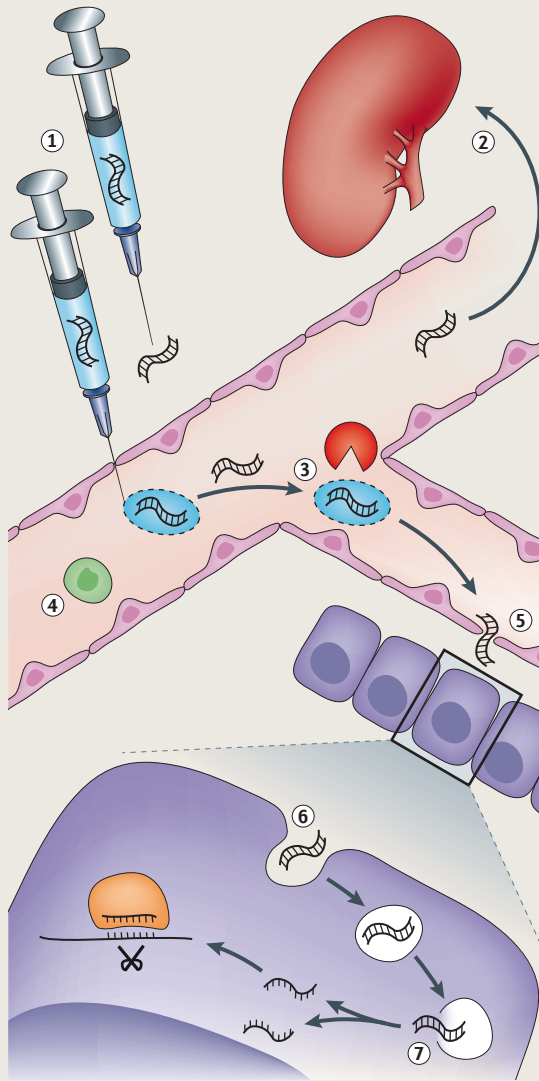
to identify releasing endosomes and to time and quantify siRNA endosomal release[17]. Only a small percentage of the siRNAs in LNPs that are taken up by the cell are released into the cytoplasm. siRNAs in LNPs are released only from maturing endosomes during a narrow time window: ~5–15 minutes after endocytosis. The releasing endosome is rapidly recognized by galectins, which are cytosolic glycoprotein sensors[41] that target the damaged endosome for autophagy. However, inhibiting autophagy does not enhance cytosolic delivery, suggesting that another mechanism limits release. The prevailing theory of endosomal release, called the 'proton sponge theory' (REF. 42), is that a build-up of positive charge in the endosome is needed to disrupt the endosomal membrane for release. Indeed, endosomal release and gene knockdown are enhanced by co- or pre-transfection of cationic polymers or their incorporation into siRNA nanoparticles that become more positively charged as the endosome acidifies[34,43]. However, it is uncertain how well tolerated these compounds are *in vivo*.

Target selection
Target selection for therapeutic gene knockdown is guided by considerations that overlap with conventional drug target selection; however, because all cells have the RNAi machinery and any gene can be knocked down, in principle any disease-causing gene can be suppressed in any cell type. This greatly expands the world of potential drug targets beyond the enzymes and receptors that

Box 2 | Barriers to siRNA therapeutics and strategies to overcome them

Small interfering RNA (siRNA) therapeutics face multiple barriers along the pathway from administration to delivery to the intracellular target site. The major barriers for both nanoparticle-formulated and targeting ligand-conjugated siRNAs are indicated with a number (see the figure). The table below provides a brief description of each barrier and suggests possible strategies to overcome them.

Approach	Description
1. Enter circulation or target tissue	
Parenteral administration	IV administration used for LNPs and other nanoparticles
Subcutaneous injection	GalNAc conjugates are administered subcutaneously and presumably reach the target tissue via lymphatics
Topical application	Used for targeting the skin, eye and mucosa
2. Avoid excretion	
PEGylation	Increases the molecular weight of siRNA or delivery vehicle to avoid renal excretion
Cholesterol conjugation	Cholesterol-conjugated siRNAs bind to circulating lipoprotein particles
Nanoparticle formulation	Nanoparticles are above the renal filtration cut-off
3. Avoid nuclease degradation	
Nucleic acid backbone modifications	Deoxynucleotides, phosphorothioate linkages and several other modifications of the ribonucleotides confer nuclease resistance
Nanoparticle formulation	Packaging within a delivery vehicle makes the RNA inaccessible to nucleases
4. Avoid immune recognition	
Nucleic acid backbone modifications	2'-O-methyl and 2'-fluoro modifications block innate immune stimulation
PEGylation	Surface charge minimizes binding to phagocytic cells and other cells
5. Extravasation	
Target tissues with leaky vessels	The liver and spleen have a fenestrated endothelium. Tumours can have leaky vessels
Endothelial transcytosis	Theoretically attractive approach to gain access to any tissue
Target endothelial or blood cells	No need to exit the vasculature
6. Cellular uptake	
Targeting ligand	Conjugate siRNA or delivery vehicle to receptor-targeting moiety (ligand, aptamer or antibody fragment) for cell-specific uptake
Association with endogenous ligand	Cholesterol-conjugated siRNAs and LNPs bind to serum apolipoproteins conferring uptake in hepatocytes
7. Endosomal release	
Membrane-destabilizing lipids	Lipid nanoparticles contain lipid bilayer-disrupting lipids that are activated by low endosomal pH
Membrane-destabilizing peptides and polymers	Masked endosomolytic peptides or polymers become unmasked (positively charged) in acidic endosomes and enhance endosomal escape of siRNA
Increase endosomal accumulation	Even if endosomal release is inefficient, efficient uptake can compensate for poor release, as only a few hundred cytosolic siRNAs are needed for maximal knockdown

IV, intravenous; GalNAc, N-acetylgalactosamine; LNP, lipid nanoparticle; PEG, polyethylene glycol.

are druggable with conventional small molecules. As most gene products have non-redundant functions in normal physiology, efficient knockdown of any gene could lead to anticipated or unanticipated direct toxicity. Target selection is key to minimize these problems. Direct toxicity is less likely to occur if humans with null or hypomorphic mutations are asymptomatic or even protected. For example, individuals with rare nonsense

mutations of the *PCSK9* gene encoding proprotein convertase subtilisin/kexin type 9, an enzyme that degrades the LDL receptor, have extremely low blood cholesterol but are healthy[44]. Knockdown of some gene targets that are needed for health in otherwise normal individuals might be well tolerated and beneficial in certain disease backgrounds, but efficiently suppressing these targets is risky. One example is the antithrombin gene *AT3*,

Box 3 | Clinically advanced siRNA delivery methods for the liver

There are several liver-targeted siRNA delivery strategies in the clinical development phase (see the figure).
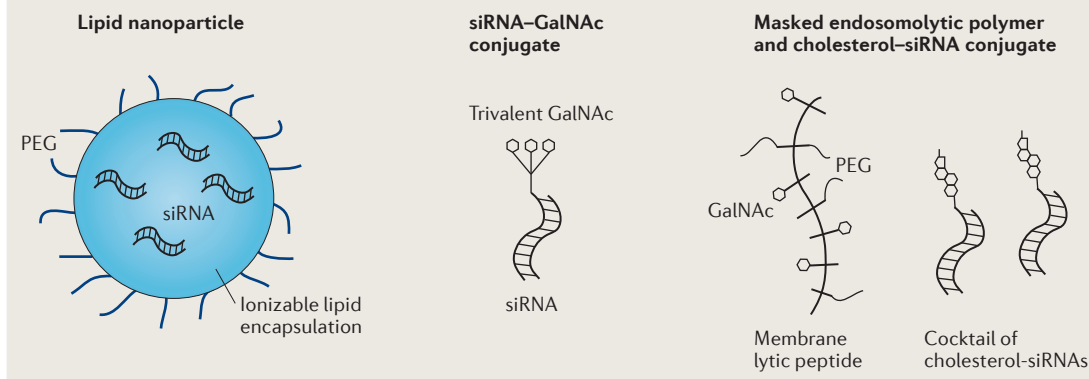
Lipid nanoparticles

Lipid nanoparticles (LNPs) are ionizable particles that are 50–100 nm in diameter, and contain mixtures of polyethylene glycol-conjugated (PEGylated) lipids, cholesterol and nucleic acids[12]. Ionizable LNPs are neutral in the circulation, where they associate with apolipoproteins (in particular, apolipoprotein E3), which mediates their endocytosis, primarily by hepatocytes[23]. The lipids become protonated at low pH in endosomes, which triggers endosomal membrane destabilization, leading to cytosolic release of some of their nucleic acid cargo.

GalNAc conjugates

Trivalent (triantennary) N-acetylgalactosamine (GalNAc), positioned via a 20 Å spacer, is a high-affinity ligand[37] for the asialoglycoprotein receptor, which is abundantly expressed on hepatocytes. The receptor is efficiently endocytosed and releases its cargo in acidic endosomes. Conjugation of triantennary GalNAc moieties to chemically stabilized small interfering RNAs (siRNAs)[33], antisense oligonucleotides (ASOs)[93] or anti-miRs[90] enables gene targeting in hepatocytes.

Masked endosomolytic compounds

Masked endosomolytic compounds are polymeric multifunctional macromolecules that incorporate both targeting ligands and endosomal escape moieties. In the original version an amphipathic poly(vinyl-ether) was conjugated to GalNAc and PEG and to an siRNA via a reversible disulfide linkage[43]. The polymer becomes positively charged in the acidic environment of endosomes, facilitating cytosolic escape of the siRNA. The multifunctional endosomolytic polymer and cholesterol-conjugated siRNA can also be injected separately and both accumulate in hepatocytes to mediate gene knockdown[34]. Co-injection of a cholesterol-conjugated siRNA and melittin-like peptide[94] (a reversibly masked amphipathic peptide polymer derived from bee venom conjugated to PEG and GalNAc) is being evaluated in clinical studies[70].

Lipid nanoparticle — PEG, siRNA, Ionizable lipid encapsulation

siRNA–GalNAc conjugate — Trivalent GalNAc, siRNA

Masked endosomolytic polymer and cholesterol–siRNA conjugate — PEG, GalNAc, Membrane lytic peptide, Cocktail of cholesterol-siRNAs

the mutation of which causes hypercoagulability. *AT3* knockdown is being investigated in Phase I clinical trials for haemophilia, in which hypercoagulability is not likely to be a problem[45]. Other anticipated direct target side effects, such as splenic atrophy from knockdown of kinesin spindle protein (*KSP*)[46] or non-alcoholic fatty liver disease from modulation of cholesterol metabolism, may be clinically well tolerated.

Potential toxicities

All oligonucleotide therapies have off-target effects that can cause unintended toxicity (BOX 4). To limit the therapeutic dose and potentially increase the therapeutic window for a therapeutic siRNA, the first step is to identify a highly potent siRNA sequence. Multiple siRNA sequences designed according to empirical rules derived from sequences that have shown high knockdown efficiency[47,48] are usually screened *in vitro*, and siRNAs with low picomolar IC_{50} (half-maximal inhibitory concentration) can generally be identified.

MicroRNA-like off-target effects. In addition to on-target effects, siRNAs exhibit sequence-specific off-target effects, including downregulation of genes with partial sequence complementarity[49]. Primarily, this effect

is mediated by inhibition of the translation of mRNAs complementary to the siRNA seed region (nucleotides at position 2–8 of the antisense strand), in a manner similar to microRNAs (miRNAs)[50,51]. Chemical modification of the siRNA antisense strand, in particular the 2′-O-methyl modification of position 2, can significantly reduce off-target effects[14]. Changing the sequence or using pools of oligonucleotides that reduce the concentration of any individual oligonucleotide[52] can also limit off-target effects. In addition, siRNA cocktails could provide the added benefit of reducing mutational escape from viral or cancer targets. However, obtaining approval for cocktails may be more difficult.

Innate immune activation. siRNAs can activate innate immune receptors, triggering interferon and inflammatory responses[53], through recognition by Toll-like receptor 3 (TLR3)[54] and TLR7 (REF. 55). In fact, TLR activation was probably responsible for the promising early clinical trial data for ocular anti-angiogenic siRNAs[54] and topically applied siRNAs against respiratory syncytial virus. Formulation of siRNAs in lipid-based delivery vehicles, which deliver siRNAs to endosomal compartments in which TLRs reside, seems to further enhance innate immune activation[55]. Limited chemical

MicroRNAs

(miRNAs). Endogenous, ~21-nucleotide-long, imperfectly paired double-stranded RNA molecules present in both plants and animals that guide the silencing of a multitude of genes bearing partially complementary sequences.

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modification of the siRNA (selective 2-O-methyl modification of one strand), however, can block binding to these receptors[12,13,24]. There may be clinical situations, such as viral infection and cancer, in which these off-target effects could be beneficial[56]. However, signs of immune activation (injection reactions, complement activation and flu-like symptoms) occur to some extent with all oligonucleotide therapies and may be a particular problem for LNPs and phosphorothioate-modified oligonucleotides[57]. There is also some evidence that cellular uptake of LNPs or other nanoparticles, even those lacking nucleic acid cargo, can trigger innate immune responses[58,59]. Pre-medication can alleviate most of these side effects. Another potential source of toxicity, which has so far not been described, could result from the accumulation of non-biodegradable chemically modified nucleotides, unnatural lipid or lipidoid components of LNPs, or polymers used to construct nanoparticles. The design of suitable biodegradable analogues[60] should address these problems if they occur.

Adaptive immune responses. Compared with protein-based therapeutics, nucleic acids have the advantage that they do not efficiently elicit antibodies, which would limit the effectiveness of repeated dosing. In fact, gene knockdown has not declined with chronic administration in the few clinical studies in which it has been tested. Although naked RNAs are poor immunogens, antibodies are easily generated against RNA–protein complexes. These are prominent in a variety of auto-immune diseases. So far, there have been no reports of antibodies against siRNAs bound to proteins, but it is unclear whether experiments to date have looked for such antibodies. Some strategies for siRNA delivery append polyethylene glycol (PEG) to the oligonucleotide to reduce excretion and improve circulating half-life. Recently, a large Phase III trial of a PEGylated RNA aptamer against factor IX, which is used as an anticoagulant during cardiac surgery, had to be terminated by Regado Biosciences because of severe anaphylactic reactions (some of which led to death) to the PEG component of the RNA[61]. This raises serious concerns about all siRNA delivery strategies that involve PEGylated oligonucleotides.

Clinical studies

Here, we discuss clinical trials of siRNA therapeutics, which have shown quantitative evidence of gene knockdown (TABLE 1).

First-generation LNPs. A first-generation LNP, 1,2-dilinoleyloxy-3-dimethylaminopropane (DLinDMA), which potently knocked down liver gene expression in rodents and non-human primates[19], showed limited liver gene knockdown in initial clinical studies and caused some toxicity (complement and innate immune activation). ALN-VSP, an LNP formulation (DLinDMA) of siRNAs targeting *KSP* and vascular endothelial growth factor A (*VEGFA*)[46], was tested in 41 patients with treatment-resistant liver metastases from a variety of tumours. Disease stabilized in three patients, and one heavily pretreated patient with endometrial carcinoma had a complete remission that lasted for the duration of the study (26 months and 50 ALN-VSP doses). However, mRNA knockdown was not convincingly shown. Administration of ALN-TTR01, which is designed to knockdown *TTR*, at a dose of 1 mg per kg knocked down TTR serum levels by 40%; TTR is expressed in the liver, and when mutated it causes familial amyloidotic neurodegeneration and cardiomyopathy[62]. Treatment was associated with infusion reactions, but none of these was serious. Ongoing Phase I/II studies of TKM-PLK1, a modified DLinDMA LNP with improved circulation time and enhanced tumour penetration and containing Polo-like kinase 1 (*PLK1*)-targeting siRNAs[63], is being evaluated in select tumours, but so far it has failed to demonstrate unequivocal sequence-specific anti-tumour effects. A Phase I trial of a similar construct that targeted two Ebola virus genes (TKM-Ebola), which protected all non-human primates tested from a lethal challenge with Zaire ebolavirus[64], was put on hold by the US Food and Drug Administration (FDA) in July 2014 because of flu-like symptoms associated with the infusion. Nonetheless, when the Ebola outbreak escalated during the summer of 2014, the FDA approved 'expanded access' to people with confirmed or suspected infections. A clinical study using a new construct designed to target the viral variant responsible for the recent West African outbreak was recently placed on hold for lack of efficacy.

Table 1 | **RNAi therapeutics with demonstrated gene knockdown or efficacy in humans***

Company	Drug	Target	Disease	Developmental phase	Mechanism	Formulation or conjugation	Efficacy	Refs
Alnylam Pharmaceuticals	ALN-TTRsc (also known as revusiran)	TTR	TTR-mediated amyloidosis	Phase III	siRNA	GalNAc conjugate	>90% serum TTR protein reduction	67
Alnylam Pharmaceuticals	ALN-PCS02	PCSK9	Hypercholesterolaemia	Phase I	siRNA	Second-generation LNP	~70% reduction of plasma PCSK9 protein	65
Alnylam Pharmaceuticals	ALN-TTR02	TTR	TTR-mediated amyloidosis	Phase III	siRNA	Second-generation LNP	>80% serum TTR protein reduction	62
Alnylam Pharmaceuticals	ALN-AT3	AT3	Haemophilia A and B, and rare bleeding disorders	Phase I	siRNA	GalNAc conjugate	>60% AT3 serum protein reduction	68
Alnylam Pharmaceuticals	ALN-TTR01	TTR	TTR-mediated amyloidosis	Phase I (terminated)	siRNA	First-generation LNP	~40% serum TTR protein reduction	62
Arrowhead Research	ARC-520	HBV	HCV	Phase II	siRNA	Dynamic PolyConjugate	~50% viral load reduction	71
Regulus Therapeutics	RG-101	miR-122	HCV	Phase II	Anti-miR	GalNAc conjugate	Viral load reductions of up to 4.8 log10	90
Santaris Pharma/Roche	SPC3649 (also known as miravirsen)	miR-122	HCV	Phase II	Anti-miR	Naked	Viral load reductions of up to 3.0 log10	89

AT3, anti-thrombin III; GalNAc, N-acetylgalactosamine; HBV, hepatitis B virus; HCV, hepatitis C virus; LNP, lipid nanoparticle; PCSK9, proprotein convertase subtilisin/kexin type 9; RNAi, RNA interference; siRNA, small interfering RNA; TTR, transthyretin. *This table summarizes RNAi therapeutics for which there is quantitative evidence of gene knockdown or inhibition.

Second-generation LNPs. Second-generation LNPs show substantially improved siRNA delivery and knockdown in the liver. Constructed with the anionic lipid dilinoleylmethyl-4-dimethylaminobutyrate (DLin-MC3-DMA), they mediate potent gene knockdown in humans at reduced doses compared with first-generation LNPs[62,65]. The furthest developed compound is ALN-TTR02 (also known as patisiran), which targets *TTR* and is being evaluated for familial amyloidotic polyneuropathy. *Ttr*-deficient mice have a mild phenotype, suggesting that *TTR* knockdown will be tolerated[66]. In a Phase I dose-escalation trial[62], 32 patients and 17 healthy volunteers who were given single intravenous injections of ALN-TTR02 (0.15–0.3 mg per kg) showed ~85% knockdown of serum TTR protein at a nadir that was durable, remaining reduced to ~60% of pretreatment levels 4 weeks later. In an open-label extension Phase II study of ALN-TTR02, patients who were given 0.3 mg per kg ALN-TTR02 every 3 weeks for 6 months demonstrated ~80% sustained knockdown of serum TTR, with little toxicity (mild to moderate infusion reactions were observed in ~15% of patients). Neurological disease stabilized and even improved over 6 months, in contrast to the deterioration normally seen in historical control patients. A Phase III clinical trial was initiated in 2013 that should be completed in 2017.

In a Phase I study in 32 healthy volunteers, ALN-PCS02, a second-generation LNP targeting *PCSK9* for the treatment of familial hypercholesterolaemia, was administered in a single dose of 0.4 mg per kg, and reduced serum PCSK9 by ~70% and serum LDL-cholesterol by ~40% at the nadir[65]. The treatment was given with corticosteroid pretreatment to suppress infusion reactions and was well tolerated. Thus, second-generation LNPs have shown impressive and durable gene knockdown in the liver at reduced doses compared with the first-generation products. Although infusion reactions are a potential problem, they can be mitigated by steroid pretreatment.

GalNAc conjugates. Although second-generation LNPs provide an acceptable delivery solution for the liver, in early-phase clinical trials GalNAc-conjugated siRNAs seem to provide robust knockdown. They also have several advantages — for instance, they can be given subcutaneously, appear to be better tolerated and do not require steroid pretreatment[33]. Moreover, conjugated siRNAs are much simpler to produce than LNP-formulated siRNAs. In a Phase I study in healthy volunteers, weekly injections of up to 10 mg per kg GalNAc-conjugated siRNA targeting *TTR* (ALN-TTRsc; also known as revusiran) resulted in sustained serum TTR protein knockdown of >90% and only mild injection-site erythemas in a minority of subjects as the main adverse reaction[67]. Similar levels of *TTR* knockdown and injection-site reactions were seen in preliminary data from a multi-dose (5 mg per kg and 7.5 mg per kg) Phase II trial of ALN-TTRsc in 2014. The study included 26 patients with TTR-mediated familial amyloid cardiomyopathy (FAC) and senile systemic amyloidosis (SSA) with mutated and wild-type *TTR*, respectively. Fifteen per cent of patients had minor (one patient had

moderate) transient liver enzyme elevations that normalized with continued drug administration, indicating a favourable safety profile.

Recent proprietary siRNA modifications developed by Alnylam Pharmaceuticals, termed enhanced stabilization chemistry (ESC), improve gene knockdown potency by as much as 50-fold[40]. GalNAc-conjugated ESC-modified siRNAs are now in Phase I studies for haemophilia (ALN-AT3, targeting AT3), complement-mediated diseases (ALN-CC5, targeting complement component 5) and porphyrias (ALN-AS1, targeting 5-aminolevulinic acid synthase 1 (ALAS1), the first enzyme in haem biosynthesis). The haemophilia study takes a new approach to treating these bleeding disorders. Instead of supplying the missing clotting factors, it will seek to enhance thrombin generation and clotting by knocking down *AT3*. Preliminary data from the Phase I dose-escalation trial in patients with severe haemophilia showed >60% reduction in circulating AT3, increased thrombin generation and improved blood clotting using a dose as low as 45 μg per kg[68]. Clinical studies are planned to begin to evaluate this platform for the treatment of hypercholesterolaemia (ALN-PCSsc) and α1-antitrypsin deficiency (ALN-AAT) in 2015, and hepatitis B virus infection (ALN-HBV) in 2016 (REF. 69). Soon, clinical trials of GalNAc-conjugated ESC-siRNAs will expand from treating small numbers of patients with orphan diseases to addressing more common metabolic diseases.

Masked endosomolytic compounds. Arrowhead Research's delivery strategy, referred to as Dynamic PolyConjugates (DPCs), seeks to improve endosomal release by using non-lipid reversibly masked endosome-disrupting peptides or polymers. The clinically most advanced DPC, called NAG–MLP, is based on a masked amphipathic melittin-like membranolytic peptide (MLP) covalently conjugated to GalNAc-targeting ligands, which bind to the ASGPR on hepatocytes and PEG (to reduce nonspecific cell adhesion and thereby improve retention in the circulation)[70]. siRNAs are conjugated to cholesterol, which facilitates cellular uptake, and injected separately. The peptide is unmasked as the endosome acidifies to enhance endosomal release. This platform is being evaluated in the clinic for treating chronic HBV infection (using separate injections of the targeted membranolytic polymer and the siRNA) and AAT deficiency. For HBV, a cocktail of two cholesterol-conjugated HBV-targeted siRNAs is co-injected with NAG–MLP (ARC-520). A Phase I dose-escalation trial in healthy volunteers and a Phase IIa dose-escalation trial in patients with chronic HBV infection have been initiated. Preliminary results[71] suggest that all NAG–MLP doses tested (0.01–4 mg per kg) were well tolerated in 36 healthy volunteers receiving the active compound, with no changes in laboratory parameters or severe drug-related adverse reactions. However, a few cases of potential hypersensitivity reactions were noted, and the risk of immune reactions against the PEGylated endosomolytic agent — especially with repeat dosing — is a potential concern. In patients with chronic HBV infection, a modest ~50% reduction in HBsAg (HBV surface antigen; a measure of viral load) at the nadir was seen after a single dose of 2 mg per kg polymer, but further dose escalation is planned. A Phase I study testing ARC-AAT for the treatment of liver-associated disease in patients with AAT deficiency has just begun[72]. The two-molecule approach has the advantage of being easily adapted to new targets and facilitates the construction of RNA cocktails. However, requiring two different molecules to accumulate in the same endosome at the same time in functional amounts may limit efficacy and increase toxicity. A single-molecule DPC that is being developed and has been tested preclinically[43] may be more practical.

In addition to the clinical studies described above, there are ongoing early-phase clinical studies by RXi Pharmaceuticals to reduce dermal scarring by targeting connective tissue growth factor[73,74], by Dicerna Pharmaceuticals to treat primary and metastatic liver cancer by targeting *MYC*[75,76], and by Silence Therapeutics to treat pancreatic cancer, with liposomes targeting protein kinase N3 in the tumour vasculature[77].

Recent clinical trials targeting the liver have shown promising, durable and well-tolerated gene knockdown, with suggestions of clinical benefit, indicating that siRNA drugs, especially GalNAc-conjugated ESC-siRNAs, are well poised to become a new class of therapeutics within the next few years.

Moving beyond the liver

Although hepatocyte delivery seems to be a largely solved problem, treatment of most diseases requires developing clinically acceptable methods to deliver siRNAs to other tissues and organs. Tissues and organs that are readily accessible to topical application or injection, such as the skin, mucosa and eye, should be the easiest to target. Indeed, cholesterol-conjugated siRNAs may be suitable for delivery to many of these tissues[78]. Similarly, RXi Pharmaceuticals' 'self-delivering' sd-rxRNAs, which are currently evaluated in clinical trials with intra-dermal administration, have shown dose-dependent (albeit rather modest) target gene knockdown in the eye following ocular administration in mice[73].

However, many commercial and academic efforts for the development of delivery platforms are still devoted to improving the designs of LNPs and other nanoparticles. There are also some efforts to use lipoproteins[79] or exosomes[80] — naturally produced lipid vesicles that carry endogenous miRNAs which are taken up by nearby and distant cells — as siRNA carriers. Particle-based delivery is not ideal for targeting most internal organs and even tumours, as LNPs and particulates are mostly trapped in the liver and extravasation is limited in other tissues with non-fenestrated endothelium. Uniform and effective siRNA delivery and gene knockdown in solid tumours by particles will probably be difficult, given the heterogeneous and aberrant (albeit leaky) tumour vasculature.

The successes of relatively simply designed siRNA conjugates in the liver and the potential toxicity of LNPs and other complex particles (and their liver trapping, increased cost and regulatory complexity) suggest that

Endosomal escape
The process of cytosolic entry of small interfering RNAs (siRNAs) from a vesicular compartment, following initial endocytosis of the siRNA (and delivery vehicle) into the target cell.

more effort should be devoted to designing conjugated siRNAs for targeted uptake. However, no small-molecule ligand–receptor pair with similar delivery potency to the GalNAc–ASGPR pair has, so far, been identified. This ligand–receptor pair may have been an especially fortuitous choice, as the receptor is highly expressed and is recycled rapidly for new rounds of internalization[40]. Antibody fragments fused to protamine or to other nucleic acid-binding peptides have been used to deliver siRNAs and knock down gene expression in tumours, disseminated blood cells and organs such as the lungs[26,27,81,82], but they are challenging to manufacture and develop into drugs. Antibodies can also be directly conjugated to siRNAs. However, a recent systematic analysis of covalently coupled siRNA–antibody conjugates with different internalization routes suggested that despite cellular internalization of the conjugates, only a subset were able to deliver functional amounts of siRNA intracellularly[83]. Thus, although internalizing siRNA–ligand conjugates can be constructed relatively easily, it may be necessary to select abundant receptors that efficiently internalize their ligands or to devote more effort to optimizing the ligand, the chemical modifications of the siRNA to enhance silencing activity and stability, or attaching modifying groups to enhance endosomal escape. Promising recent efforts to enhance the cytosolic bioavailability of siRNAs include various endosome-destabilizing compounds[84] and chemical modifications that increase the hydrophobicity of the oligonucleotide[73,85].

One especially attractive and flexible 'conjugate' platform for targeted delivery is RNA chimaeras composed of an RNA aptamer, selected for high-affinity binding to a cell surface receptor, and covalently linked to one strand of an siRNA[29–31]. These are taken up by receptor-mediated endocytosis and cleaved by Dicer to liberate an active siRNA. Recent work with breast cancer homing epithelial cell adhesion molecule (EpCAM) aptamer–siRNA chimaeras suggests that, like GalNAc conjugates, they concentrate in targeted tissues after subcutaneous injection[95]. Application of improved chemistries for nuclease stability and novel endosomal escape strategies may further improve this versatile siRNA delivery platform.

Conclusions

siRNA therapeutics are now well poised to enter the clinical formulary as a new class of drugs in the near future. Clinical studies conducted in the past 2 years indicate that safe and effective liver gene knockdown is achievable. Although targeting any individual gene might lead to unanticipated clinical toxicity that could stop the development of any individual siRNA drug, we anticipate a rapid expansion of clinical trials for multiple clinical indications. The first siRNA drug approval could occur in the next few years. The lessons learned about delivering siRNAs are likely to facilitate the development of ways to deliver the larger cargo that is being contemplated for therapeutics using modified mRNAs[86] or CRISPR–Cas9 (clustered regularly interspaced short palindromic repeats–CRISPR-associated 9)-mediated[87] gene editing. However, the delivery obstacles for these other approaches are greater than for siRNAs and should not be underestimated.

1. Zamecnik, P. C. & Stephenson, M. L. Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. *Proc. Natl Acad. Sci. USA* **75**, 280–284 (1978).
2. Stephenson, M. L. & Zamecnik, P. C. Inhibition of Rous sarcoma viral RNA translation by a specific oligodeoxyribonucleotide. *Proc. Natl Acad. Sci. USA* **75**, 285–288 (1978).
   First demonstration of the possibility of inhibiting gene expression using oligonucleotides.
3. Kole, R., Krainer, A. R. & Altman, S. RNA therapeutics: beyond RNA interference and antisense oligonucleotides. *Nat. Rev. Drug Discov.* **11**, 125–140 (2012).
4. Bennett, C. F. & Swayze, E. E. RNA targeting therapeutics: molecular mechanisms of antisense oligonucleotides as a therapeutic platform. *Annu. Rev. Pharmacol. Toxicol.* **50**, 259–293 (2010).
5. Sharma, V. K., Sharma, R. K. & Singh, S. K. Antisense oligonucleotides: modifications and clinical trials. *Med. Chem. Commun.* **5**, 1454–1471 (2014).
6. Raal, F. J. *et al.* Mipomersen, an apolipoprotein B synthesis inhibitor, for lowering of LDL cholesterol concentrations in patients with homozygous familial hypercholesterolaemia: a randomised, double-blind, placebo-controlled trial. *Lancet* **375**, 998–1006 (2010).
7. Fire, A. *et al.* Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806–811 (1998).
   Initial report describing mediation of RNAi by double-stranded RNAs.
8. Elbashir, S. M. *et al.* Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**, 494–498 (2001).
   Demonstration that exogenously supplied short double-stranded RNAs can specifically and efficiently inhibit gene expression in mammalian cells.
9. Song, E. *et al.* RNA interference targeting Fas protects mice from fulminant hepatitis. *Nat. Med.* **9**, 347–351 (2003).
   First proof of principle that siRNAs can be harnessed to treat disease in an *in vivo* model.
10. Morrissey, D. V. *et al.* Activity of stabilized short interfering RNA in a mouse model of hepatitis B virus replication. *Hepatology* **41**, 1349–1356 (2005).
11. Chiu, Y.-L. & Rana, T. M. siRNA function in RNAi: a chemical modification analysis. *RNA* **9**, 1034–1048 (2003).
12. Morrissey, D. V. *et al.* Potent and persistent *in vivo* anti-HBV activity of chemically modified siRNAs. *Nat. Biotechnol.* **23**, 1002–1007 (2005).
    First demonstration that siRNAs can be chemically modified to attenuate nuclease degradation and immune stimulation to allow for efficient lipid-based systemic administration.
13. Judge, A. D., Bola, G., Lee, A. C. H. & MacLachlan, I. Design of noninflammatory synthetic siRNA mediating potent gene silencing *in vivo*. *Mol. Ther.* **13**, 494–505 (2006).
14. Jackson, A. L. *et al.* Position-specific chemical modification of siRNAs reduces 'off-target' transcript silencing. *RNA* **12**, 1197–1205 (2006).
15. Bartlett, D. W. & Davis, M. E. Insights into the kinetics of siRNA-mediated gene silencing from live-cell and live-animal bioluminescent imaging. *Nucleic Acids Res.* **34**, 322–333 (2006).
16. Gilleron, J. *et al.* Image-based analysis of lipid nanoparticle-mediated siRNA delivery, intracellular trafficking and endosomal escape. *Nat. Biotechnol.* **31**, 638–646 (2013).
17. Wittrup, A. *et al.* Visualizing lipid-formulated siRNA release from endosomes and target gene knockdown. *Nat. Biotechnol.* http://dx.doi.org/10.1038/nbt.3298 (2015).
18. Kanasty, R., Dorkin, J. R., Vegas, A. & Anderson, D. Delivery materials for siRNA therapeutics. *Nat. Mater.* **12**, 967–977 (2013).
19. Zimmermann, T. S. *et al.* RNAi-mediated gene silencing in non-human primates. *Nature* **441**, 111–114 (2006).
    First report of potent RNAi-mediated gene silencing in non-human primates after systemic administration of siRNA formulated in LNPs.
20. Akinc, A. *et al.* A combinatorial library of lipid-like materials for delivery of RNAi therapeutics. *Nat. Biotechnol.* **26**, 561–569 (2008).
21. Semple, S. C. *et al.* Rational design of cationic lipids for siRNA delivery. *Nat. Biotechnol.* **28**, 172–176 (2010).
22. Shi, B. *et al.* Biodistribution of small interfering RNA at the organ and cellular levels after lipid nanoparticle-mediated delivery. *J. Histochem. Cytochem.* **59**, 727–740 (2011).
23. Akinc, A. *et al.* Targeted delivery of RNAi therapeutics with endogenous and exogenous ligand-based mechanisms. *Mol. Ther.* **18**, 1357–1364 (2010).
24. Judge, A. D. *et al.* Confirming the RNAi-mediated mechanism of action of siRNA-based cancer therapeutics in mice. *J. Clin. Invest.* **119**, 661–673 (2009).
25. Bartlett, D. W., Su, H., Hildebrandt, I. J., Weber, W. A. & Davis, M. E. Impact of tumor-specific targeting on the biodistribution and efficacy of siRNA nanoparticles measured by multimodality *in vivo* imaging. *Proc. Natl Acad. Sci. USA* **104**, 15549–15554 (2007).
26. Song, E. *et al.* Antibody mediated *in vivo* delivery of small interfering RNAs via cell-surface receptors. *Nat. Biotechnol.* **23**, 709–717 (2005).
27. Peer, D., Zhu, P., Carman, C. V., Lieberman, J. & Shimaoka, M. Selective gene silencing in activated leukocytes by targeting siRNAs to the integrin lymphocyte function-associated antigen-1. *Proc. Natl Acad. Sci. USA* **104**, 4095–4100 (2007).
28. Peer, D., Park, E. J., Morishita, Y., Carman, C. V. & Shimaoka, M. Systemic leukocyte-directed siRNA delivery revealing cyclin D1 as an anti-inflammatory target. *Science* **319**, 627–630 (2008).
29. McNamara, J. O. *et al.* Cell type-specific delivery of siRNAs with aptamer-siRNA chimeras. *Nat. Biotechnol.* **24**, 1005–1015 (2006).
30. Berezhnoy, A., Castro, I., Levay, A., Malek, T. R. & Gilboa, E. Aptamer-targeted inhibition of mTOR in T cells enhances antitumor immunity. *J. Clin. Invest.* **124**, 188–197 (2014).

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31. Wheeler, L. A. *et al.* Inhibition of HIV transmission in human cervicovaginal explants and humanized mice using CD4 aptamer-siRNA chimeras. *J. Clin. Invest.* **121**, 2401–2412 (2011).
32. Davis, M. E. *et al.* Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. *Nature* **464**, 1067–1070 (2010).
33. Nair, J. K. *et al.* Multivalent *N*-acetylgalactosamine-conjugated siRNA localizes in hepatocytes and elicits robust RNAi-mediated gene silencing. *J. Am. Chem. Soc.* **136**, 16958–16961 (2014).
    Description of triantennary GalNAc-conjugated siRNA for liver-targeted gene knockdown.
34. Wong, S. C. *et al.* Co-injection of a targeted, reversibly masked endosomolytic polymer dramatically improves the efficacy of cholesterol-conjugated small interfering RNAs *in vivo*. *Nucleic Acid Ther.* **22**, 380–390 (2012).
35. Kortylewski, M. *et al. In vivo* delivery of siRNA to immune cells by conjugation to a TLR9 agonist enhances antitumor immune responses. *Nat. Biotechnol.* **27**, 925–932 (2009).
36. Soutschek, J. *et al.* Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature* **432**, 173–178 (2004).
37. Biessen, E. A. *et al.* Synthesis of cluster galactosides with high affinity for the hepatic asialoglycoprotein receptor. *J. Med. Chem.* **38**, 1538–1546 (1995).
38. Matsuda, S. *et al.* siRNA conjugates carrying sequentially assembled trivalent *N*-acetylgalactosamine linked through nucleosides elicit robust gene silencing *in vivo* in hepatocytes. *ACS Chem. Biol.* **10**, 1181–1187 (2015).
39. Rajeev, K. G. *et al.* Hepatocyte-specific delivery of siRNAs conjugated to novel non-nucleosidic trivalent *N*-acetylgalactosamine elicits robust gene silencing *in vivo*. *ChemBioChem* **16**, 903–908 (2015).
40. Manoharan, M. GalNAc-siRNA with enhanced stabilization chemistry: ESC-GalNAc-siRNA. *Alnylam* [online], http://www.alnylam.com/web/assets/ALNY-ESC-GalNAc-siRNA-TIDES-May2014-Capella.pdf (2014).
41. Thurston, T. L. M., Wandel, M. P., von Muhlinen, N., Foeglein, Á. & Randow, F. Galectin 8 targets damaged vesicles for autophagy to defend cells against bacterial invasion. *Nature* **482**, 414–418 (2012).
42. Boussif, O., Zanta, M. A. & Behr, J. P. Optimized galenics improve *in vitro* gene transfer with cationic molecules up to 1000-fold. *Gene Ther.* **3**, 1074–1080 (1996).
43. Rozema, D. B. *et al.* Dynamic PolyConjugates for targeted *in vivo* delivery of siRNA to hepatocytes. *Proc. Natl Acad. Sci. USA* **104**, 12982–12987 (2007).
44. Cohen, J. C., Boerwinkle, E., Mosley, T. H. Jr & Hobbs, H. H. Sequence variations in *PCSK9*, low LDL, and protection against coronary heart disease. *N. Engl. J. Med.* **354**, 1264–1272 (2006).
45. Sorensen, B. *et al.* A subcutaneously administered RNAi therapeutic (ALN-AT3) targeting antithrombin for treatment of hemophilia: interim Phase 1 study results in healthy volunteers and patients with hemophilia A or B. Oral Poster Abstract 693. *56th American Society of Hematology* [online], https://ash.confex.com/ash/2014/webprogram/Paper75077.html (2014).
46. Tabernero, J. *et al.* First-in-humans trial of an RNA interference therapeutic targeting VEGF and KSP in cancer patients with liver involvement. *Cancer Discov.* **3**, 406–417 (2013).
47. Reynolds, A. *et al.* Rational siRNA design for RNA interference. *Nat. Biotechnol.* **22**, 326–330 (2004).
48. Elbashir, S. M., Harborth, J., Weber, K. & Tuschl, T. Analysis of gene function in somatic mammalian cells using small interfering RNAs. *Methods* **26**, 199–213 (2002).
49. Jackson, A. L. *et al.* Expression profiling reveals off-target gene regulation by RNAi. *Nat. Biotechnol.* **21**, 635–637 (2003).
50. Jackson, A. L. *et al.* Widespread siRNA 'off-target' transcript silencing mediated by seed region sequence complementarity. *RNA* **12**, 1179–1187 (2006).
51. Birmingham, A. *et al.* 3′ UTR seed matches, but not overall identity, are associated with RNAi off-targets. *Nat. Methods* **3**, 199–204 (2006).
52. Huang, L. *et al.* Efficient and specific gene knockdown by small interfering RNAs produced in bacteria. *Nat. Biotechnol.* **31**, 350–356 (2013).
53. Judge, A. D. *et al.* Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. *Nat. Biotechnol.* **23**, 457–462 (2005).
54. Kleinman, M. E. *et al.* Sequence- and target-independent angiogenesis suppression by siRNA via TLR3. *Nature* **452**, 591–597 (2008).
55. Hornung, V. *et al.* Sequence-specific potent induction of IFN-α by short interfering RNA in plasmacytoid dendritic cells through TLR7. *Nat. Med.* **11**, 263–270 (2005).
56. Poeck, H. *et al.* 5′-triphosphate-siRNA: turning gene silencing and Rig-I activation against melanoma. *Nat. Med.* **14**, 1256–1263 (2008).
57. Roberts, T. L., Sweet, M. J., Hume, D. A. & Stacey, K. J. Cutting edge: species-specific TLR9-mediated recognition of CpG and non-CpG phosphorothioate-modified oligonucleotides. *J. Immunol.* **174**, 605–608 (2005).
58. Kedmi, R., Ben-Arie, N. & Peer, D. The systemic toxicity of positively charged lipid nanoparticles and the role of Toll-like receptor 4 in immune activation. *Biomaterials* **31**, 6867–6875 (2010).
59. Landesman-Milo, D. & Peer, D. Toxicity profiling of several common RNAi-based nanomedicines: a comparative study. *Drug Deliv. Transl. Res.* **4**, 96–103 (2014).
60. Maier, M. A. *et al.* Biodegradable lipids enabling rapidly eliminated lipid nanoparticles for systemic delivery of RNAi therapeutics. *Mol. Ther.* **21**, 1570–1578 (2013).
61. Rusconi, C. Phase 3 evaluation of revolixys kit: study summary and lessons learned. *Celebrating the 25th Anniversary of Selex, ASGCT meeting May 10–12, 2015, New Orleans* (2015).
62. Coelho, T. *et al.* Safety and efficacy of RNAi therapy for transthyretin amyloidosis. *N. Engl. J. Med.* **369**, 819–829 (2013).
    First demonstration of highly potent siRNA-mediated gene knockdown in humans.
63. Degenhardt, Y. & Lampkin, T. Targeting Polo-like kinase in cancer therapy. *Clin. Cancer Res.* **16**, 384–389 (2010).
64. Geisbert, T. W. *et al.* Postexposure protection of non-human primates against a lethal Ebola virus challenge with RNA interference: a proof-of-concept study. *Lancet* **375**, 1896–1905 (2010).
65. Fitzgerald, K. *et al.* Effect of an RNA interference drug on the synthesis of proprotein convertase subtilisin/kexin type 9 (PCSK9) and the concentration of serum LDL cholesterol in healthy volunteers: a randomised, single-blind, placebo-controlled, phase 1 trial. *Lancet* **383**, 60–68 (2014).
66. Adams, D. *et al.* Phase 2 open-label extension study of patisiran, an RNAi therapeutic for the treatment of familial amyloidotic polyneuropathy. *Alnylam* [online], http://www.alnylam.com/web/assets/Patisiran_Phase2_OLE_ANA_POSTER-3.pdf (2014)
67. Zimmermann, T. *et al.* Phase I first-in-human trial of ALN-TTRsc, a novel RNA interference therapeutic for the treatment of familial amyloidotic cardiomyopathy (FAC). *Alnylam* [online], http://www.alnylam.com/web/wp-content/uploads/2013/09/ALN-TTRsc-PhI-HFSA-Poster-Sep2013.pdf (2013).
68. Akinc, A. A. Subcutaneously administered investigational RNAi therapeutic (ALN-AT3) targeting antithrombin for treatment of hemophilia: interim Phase 1 study results in healthy volunteers and hemophilia A and B subjects. *Alnylam* [online], http://www.alnylam.com/web/assets/2015-Goring-meeting-Akinc_Jan2015.pdf (2015).
69. [No authors listed.] RNAi Roundtable: ALN-PCSsc for the treatment of hypercholesterolemia. *Alnylam* [online], http://www.alnylam.com/web/assets/RNAi_Roundtable_PCSsc_081414.pdf (2014).
70. Wooddell, C. I. *et al.* Hepatocyte-targeted RNAi therapeutics for the treatment of chronic hepatitis B virus infection. *Mol. Ther.* **21**, 973–985 (2013).
71. Yuen, M.-F. *et al.* Phase II, dose ranging study of ARC-520, a siRNA-based therapeutic, in patients with chronic hepatitis B virus infection. *American Association for the Study of Liver Diseases* [online], http://files.shareholder.com/downloads/AMDA-2OTJP1/3374437865x0x793199/06940176-e88b-4bfd-9ded-aacd216c7e9a/AASLD%202014%20ARC-520.pdf (2014).
72. [No authors listed.] Arrowhead begins Phase 1 trial of ARC-AAT for treatment of liver disease associated with alpha-1 antitrypsin deficiency. *Arrowhead Research* [online], http://ir.arrowheadresearch.com/releasedetail.cfm?ReleaseID=897578 (2015).
73. Byrne, M. *et al.* Novel hydrophobically modified asymmetric RNAi compounds (sd-rxRNA) demonstrate robust efficacy in the eye. *J. Ocul. Pharmacol. Ther.* **29**, 855–864 (2013).
74. [No authors listed.] Clinical trials — overview. *RXI Pharmaceuticals* [online], http://www.rxipharma.com/clinical-trials/#additional
75. Tolcher, A. W. *et al.* Safety and activity of DCR-MYC, a first-in-class Dicer-substrate small interfering RNA (DsiRNA) targeting MYC, in a phase I study in patients with advanced solid tumors. *J. Clin. Oncol.* **33**, 915_suppl 11006 (2015).
76. Dudek, H. *et al.* Knockdown of β-catenin with dicer-substrate siRNAs reduces liver tumor burden *in vivo*. *Mol. Ther.* **22**, 92–101 (2014).
77. Schultheis, B. *et al.* First-in-human phase I study of the liposomal RNA interference therapeutic Atu027 in patients with advanced solid tumors. *J. Clin. Oncol.* **32**, 4141–4148 (2014).
78. Wu, Y. *et al.* Durable protection from herpes simplex virus-2 transmission following intravaginal application of siRNAs targeting both a viral and host gene. *Cell Host Microbe* **5**, 84–94 (2009).
79. Nakayama, T. *et al.* Harnessing a physiologic mechanism for siRNA delivery with mimetic lipoprotein particles. *Mol. Ther.* **20**, 1582–1589 (2012).
80. Alvarez-Erviti, L. *et al.* Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nat. Biotechnol.* **29**, 341–345 (2011).
81. Kumar, P. *et al.* T cell-specific siRNA delivery suppresses HIV-1 infection in humanized mice. *Cell* **134**, 577–586 (2008).
82. Yao, Y.-D. *et al.* Targeted delivery of PLK1-siRNA by ScFv suppresses Her2+ breast cancer growth and metastasis. *Sci. Transl Med.* **4**, 130ra48 (2012).
83. Cuellar, T. L. *et al.* Systematic evaluation of antibody-mediated siRNA delivery using an industrial platform of THIOMAB-siRNA conjugates. *Nucleic Acids Res.* **43**, 1189–1203 (2015).
84. Rozema, D. B. *et al.* Protease-triggered siRNA delivery vehicles. *J. Control. Release* **209**, 57–66 (2015).
85. Meade, B. R. *et al.* Efficient delivery of RNAi prodrugs containing reversible charge-neutralizing phosphotriester backbone modifications. *Nat. Biotechnol.* **32**, 1256–1261 (2014).
86. Warren, L. *et al.* Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. *Cell Stem Cell* **7**, 618–630 (2010).
87. Yin, H. *et al.* Genome editing with Cas9 in adult mice corrects a disease mutation and phenotype. *Nat. Biotechnol.* **32**, 551–553 (2014).
88. Goemans, N. M. *et al.* Systemic administration of PRO051 in Duchenne's muscular dystrophy. *N. Engl. J. Med.* **364**, 1513–1522 (2011).
89. Janssen, H. L. A. *et al.* Treatment of HCV infection by targeting microRNA. *N. Engl. J. Med.* **368**, 1685–1694 (2013).
90. Bhat, B. *et al.* RG-101, a GalNAC-conjugated anti-miR employing aunique mechanism of action by targeting host factor microRNA-122 (miR-122), demonstrates potent activity and reduction of HCV in preclinical studies. *Hepatology* **58**, 1393A (2013).
91. Hata, A. & Lieberman, J. Dysregulation of microRNA biogenesis and gene silencing in cancer. *Sci. Signal.* **8**, re3 (2015).
92. Daige, C. L. *et al.* Systemic delivery of a miR34a mimic as a potential therapeutic for liver cancer. *Mol. Cancer Ther.* **13**, 2352–2360 (2014).
93. Prakash, T. P. *et al.* Targeted delivery of antisense oligonucleotides to hepatocytes using triantennary *N*-acetyl galactosamine improves potency 10-fold in mice. *Nucleic Acids Res.* **42**, 8796–8807 (2014).
94. Rozema, D. B., Ekena, K., Lewis, D. L., Loomis, A. G. & Wolff, J. A. Endosomolysis by masking of a membrane-active agent (EMMA) for cytoplasmic release of macromolecules. *Bioconjug. Chem.* **14**, 51–57 (2003).
95. Gilboa-Geffen, A. *et al.* Gene knockdown by EpCAM aptamer–siRNA chimeras suppresses epithelial breast cancers and their tumor-initiating cells. *Mol. Cancer Ther.* (in the press).

Competing interests statement
The authors declare competing interests: see Web version for details.