siRNAs: APPLICATIONS IN FUNCTIONAL GENOMICS AND POTENTIAL AS THERAPEUTICS

Yair Dorsett and Thomas Tuschl

Molecules that can specifically silence gene expression are powerful research tools. Much effort has been put into the development of such molecules and has resulted in the creation of different classes of potential therapeutic agents. Small interfering RNA (siRNA) is one of the latest additions to the repertoire of sequence-specific gene-silencing agents. The robustness of this approach has motivated numerous biotechnology organizations and academic institutions to develop siRNA libraries for high-throughput genome-wide screening in mammalian cells. This article first overviews current nucleic-acid-based approaches for gene silencing, and then focuses on the application of siRNAs in particular in functional genomics and as potential therapeutics.

INTERFERON RESPONSE
A cellular response to dsRNA longer than 30 base pairs that results in global post-transcriptional gene silencing.

Laboratory of RNA Molecular Biology, Rockefeller University, 1230 York Avenue, Box 186, New York, New York 10021, USA. Correspondence to T.T. e-mail: ttuschl@rockefeller.edu
doi:10.1038/nrd1345

Classical genetic approaches identify gene mutations that disrupt the function or pathway being studied. The recovery and mapping of mutations affecting phenotypes is time-consuming and usually not easily applied to mammalian systems. Reverse genetic approaches involve the disruption of a gene with an unknown or suspected function to determine the effect on a function or pathway; in many cases, this is also expensive and time-consuming. Now that the genomes of many key model organisms have been largely sequenced, nucleic-acid-based approaches that act to silence gene expression in a sequence-specific manner have become powerful tools for investigating gene function. These nucleic acid molecules are also being developed as therapeutic agents that target viruses and disease-causing genes.

Small interfering RNAs (siRNAs) are one of the most recent additions to the wide repertoire of nucleic acid molecules used to silence gene expression. siRNAs are the effector molecules of the RNA interference (RNAi) pathway[1,2], which was discovered in 1998 when Andrew Fire and Craig Mello injected double-stranded RNA (dsRNA) into the nematode *Caenorhabditis elegans*, initiating a potent sequence-specific degradation of cytoplasmic mRNAs containing the same sequence as the dsRNA trigger[3,4]. The discovery of RNAi in nematodes made it apparent that post-transcriptional gene silencing (PTGS) in plants, and quelling in fungi, were fundamentally related processes that were also triggered by dsRNA. RNAi was rapidly developed as a tool to study gene function, and was found to occur in protozoa and almost all higher eukaryotes tested[4–8].

These early applications used long dsRNA, but long dsRNA was not effective in most mammalian cells because it induced the antiviral INTERFERON (IFN) response[9], which usually leads to cell death. Genetic and biochemical investigations of the mechanisms guiding RNAi in different organisms revealed the conservation of cellular machinery that cleaves long dsRNA into duplexes of 21- to 28-nucleotide siRNAs, which guide the sequence-specific degradation of mRNAs[2,10–12] (FIG. 1). The elucidation of siRNA structure led to the discovery that siRNAs can effectively reduce gene expression in many mammalian cell types without triggering the IFN response[13–15].

RNAi provides a new, reliable method to investigate gene function that has many advantages over other nucleic-acid-based approaches, and which is therefore currently the most widely used gene-silencing technique in functional genomics. Previous extensive research on the development of therapeutic antisense nucleic acids should facilitate development of therapeutic siRNAs. This review will give a brief overview of the most popular

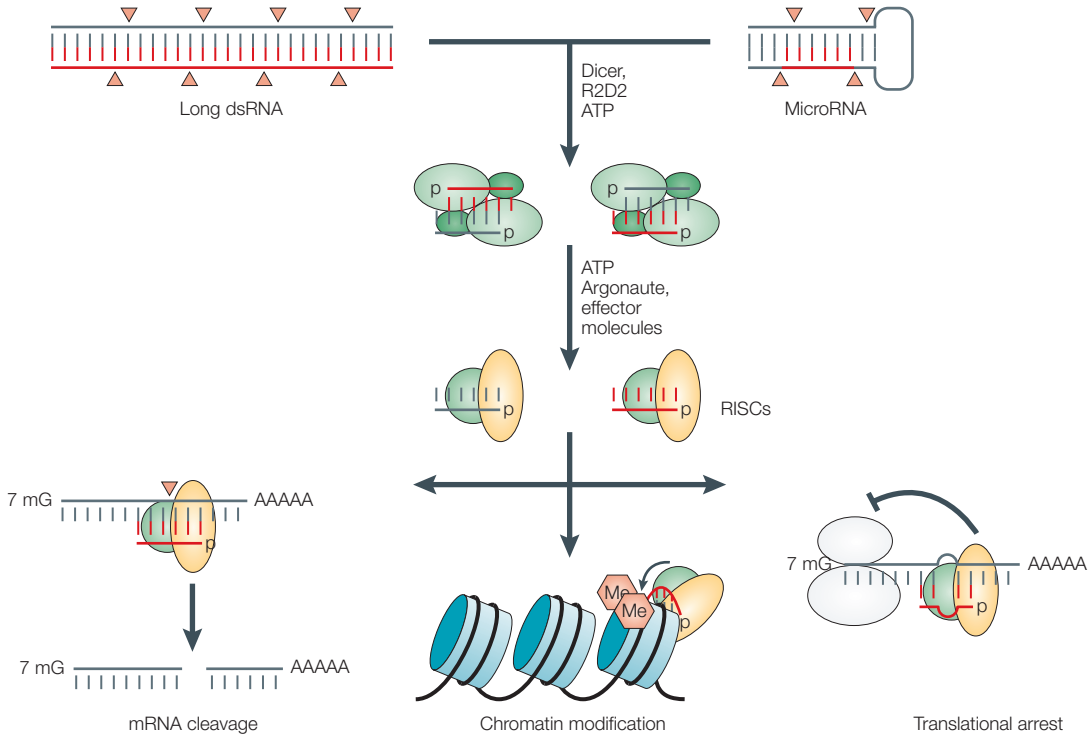

Figure 1 | **Mechanisms of nucleic-acid-based approaches for gene silencing: RNA silencing.** Double-stranded (ds) RNA can be produced endogenously within the cell, as in the case of microRNAs (miRNAs) and long dsRNA produced by genomic transcription of long sense and antisense RNAs. Alternatively, dsRNA can be introduced directly into the cell through a dsRNA virus or by experimental manipulation. The dsRNA present is cleaved by the Dicer enzyme within the cell into 21- to 28-nucleotide small interfering RNAs (siRNAs) or miRNAs that are passed on to protein complexes by the dsRNA-binding protein R2D2, forming RNA-induced silencing complexes (RISCs). There are probably different types of RISCs that direct mRNA degradation, translational inhibition or chromatin modification. 7 mG, 7-methyl guanine; AAAAA, poly-adenosine tail; Me, methyl group; p, 5′ phosphate.

nucleic-acid-based gene-silencing approaches available and discuss the applications of siRNAs in functional genomics and their potential as therapeutic agents.

Nucleic-acid-based gene silencing

Several different types of molecule that act to inhibit gene expression by sequence-specific targeting of mRNAs have been developed in the hope of creating therapeutic agents. The three major nucleic-acid-based gene-silencing molecules are chemically modified antisense oligodeoxyribonucleic acids (ODNs), ribozymes and siRNAs[16]. Less-utilized antisense molecules include peptide nucleic acids (PNAs)[17], morpholino phosphorodiamidates[18], DNAzymes[19–21] and the recently developed 5′-end-mutated U1 small nuclear RNAs[22]. siRNAs, ODNs and ribozymes silence gene expression through different mechanisms, as shown in FIGS 1,2,3.

ODNs. ODNs are generally ~20 nucleotides in length and act by hybridizing to pre-mRNA and mRNA to produce a substrate for ribonuclease H (RNase H), which specifically degrades the RNA strand of the formed RNA–DNA duplexes[23] (FIG. 2). If modified in a way to prevent the action of RNase H, ODNs can inhibit translation of mRNA via steric hindrance[24], or inhibit splicing of pre-mRNAs[25] (FIG. 2). ODNs and modifications thereof have been used to target dsDNA for the inhibition of transcription by the formation of triple helices[26].

Ribozymes. Ribozymes bind to RNA through Watson–Crick base pairing and act to degrade target RNA by catalysing the hydrolysis of the phosphodiester backbone[27] (FIG. 3). There are several different classes of ribozymes, with the 'hammerhead' ribozyme being the most widely studied. As its name implies, the hammerhead ribozyme forms a unique secondary structure when hybridized to its target mRNA. The catalytically important residues within the ribozyme are flanked by target-complementary sequences that flank the target RNA cleavage site. Cleavage by a ribozyme requires divalent ions, such as magnesium, and is also dependent on target RNA structure and accessibility[28]. Co-localizing a ribozyme with a target RNA within the cell through the use of localization signals greatly increases their silencing efficiency[29]. The hammerhead ribozymes are short enough to be chemically synthesized or can be transcribed from vectors[30], allowing for the continuous production of ribozymes within cells.

siRNAs. siRNAs found in nature are derived from the cytoplasmic processing of long dsRNA by the RNase-III-type enzyme termed Dicer[31]. Dicer cleaves long dsRNA

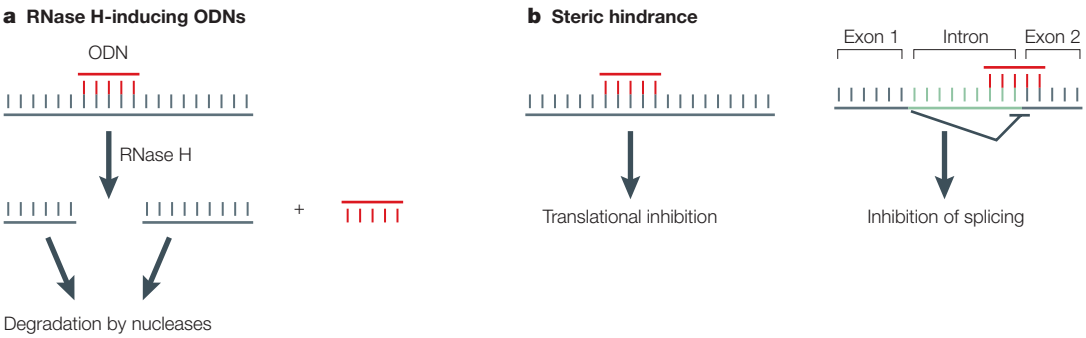

Figure 2 | **Mechanisms of nucleic-acid-based approaches for gene silencing: antisense compounds.** Two mechanisms by which antisense compounds sequence-specifically alter gene expression. Oligodeoxyribonucleic acids (ODNs) can be introduced into the cell through experimental manipulation. The antisense molecules can hybridize to either mRNA or pre-mRNA. The RNA strand of DNA–RNA duplexes is degraded by RNase H. Certain chemically modified antisense molecules complexed with RNA are not recognized by RNase H. These types of compound can be used to inhibit translation of mRNAs or inhibit or alter splicing pathways of pre-mRNAs.

into 21- to 28-nucleotide siRNA duplexes that contain 2-nucleotide 3′ overhangs with 5′ phosphate and 3′ hydroxyl termini (FIG. 4). Components of the RNAi machinery specifically recognize the siRNA duplex and incorporate a single siRNA strand into a protein complex[32] termed the RNA-induced silencing complex (RISC)[10]. RISC cleaves mRNAs containing perfectly complementary sequences, 10 nucleotides from the 5′ end of the incorporated siRNA strand[12]. Like ribozymes, siRNAs can be synthetically produced or expressed from vectors transcribing short double-stranded hairpin-like RNAs that are processed into siRNAs inside the cell. Unlike ODNs and ribozymes, siRNAs cannot effectively target pre-mRNAs for degradation in mammalian cells[33]. Evidence exists that several organisms use RNAi-related mechanisms to also target CHROMATIN modifications and transcriptionally silence genes[34–44].

siRNAs resemble non-coding RNA molecules termed microRNAs (miRNAs) that are naturally used by cells to regulate gene expression[45,46]. A mature miRNA is a single-stranded molecule of 21- to 22-nucleotides that is excised in the cytoplasm from a 70-nucleotide hairpin precursor[47]. The mature miRNAs are incorporated into a protein complex (miRNP) that associates with ribosomes and inhibits translation of mRNAs containing sequences partially complementary to the miRNA in their 3′ untranslated region (UTR)[48–52]. If presented with a substrate with perfect complementarity, an miRNA molecule can act like an siRNA and guide multiple rounds of mRNA degradation[53].

Comparison of gene-silencing approaches

Several groups have compared different aspects of gene silencing mediated by ODNs and siRNAs in tissue culture models[54–60]. Drawing conclusions from these studies is not straightforward, because the effectiveness of gene silencing depends on the concentration of silencing reagent, transfection technique, cell type, target site selection, chemical modifications and the time point at which data were analysed. None of the analyses conducted so far has taken all of these parameters into consideration. The finding that the sequence of an siRNA molecule itself affects silencing efficiency independently of target site accessibility further complicates comparison of ODNs with siRNAs[61–63].

How crucial target accessibility is for the various gene silencing techniques remains a matter of debate. RNA-binding proteins and extensive secondary or tertiary structures within mRNA are suggested to interfere with the hybridization of ODNs to their target RNA molecules. Several groups have investigated whether these variables also affect the efficiency of siRNAs[56,58,59,63]. Most of these studies have found a direct correlation between the efficiency of an ODN and an siRNA relative to the target position on mRNA. All studies except two[59,64] have also suggested that siRNAs are far more potent and longer-lasting than various types of ODN[55–58,60]. It is estimated that the half-maximal inhibition levels (IC_{50}) of siRNAs are some 100- to 1,000-fold lower than an optimal phosphorothioate-modified oligodeoxynucleotide[65] directed against the same target[55–57]. Although a systematic and extensive comparison of the gene silencing efficiency mediated by ribozymes and/or DNAzymes and siRNAs has yet to be done, several experiments have indicated that siRNAs are also more effective than ribozymes and DNAzymes[66,67]. Long hairpin loops that seem to silence gene expression by RNAi are also more potent than hammerhead ribozymes[68].

All three major approaches for targeting mRNA degradation have the potential for nonspecific effects on gene expression. ODNs, especially when phosphorothioate-modified, can be toxic because they act non-specifically by binding endogenous proteins[69]. The high concentration at which ODNs must be used to elicit gene-silencing activity further compounds this problem. ODNs with the CpG motif have also been shown to induce expression of IFNs or other innate immune responses through the binding of Toll-like receptors (TLRs)[70–72]. This nonspecific property of ODNs has actually been discovered to be the reason for the therapeutic properties of several successful ODNs[73,74].

CHROMATIN
Complex of DNA, histones and non-histone proteins from which eukaryotic chromosomes are formed.

Because ribozymes, like ODNs, hybridize to their targets without assistance, relatively high concentrations are also needed to silence genes, and unspecific effects can occur, especially when the ribozymes are chemically modified. The use of RNA localization signals or RNA chaperones can overcome this problem, allowing for potent silencing with a relatively low concentration of ribozyme[75]. Recent data have demonstrated that humans and mice express TLRs that are activated by uridine/guanosine- and uridine-rich single-stranded RNA oligonucleotides, respectively[76,77]. Activation of these TLRs by single-stranded RNA seems to occur in the endosomal compartment of plasmacytoid dendritic cells, and results in the expression of IFN-γ and other cytokines. If chemically modified siRNAs or ribozymes delivered *in vivo* are endocytosed and denatured, they can, depending on the siRNA sequence, activate these particular TLRs. This potential side effect could, like CpG motifs in antisense ODNs, be beneficial for therapy of viral infections or cancer.

The low concentration of siRNA required to elicit effective gene silencing, and the fact that siRNAs are specifically and rapidly incorporated into RISC, diminishes the potential for the nonspecific binding of proteins. Indeed, several reports have demonstrated that transfection of siRNAs at moderate concentrations does not cause global nonspecific effects on gene expression[78–80]. Three recent reports, however, have demonstrated that the application of RNAi in mammalian cells can affect gene expression nonspecifically, depending on siRNA concentration, cell type, delivery reagent and mode of siRNA expression[81–83]. These nonspecific effects include the stimulation of subsets of genes involved in the IFN response, although the induction of IFN response genes in these studies did not cause cellular growth arrest, as would be expected if a true IFN response were activated. In agreement, the microarray gene profiles of HeLa cells transfected with long dsRNA, or treated with IFN type 1 or a high concentration of luciferase siRNA (200 nM), only partly overlap[81]. These studies describe the potential for side effects in the application of siRNAs in therapeutics and investigative applications, and emphasize the importance of identifying effective siRNAs so that the lowest possible concentration of siRNA is used for gene silencing. It will be interesting to see if mice treated with short-hairpin-RNA-producing vectors, or siRNAs, display induction of IFN-response genes.

Besides their nonspecific effects, nucleic-acid-based gene-silencing molecules are also prone to inducing off-target effects by targeting sequences closely related to the target of interest. The level of off-target effect is dependent on the mode of silencing and the stability of the nucleic acid hybrid. ODNs are particularly likely to induce off-target effects, because as few as six or seven contiguous DNA/RNA base pairs can be recognized by RNase H[84]. To circumvent this problem, antisense oligonucleotide gapmers were developed, resulting in only one stretch of ~10 nucleotides of ODNs that can elicit an RNase H response[85]. Detailed investigations of how siRNAs function have revealed artefacts caused by unintentional targeting of mRNAs. If siRNAs are not carefully selected, siRNAs having partial complementarity to an mRNA target can act like endogenous miRNAs and repress translation[86–88] or subject mRNAs to degradation[78]. The latter study, which compared the gene-expression profiles created by different siRNAs targeted against the same transcript, revealed that in extreme cases as little as 11- to 14-nucleotide complementarity between the 5′ end of either siRNA strand to an mRNA can cause a reproducible reduction in transcript levels[78]. Phenotypes identified in RNAi screens should later be confirmed with different siRNAs targeting the same transcript[89]. If antisense sequences are carefully selected, ODNs, ribozymes, DNAzymes and siRNAs are able to selectively target a particular allele that differs from another by as little as a single nucleotide[66,90–94]. The remainder of this review will focus on aspects of siRNA-mediated gene silencing.

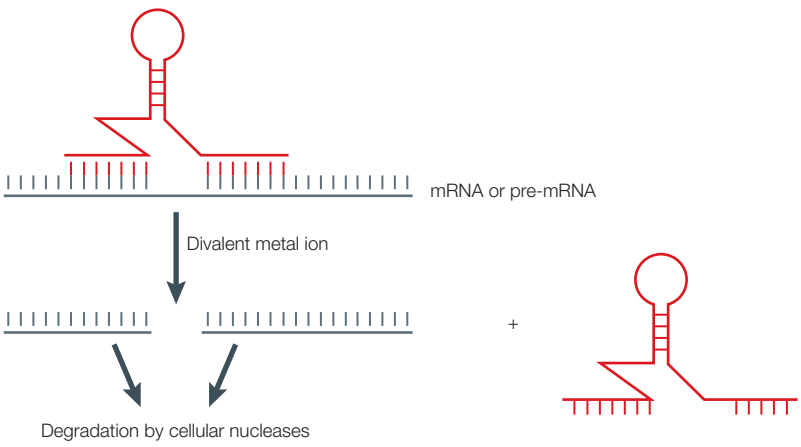

Figure 3 | **Mechanisms of nucleic-acid-based approaches for gene silencing: ribozymes.** General mechanism by which ribozymes silence gene expression. Ribozymes can be produced within the cell through transcription or can be directly introduced into the cell through experimental manipulation. For the hammerhead ribozyme, two arms are used to direct the catalytic centre to target the hydrolysis of the phosphodiester backbone of the mRNA.

Vectors for production of siRNAs

After the discovery of catalytic RNA, and the very small hammerhead ribozyme in particular, synthetic ribozyme-based therapeutics were intensively explored. Because small RNA molecules can either be chemically synthesized or expressed from DNA vectors, they were also examined as targeting reagents in gene therapy. The development of vectors that produce hammerhead ribozymes from an RNA polymerase III promoter (pol III) facilitated the development of similar vectors for production of siRNAs[95]. Production of siRNAs from a vector has predominantly been done through the transcription of a hairpin RNA that structurally mimics an miRNA precursor, allowing it to be processed into an siRNA inside the cell. TABLE 1 provides a comparison of the advantages and disadvantages of vectors versus synthetic siRNAs.

Vectors can stably integrate into the genome and mediate the long-term knockdown of endogenous transcripts in cell culture and *in vivo*. Several groups have developed adenoviral[96–99], adeno-associated viral

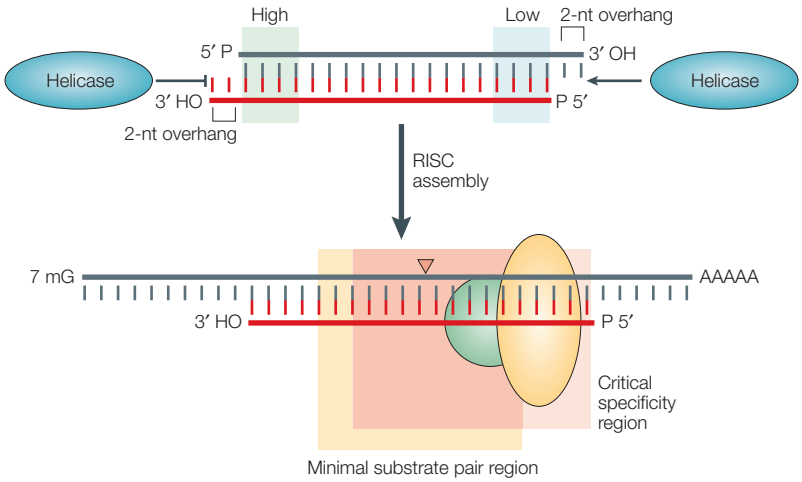

Figure 4 | **Features of efficient and specific siRNAs.** The thermodynamic stability of the first few base pairs of either siRNA strand can affect the ratio of RISCs containing the antisense (red line) or sense strands of siRNAs. The relatively low thermodynamic stability (blue shaded box) in the 5′ end of the antisense strand compared with the high thermodynamic stability (green shaded box) in the 5′ end of the sense strand leads to a bias for the incorporation of the antisense strand into RISC. More RISCs containing antisense strands means a more effective siRNA and also diminishes the chance of off-target effects caused by the sense strand. The 5′ half of siRNAs have a more significant role in target recognition than the 3′ half. As few as 11 to 14 contiguous base pairs from the 5′ end of the siRNA and an mRNA have been observed to target gene silencing. The minimal substrate for a siRNA observed so far is comprised of the central 13 nucleotides (J. Martinez, personal communication) The orange triangle indicates the site of mRNA cleavage. nt, nucleotide; RISC, RNA-induced silencing complex; siRNA, small interfering RNA.

(AAV)[100,101], retroviral[102] and lentiviral vectors[103–105] that initiate RNAi in transduced tissue culture cells and *in vivo* by transcription of a hairpin RNA from a pol II or pol III promoter. These viral vectors could one day be applied as an alternative mode of gene therapy (see below). To increase their utility for cell culture studies, vectors that mediate inducible pol III expression of siRNAs were developed[103,106–108]. The development of a pol-II-based vector that can produce a several-hundred-base-pair hairpin RNA *in vivo* without inducing the IFN response has provided an alternative method for RNAi in mammals, while also permitting the creation of tissue-specific 'knockdown' mice[109]. To overcome induction of the IFN response owing to the presence of long dsRNA in the cytoplasm, efficient export of the RNA to the cytoplasm is prevented.

In vivo delivery of siRNAs

ODNs and ribozymes have been successfully delivered *in vivo* using a variety of strategies. Intravenous injection is currently the most popular mode of delivery of ODNs in ongoing clinical trials. Successful delivery of siRNAs, siRNA-producing plasmids or siRNA-producing viruses into mammalian model organisms has been carried out using various methods. These methods include electroporation[110–113] and both local[96,101,114–116] and systemic injection[96,115,117–123] procedures. It is difficult to make generalizations about which delivery method leads to the most effective silencing, however, because different tissues have different requirements for effective delivery, especially for animals of different sizes.

High-pressure tail vein injection of siRNAs in physiological solution was the first procedure to successfully deliver siRNAs into highly vascularized mouse tissue[115,117–119,121–123], causing up to 90% reduction in target gene expression in the liver and, to a lesser extent, in the lung, kidney, spleen and pancreas. The silencing is transient, in certain cases lasting more than a week, and the levels of silencing are not absolute because there is significant animal-to-animal variation.

Development of siRNA-producing viruses holds great promise as an alternative mode of gene therapy for DOMINANT human diseases, as well as for studying gene function in mammalian model systems[96,101,102,104]. Several different types of virus have been engineered to produce siRNAs. Recombinant AAV can mediate the delivery and long-term expression of a transgene in both dividing and non-dividing mammalian cells. The virus is mostly found in an episomal form that integrates randomly and at a low frequency into the host genome. Incredibly, injection of siRNA-producing AAV into mouse brain resulted in effective silencing near the injection site for up to seven weeks after infection[101]. Delivery of siRNA-producing adenovirus to mouse liver by injection into the tail vein, or to mouse brain by direct injection, has also resulted in effective silencing of gene expression[96]. siRNA-producing lentiviruses that are able to transduce non-dividing cells and that escape transcriptional silencing during development have been used to deliver siRNAs into embryonic stem cells to create knockdown mice (see below)[104]. At the moment, there is no obvious reason why siRNA-producing viral vectors cannot be applied to gene therapy by using a strategy similar to that used to deliver ribozymes for the treatment of HIV currently in Phase I and II clinical trials[124]. siRNAs have been shown to successfully target HIV in tissue culture models[125].

If siRNAs are to be used for therapeutic purposes, methods must be developed that will allow the gentle delivery of siRNAs *in vivo*. Such methods, although still imperfect, have been developed for the delivery of ODNs[126], including ingestion of chemically modified ODNs[127,128], one of which has been given Orphan Drug status by the US FDA[129]. The recently discovered small molecules that enhance the transdermal penetration of several macromolecules[130], including ODNs, could potentially be used for the systemic delivery of siRNAs through a transdermal patch. Aerosol methods similar to those used for gene delivery in the lungs[131] might also be used for the gentle delivery of siRNAs in the near future. It remains to be seen whether and which chemically modified siRNAs enhance *in vivo* delivery. In order not to be limited to the current repertoire of ODN and ribozyme chemical modifications, new types of chemical modification are currently being developed for siRNAs.

siRNAs to investigate gene function *in vivo*

RNAi is a promising tool for mouse and rat gene function analysis, and has allowed for the creation of knockdown mice that in certain contexts offer advantages over the classical method of homologous recombination

DOMINANT DISEASE
A disease caused by a dominant genetic mutation.

Table 1 | **Vector-based versus synthetic siRNA-based RNAi in mammalian cells**

Approach	Advantages	Disadvantages
Vector-based	Delivery to non-transfectable cells. Stable silencing for non-essential genes. Inducible expression. Enzymatic preparation of hairpin libraries using cDNAs and cDNA libraries. Flexibility of shuttling of hairpin insert between different vectors (for example, between lenti-, retro- or adenoviral expression vectors; variation of promoters). Stable positive-readout screening using complex polyclonal libraries.	Prone to nonspecific interferon-response-related effects caused by high expression of hairpin RNA. Difficult to select and construct highly effective hairpin RNAs. Decreased potential for systemic delivery in therapeutic applications.
siRNA-based	Less prone to induce nonspecific side effects due to greater control over amount of transfected reagent. Ease of chemical synthetic production and quality control. Small size and chemical modifications hold best potential for therapeutic applications. Useful for structural functional studies of RNAi machinery.	Duration of silencing is dependent on rate of cell division.

The overall costs for both approaches are similar if arrayed libraries are produced targeting individual genes. RNAi, RNA interference; siRNA, small interfering RNA.

(FIG. 5). The silencing mediated by RNAi constructs in mice is stably passed on through the germline[132]. With RNAi, one could easily target particular splice variants of a gene for destruction[63]. Multicopy genes that are functionally redundant can theoretically all be knocked-down with one transgene construct. By targeting a conserved domain, an entire gene family can be knocked down. RNAi can also overcome the current difficulties in creating double-knockout mice of two genes that are in close proximity on the same chromosome.

siRNAs have also broadened the horizons of the types of experiments that can be done in mammalian model systems. For example, to determine the relative amount of gene product needed for certain processes at particular developmental stages, it is now possible to modulate gene dosage in a spatial and temporal manner by simply varying the amount of siRNA expressed in the cell[133]. Current difficulties in creating temporally and spatially restricted knockout mice include identifying regulatory regions that can express recombinase proteins in the desired patterns. Several groups have used RNAi to rapidly circumvent this problem by the local injection or electroporation of siRNA-producing plasmids or viruses[96,101,112,134].

The refinement of selection methods for effective and specific hairpins, as well as the refinement of expression and delivery techniques for siRNAs, will make mouse knockdowns a useful technique for future research. It should, however, be cautioned that it is uncertain whether long-term expression of siRNAs in mice can cause side effects. Long-term expression of high levels of hairpin RNAs could, in theory, compete with endogenously expressed miRNAs for incorporation into miRNPs.

siRNAs as tools for genome-wide screening

RNAi has become the preferred approach for functional genomics in several model systems[135]. Several near-genome-wide RNAi screens have been conducted using long dsRNA in *C. elegans*[136–146] and *Drosophila melanogaster*[147–149]. These screens have identified genes involved in fundamental processes such as cell division, apoptosis and cell morphology, and physiological processes such as fat metabolism.

Until recently, genetic screens were mostly limited to non-mammalian model organisms, and only a few non-saturating genetic screens had been conducted in mice[150–154]. This prompted the use of other vertebrate model organisms, such as zebrafish, in which SATURATING GENETIC SCREENS could be carried out relatively easily and affordably. Current reverse genetic approaches for studying embryonic development in zebrafish use antisense molecules, termed morpholino phosphorodiamidates[18], because RNAi using long dsRNA does not seem to work in zebrafish[155], even though zebrafish do contain miRNAs[156]. With the advent of siRNAs, it is now feasible to carry out reverse genetic screens in mammalian tissue culture cells, *Xenopus* oocytes[157], chicken embryos[158] and, potentially, mouse and rat embryos[112,159,160]. It is not established whether siRNAs are functional in zebrafish. Such screens will bypass the time-consuming task of identifying and validating clear mammalian homologues, as well as providing a means to easily discern new mechanisms that are specific to mammals. It has finally become feasible to conduct a relatively rapid identification of human-specific processes through targeting of human-specific genes in different cell types.

Reverse genetic approaches that used either ODNs or ribozymes to reduce the expression of specific genes have proven successful for use in drug target validation, but have never passed the hurdle of genome-wide target identification. RNAi has become the preferred approach for functional genomics in mammalian tissue culture. There are several reasons for this choice, including the high gene-silencing efficiency at low concentration, ease of finding accessible target sites, high specificity, good stability and custom siRNA synthesis at moderately low cost. Several large-scale RNAi screens have been conducted in mammalian tissue culture cells using synthetic siRNAs[161] or hairpin expression[162–164]; these screens have identified genes involved in apoptosis, signalling, regulation of protein stability and the ultraviolet radiation damage response.

SATURATING GENETIC SCREEN
A screen of sufficient scale to identify all possible target genes.

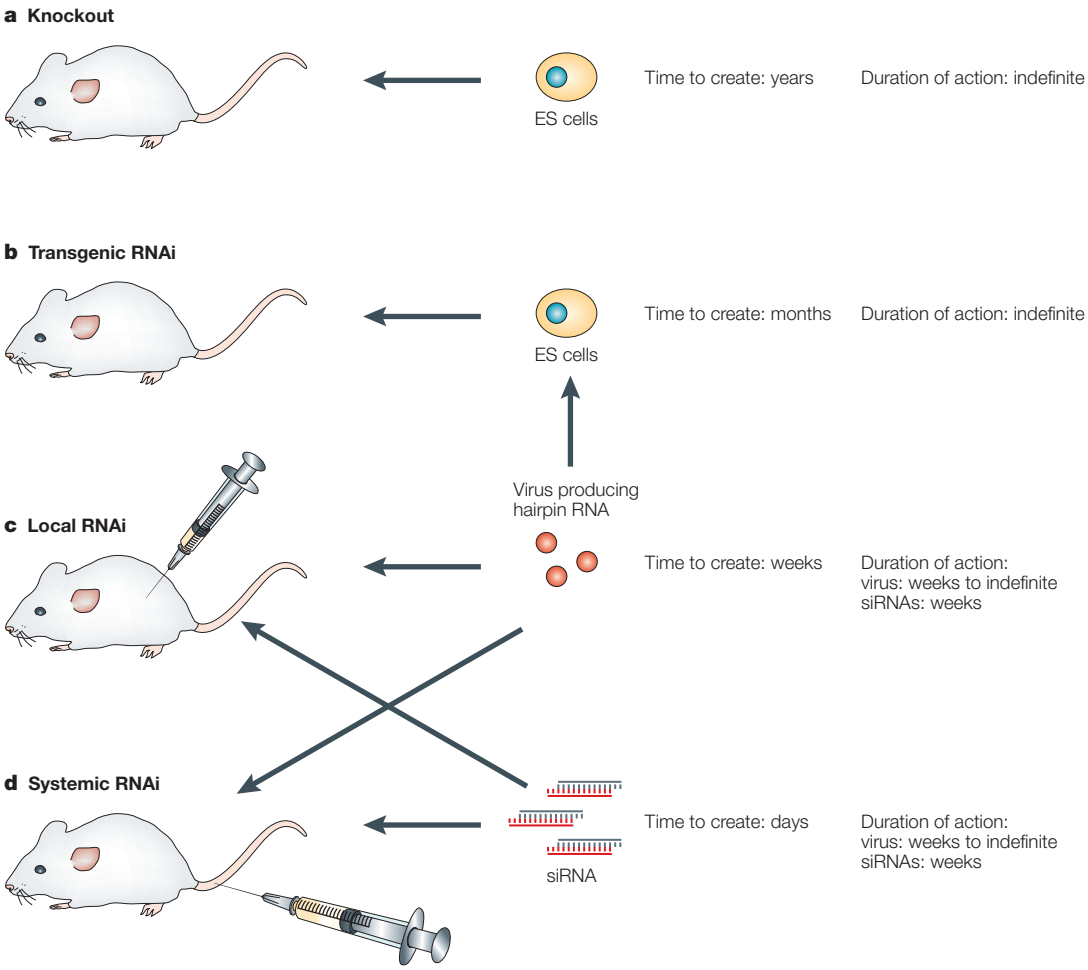

Figure 5 | ***In vivo* mammalian gene silencing.** The figure outlines several methods of gene silencing in the mouse, and compares their time of preparation and duration of action. ES, embryonic stem; RNAi, RNA interference; siRNA, small interfering RNA.

The potential of genome-wide screening by RNAi in mammals for identifying new therapeutic drug targets is only limited by the types of screens one can do: a mammalian RNAi-based screen can be carried out for any process for which a tissue culture model exists. As with classical genetic approaches, modifier screens, which look to identify suppressors or enhancers of particular processes, can be conducted, depending on the experimental setup. The mammalian siRNA-based screens conducted so far have also accommodated the use of extracellular agents to induce particular processes. For the most part, RNAi-based screening in mammalian cells has been limited to easily transfectable, rapidly dividing adherent cell types. However, one could imagine conducting a screen for factors that mediate cellular differentiation using totipotent or pluripotent stem cell lines. Electroporation techniques currently used to deliver siRNAs to non-adherent cells could potentially be used for high-throughout screening (HTS)[165]. With regard to electroporation, siRNAs are preferable to hairpin-producing vectors, as the conditions for effective delivery of siRNAs are milder than for plasmids and result in less cell death[166]. New modes of siRNA delivery could arise from the identification of siRNA chemical modifications that facilitate uptake in primary and/or non-adherent cells. Future small-molecule drug screens might identify molecules that stimulate siRNA uptake in particular cell types. Viral vectors that are able to transduce primary cells have recently been applied as a mode of delivery for RNAi-based screens[164]. With the development of such technologies, the rate-limiting steps for RNAi-based screening will soon be screen design and data analysis.

RNAi is proving helpful in the validation of potential drug targets identified by the use of cDNA microarrays[167,168]. Large amounts of microarray data have been produced in an effort to identify genes whose expression in disease tissue deviates from that of normal-tissue gene expression. These microarray studies usually identify hundreds, if not thousands, of genes that have altered expression, making it difficult to identify the relevant drug targets; siRNAs designed to target genes that are overexpressed in disease tissue can now be used to rapidly identify suitable drug targets for a particular disease.

The current rate-limiting step for genome-wide screening in mammalian cells is resource availability. Technologies that combine 96- or 384-well plate formats,

Table 2 | **Terminal modifications of siRNA duplexes**

Modification	Gene silencing	Cell system	References
Sense strand 5′ or 3′ termini			
Aminolinker	++++	HeLa, HeLa extract	32,189,193
Puromycin, biotin	++++	HeLa	193
Fluorescein	++++	HeLa	63
Antisense strand 3′ terminus			
Aminolinker	++++	HeLa, HeLa extract	32,189
Puromycin, biotin	++++	HeLa	193
Fluorescein	+++	HaCaT	194
Fluorescein, Alexa488	–	HeLa	63
Inverted 2′-deoxy abasic cap	++++	HeLa	189
Antisense strand 5′ terminus			
Aminolinker	–	HeLa, HeLa extract	32,189,193
Fluorescein	++++	HeLa	63
Inverted 2′-deoxy abasic cap	–	HeLa	189

Scale of the silencing effect as compared with the efficiency of unmodified siRNA duplex: –, modification rendering the duplex inactive; +, 20–40%; ++, 40–60%; +++, 60–80%; ++++, >80% of efficiency of unmodified duplex. siRNA, small interfering RNA.

cationic transfection, robotics and image recognition software are already being used to conduct near-genome-wide screens in mammalian tissue culture cells. New types of hardware platforms for the HTS process are also being developed. Most recently, microarray chips have been developed that use plasmids or siRNAs spotted on glass slides and reverse transfection to allow for rapid screening of mammalian cells[169–171]. The drawbacks of RNAi microarrays are that only adherent cells can be analysed, the cationic transfection reagent used is restricted to specific cell types and technology must be developed to allow the long-term storage of RNAi microarrays.

As the probability of having off-target effects increases with genome size, the importance of careful siRNA design increases. Recent experimental findings[61,62,172–174] have aided in the development of siRNA libraries by refining the standard parameters[175] for selecting effective siRNAs (FIG. 4). All of these findings converge on the conclusion that the thermodynamic stability of the first few base pairs of both ends of an siRNA duplex are crucial for determining which siRNA strand will be incorporated into RISC. Several groups are designing methods for the high-throughput development of hairpin-producing plasmid libraries[162,176,177]. Although the parameters for selecting effective siRNAs could also be applied for construction of hairpin vectors, they might be less reliable because the positions at which Dicer RNase III cleaves a hairpin are not well defined. Following the refined parameters for siRNA design does not, however, guarantee an effective siRNA, because the target position within a mRNA might also affect silencing efficiency[56,59,63]. Besides good siRNA design, pooling of effective siRNAs against the same target also decreases off-target effects. The pooling strategy dilutes out the effect of any siRNAs having off-target effects, while keeping the total of target-gene-specific siRNAs constant. Pooling of siRNAs that are randomly generated *in vitro* from Dicer cleavage of long dsRNA would also have this advantage, although the fraction of effective siRNAs cannot be controlled[178]. siRNAs produced with this procedure should be further purified, to remove small amounts of unprocessed dsRNA that could induce the IFN response.

Although screening by RNAi is relatively fast and easy, it has several disadvantages when compared with classical genetic screens. Most significantly, classical genetic screens can identify mutations that are not in coding regions. Classical genetic screens can also produce dominant-negative or gain-of-function mutations, which are often useful, and sometimes essential, for understanding gene function. To overcome some of these pitfalls, arrayed adenovirus cDNA expression libraries (knock-ins) are being used in combination with arrayed adenovirus libraries that express short hairpin RNAs[97]. Another pitfall of screening with RNAi is that siRNAs almost never fully deplete the target mRNA and usually several different siRNAs must be screened before an effective siRNA is identified. Transient RNAi in rapidly dividing tissue culture cells usually lasts three to five days. However, even if applied to slowly dividing cells, it is possible that an effective siRNA will have difficulty in depleting a stable protein. Another layer of complexity to consider when designing siRNA-based screens is cell type. The amount of available RISC can vary between cell types, possibly reflecting the relative levels of endogenous miRNAs competing for the RNAi machinery, and is a limiting factor for RNAi efficiency[63,139]. In the long term, the identification of specific and effective siRNAs for each gene will help to overcome some of these problems.

Mammalian RNAi-based genomic screens offer great opportunities. The institutions that are developing platforms for high-throughput, genome-wide RNAi screens in mammalian cells will have a competitive advantage in biomedical research. Complementing these siRNA-based screens with proteomic methods will yield a relatively descriptive outlook on particular cellular processes that can be further studied.

siRNA-based therapeutics

Several ODN and ribozyme molecules are already being tested in clinical trials, and one antisense ODN — fomivirsen (Vitravene; Isis) — has been approved by the US FDA for the treatment of cytomegalovirus infection of the eye. So far, most of the antisense oligonucleotides in clinical trials are phosphorothioate-modified ODNs[65] or phosphorothioate-modified ODN gapmers, which have problems such as toxicity at high concentration and a low affinity for their target RNAs. Several second-generation antisense constructs containing additional types of chemical modifications are also currently in clinical trials and are predicted to do better than their phosphorothioate ODN predecessors. A number of recent reviews have covered these different drugs and their targets, so they will not be discussed here further[23,124,126].

As siRNAs and their functionality in mammalian cells were discovered only three years ago, they have not yet had time to enter clinical trials. There is, however, no

Table 3 | **Modification of the ribose 2′ position**

Modification	Position	Gene silencing	Cells	References
3′ overhangs				
2′-deoxy		++++	HeLa S3, HEK 293, COS-7, NIH3T3, HaCaT, HeLa	13,175,189,194
2′-Ome		++++	HaCaT	191
2′-Oal		++++	HaCaT	191
LNA		++++	HeLa	192
5′ phosphate		++++	HaCaT	191
Base-paired region				
2′-deoxy	Fully modified s	+	HeLa	190
2′-deoxy	Fully modified as	–	HeLa	190
2′-Ome	2 to 4 nt terminal nt of both strands	++++	HaCaT	191
2′-Ome	50% nt	+++/++/+*	HaCaT, HeLa	189,195
2′-Ome	Fully modified s	+/–	HeLa	189,190
2′-Ome	Fully modified as	–	HeLa	189,190
2′-Oal	1 nt at both 5′ ends	+++	HaCaT	191
LNA	4 to 8 nt of both strands	++++ to – *	HeLa	191
LNA	1 nt at both 5′ ends	++++	HeLa	192
2′-fU, 2′-fC	One or both strands	++++	293T, CD4+ T, HeLa	63,190,192,196
2′-fU, 2′-fC and 2′-deoxy	3 to 13 2′-deoxy nt in as strand	++++/+++/++*	HeLa	190
2′-fU, 2′-fC and pS	3 pS linkages at both 3′ ends	++++	HeLa	63
2′-fU, 2′-fC and pS	Fully pS modified as	+	HeLa	190
pS	25–50% linkages	++++	HeLa, HaCaT, SW3T3	63,191,192
pS	Both strands	+++/++	HeLa	190,192

*The effect is dependent on the position of modifications within the siRNA duplex. Scale of the silencing effects is the same as described in TABLE 2. 2′-Ome, 2′-*O*-methylribose; 2′-Oal; 2′-*O*-allylribose; 2′-fU, 2′-fluoro-2′-deoxyuridine; 2′-fC, 2′-fluoro-2′-deoxycytidine; as, antisense strand of siRNA duplex; LNA, locked nucleotides (2′-*O*,4′-methylene nucleotides); pS, phosphorothioate internucleotidic linkage; s, sense strand of siRNA duplex; siRNA, small interfering RNA.

obvious scientific reason why siRNAs will not be used as therapeutics with strategies similar to those that are now used for ODNs and ribozymes. siRNAs are rapidly catching up with ODNs and ribozymes for development as therapeutics after the establishment of siRNA-based biotechnology companies that focus on the development of clinical programmes[179]. Several proof-of-principle experiments have demonstrated the therapeutic potential of siRNAs: siRNAs protected mice from fulminant hepatitis[121,122], viral infection[123,180], sepsis[115], tumour growth[181–185] and ocular neovascularization causing macular degeneration[114].

Given that siRNAs delivered by high-pressure tail vein injection are most effective in the mouse liver, several groups have tested the potential of siRNAs as therapeutic agents for a wide variety of liver diseases. By targeting endogenous genes expressed in the liver that mediate apoptosis, mice pre-treated with siRNAs targeting either caspase 8 (REF.121) or the FAS cell death receptor[122] were protected from acute liver failure induced by a variety of reagents. The treatment of mice with the same siRNAs after insult of the liver by apoptosis-inducing reagents also protected mice from liver breakdown. Other groups have successfully demonstrated the therapeutic potential of siRNAs for the treatment of hepatitis B virus (HBV) infection by directly targeting the virus[119,120,123]. A replication-competent HBV genome was co-delivered with siRNAs targeting portions of the HBV genome to effectively reduce viral replication and protein production. Although promising, it has yet to be demonstrated whether siRNAs can effectively reduce virus levels when applied to a real, ongoing infection. These results demonstrate the therapeutic potential of siRNAs and should stimulate research into delivery methods that are also suitable for therapeutic applications.

Optimizing the effectiveness of nucleic-acid-based gene silencing *in vivo* requires that numerous parameters be considered. The silencing molecule must be stable in the circulatory system as well as in tissues, and should bind blood proteins to a degree that is non-toxic, but that prevents immediate loss of the molecule through excretion. Much effort has been put into identifying chemical modifications of nucleic acids that decrease their susceptibility to nuclease attack, while allowing them to maintain gene-silencing activity sufficient for therapeutic use[57,186,187]. The compromises that need to be made for systemic delivery are best illustrated for the

phosphorothioate-modified ODNs currently in clinical trials. Even though the modification decreases the affinity of the ODN to its target RNA, it increases the effectiveness of the molecules *in vivo* by increasing their stability, retention, cellular uptake and biodistribution. This is because phosphorothioate modifications increase the affinity of ODNs to blood proteins and also prevent the direct action of nucleases that would otherwise degrade the ODNs[188].

siRNA duplexes are protected from single-strand-specific endonucleases, making them more stable than either ODNs or ribozymes in serum[60]. However, because stability in sera does not always translate to stability in the blood, and because unmodified siRNAs are not readily taken up by cells nor have a sufficient affinity for blood proteins, siRNAs must also be chemically modified if they are to be used for therapeutic purposes without a gene-therapy-based platform that includes the use of viruses.

The modification of siRNAs could interfere with incorporation of the siRNA into RISC, unwinding of the siRNA duplex by helicase activities and/or the rate of target cleavage and product release. Several groups have attempted to identify chemical modifications that increase stability of siRNAs while maintaining good silencing efficiency[63,175,189–192]. TABLES 2 and 3 summarize the various chemical modifications of siRNAs and their effect on gene silencing. Of note, phosphorothioate modifications are well tolerated within siRNA duplexes, suggesting that cells will take up these types of siRNAs, similarly to their ODN and ribozyme counterparts. There are, however, no reports on the effectiveness of chemically modified siRNAs *in vivo*. A high potential for siRNAs as therapeutic agents has initiated efforts to develop new types of nucleic acid chemical modifications, some of which are specific to siRNA structure. Numerous clinical trials involving therapeutic siRNAs are anticipated in the near future.

Conclusion

We are in the dawn of a new age in functional genomics driven by RNAi methods. Although there are technical challenges associated with the therapeutic application of siRNAs, such as synthesis, delivery and specificity, they currently offer numerous advantages over other gene-silencing approaches. The siRNA approach for gene silencing holds great therapeutic promise, as siRNAs, like miRNAs, are naturally used by cells to regulate gene expression and are therefore non-toxic and highly effective. One potential drawback of using siRNAs for therapeutics is that if used long term, siRNAs could theoretically out-compete the function of endogenous miRNA genes in certain cell types.

The years of research done on antisense therapeutics will greatly facilitate the development of therapeutic siRNAs. Further research into the fundamental mechanisms of RNAi could unveil new dimensions of siRNA-mediated gene silencing that will have profound implications for understanding gene regulation, and which could also affect the development of functional genomics and therapeutic applications.

1. Elbashir, S. M., Lendeckel, W. & Tuschl, T. RNA interference is mediated by 21 and 22 nt RNAs. *Genes Dev.* **15**, 188–200 (2001).
2. Hamilton, A. J. & Baulcombe, D. C. A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* **286**, 950–952 (1999).
3. Fire, A. *et al*. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806–811 (1998).
The landmark paper describing the discovery of RNAi.
4. Montgomery, M. K., Xu, S. & Fire, A. RNA as a target of double-stranded RNA-mediated genetic interference in *Caenorhabditis elegans*. *Proc. Natl Acad. Sci. USA* **95**, 15502–15507 (1998).
5. Waterhouse, P. M., Graham, M. W. & Wang, M. B. Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. *Proc. Natl Acad. Sci. USA* **95**, 13959–13964 (1998).
6. Kennerdell, J. R. & Carthew, R. W. Use of dsRNA-mediated genetic interference to demonstrate that *frizzled* and *frizzled 2* act in the wingless pathway. *Cell* **95**, 1017–1026 (1998).
7. Ngo, H., Tschudi, C., Gull, K. & Ullu, E. Double-stranded RNA induces mRNA degradation in *Trypanosoma brucei*. *Proc. Natl Acad. Sci. USA* **95**, 14687–14692 (1998).
8. Cogoni, C. & Macino, G. Homology-dependent gene silencing in plants and fungi: a number of variations on the same theme. *Curr. Opin. Microbiol.* **2**, 657–662 (1999).
9. Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, R. H. & Schreiber, R. D. How cells respond to interferons. *Annu. Rev. Biochem.* **67**, 227–264 (1998).
10. Hammond, S. M., Bernstein, E., Beach, D. & Hannon, G. J. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* **404**, 293–296 (2000).
11. Zamore, P. D., Tuschl, T., Sharp, P. A. & Bartel, D. P. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101**, 25–33 (2000).
12. Elbashir, S. M., Martinez, J., Patkaniowska, A., Lendeckel, W. & Tuschl, T. Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. *EMBO J.* **20**, 6877–6888 (2001).
13. Elbashir, S. M. *et al*. Duplexes of 21-nucleotide RNAs mediate RNA interference in mammalian cell culture. *Nature* **411**, 494–498 (2001).
The landmark paper describing the first application RNAi in mammalian cell culture.
14. Caplen, N. J., Parrish, S., Imani, F., Fire, A. & Morgan, R. A. Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. *Proc. Natl Acad. Sci. USA* **98**, 9742–9747 (2001).
15. Bitko, V. & Barik, S. Phenotypic silencing of cytoplasmic genes using sequence-specific double-stranded short interfering RNA and its application in the reverse genetics of wild type negative-strand RNA viruses. *BMC Microbiol.* **1**, 34 (2001).
16. Scherer, L. J. & Rossi, J. J. Approaches for the sequence-specific knockdown of mRNA. *Nature Biotechnol.* **21**, 1457–1465 (2003).
A comprehensive review comparing the different approaches for silencing of mRNA.
17. Braasch, D. A. & Corey, D. R. Novel antisense and peptide nucleic acid strategies for controlling gene expression. *Biochemistry* **41**, 4503–4510 (2002).
18. Heasman, J. Morpholino oligos: making sense of antisense? *Dev. Biol.* **243**, 209–214 (2002).
19. Schubert, S. *et al*. RNA cleaving '10–23' DNAzymes with enhanced stability and activity. *Nucleic Acids Res.* **31**, 5982–5992 (2003).
20. Chakraborti, S. & Banerjea, A. C. Inhibition of HIV-1 gene expression by novel DNA enzymes targeted to cleave HIV-1 TAR RNA: potential effectiveness against all HIV-1 isolates. *Mol. Ther.* **7**, 817–826 (2003).
21. Santoro, S. W. & Joyce, G. F. A general purpose RNA-cleaving DNA enzyme. *Proc. Natl Acad. Sci. USA* **94**, 4262–4266 (1997).
22. Fortes, P. *et al*. Inhibiting expression of specific genes in mammalian cells with 5′ end-mutated U1 small nuclear RNAs targeted to terminal exons of pre-mRNA. *Proc. Natl Acad. Sci. USA* **100**, 8264–8269 (2003).
23. Kurreck, J. Antisense technologies. Improvement through novel chemical modifications. *Eur. J. Biochem.* **270**, 1628–1644 (2003).
24. Baker, B. F. *et al*. 2′-*O*-(2-methoxy)ethyl-modified anti-intercellular adhesion molecule 1 (ICAM-1) oligonucleotides selectively increase the ICAM-1 mRNA level and inhibit formation of the ICAM-1 translation initiation complex in human umbilical vein endothelial cells. *J. Biol. Chem.* **272**, 11994–2000 (1997).
25. Lu, Q. L. *et al*. Functional amounts of dystrophin produced by skipping the mutated exon in the mdx dystrophic mouse. *Nature Med.* **9**, 1009–1014 (2003).
26. Uil, T. G., Haisma, H. J. & Rots, M. G. Therapeutic modulation of endogenous gene function by agents with designed DNA-sequence specificities. *Nucleic Acids Res.* **31**, 6064–6078 (2003).
27. Doudna, J. A. & Cech, T. R. The chemical repertoire of natural ribozymes. *Nature* **418**, 222–228 (2002).
A comprehensive review on ribozymes.
28. Kuwabara, T., Warashina, M. & Taira, K. Cleavage of an inaccessible site by the maxizyme with two independent binding arms: an alternative approach to the recruitment of RNA helicases. *J. Biochem. (Tokyo)* **132**, 149–155 (2002).
29. Michienzi, A. & Rossi, J. J. Intracellular applications of ribozymes. *Methods Enzymol.* **341**, 581–596 (2001).
30. Good, P. D. *et al*. Expression of small, therapeutic RNAs in human cell nuclei. *Gene Ther.* **4**, 45–54 (1997).
31. Bernstein, E., Caudy, A. A., Hammond, S. M. & Hannon, G. J. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**, 363–366 (2001).
32. Martinez, J., Patkaniowska, A., Urlaub, H., Lührmann, R. & Tuschl, T. Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. *Cell* **110**, 563–574 (2002).
33. Zeng, Y. & Cullen, B. R. RNA interference in human cells is restricted to the cytoplasm. *RNA* **8**, 855–860 (2002).
34. Wassenegger, M. & Pelissier, T. A model for RNA-mediated gene silencing in higher plants. *Plant Mol. Biol.* **37**, 349–362 (1998).
35. Mette, M. F., Aufsatz, W., van der Winden, J., Matzke, M. A. & Matzke, A. J. M. Transcriptional silencing and promoter methylation triggered by double-stranded RNA. *EMBO J.* **19**, 5194–5201 (2000).
36. Jones, A. L., Thomas, C. L. & Maule, A. J. De novo methylation and co-suppression induced by a cytoplasmically replicating plant RNA virus. *EMBO J.* **17**, 6385–6393 (1998).

37. Wang, M.-B., Wesley, S. V., Finnegan, E. J., Smith, N. A. & Waterhouse, P. M. Replicating satellite RNA induces sequence-specific DNA methylation and truncated transcripts in plants. *RNA* **7**, 16–28 (2001).
38. Llave, C., Kasschau, K. D., Rector, M. A. & Carrington, J. C. Endogenous and silencing-associated small RNAs in plants. *Plant Cell* **14**, 1605–1619 (2002).
39. Volpe, T. A. *et al*. Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. *Science* **297**, 1833–1837 (2002).
The landmark paper describing the link between RNAi and heterochromatin formation in *Schizosaccharomyces pombe*.
40. Reinhart, B. J. & Bartel, D. P. Small RNAs correspond to centromere heterochromatic repeats. *Science* **297**, 1831 (2002).
41. Hall, I. M. *et al*. Establishment and maintenance of a heterochromatin domain. *Science* **297**, 2232–2237 (2002).
42. Verdel, A. *et al*. RNAi-mediated targeting of heterochromatin by the RITS complex. *Science* **303**, 672–676 (2004).
43. Pal-Bhadra, M., Bhadra, U. & Birchler, J. A. RNAi related mechanism affect both transcriptional and posttranscriptional transgene silencing in *Drosophila*. *Mol. Cell* **9**, 315–327 (2002).
44. Pal-Bhadra, M. *et al*. Heterochromatic silencing and HP1 localization in *Drosophila* are dependent on the RNAi machinery. *Science* **303**, 669–672 (2004).
45. Carrington, J. C. & Ambros, V. Role of microRNAs in plant and animal development. *Science* **301**, 336–338 (2003).
46. Bartel, D. P. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**, 281–297 (2004).
A comprehensive review on miRNAs.
47. Lee, Y., Jeon, K., Lee, J. T., Kim, S. & Kim, V. N. MicroRNA maturation: stepwise processing and subcellular localization. *EMBO J.* **21**, 4663–4670 (2002).
48. Olsen, P. H. & Ambros, V. The lin-4 regulatory RNA controls developmental timing in *Caenorhabditis elegans* by blocking LIN-14 protein synthesis after the initiation of translation. *Dev. Biol.* **216**, 671–680 (1999).
49. Seggerson, K., Tang, L. & Moss, E. G. Two genetic circuits repress the *Caenorhabditis elegans* heterochronic gene lin-28 after translation initiation. *Dev. Biol.* **243**, 215–225 (2002).
50. Wightman, B., Ha, I. & Ruvkun, G. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in *C. elegans*. *Cell* **75**, 855–862 (1993).
51. Nelson, P. T., Hatzigeorgiou, A. G. & Mourelatos, Z. miRNP:mRNA association in polyribosomes in a human neuronal cell line. *RNA* **10**, 387–394 (2004).
52. Kim, J. *et al*. Identification of many microRNAs that copurify with polyribosomes in mammalian neurons. *Proc. Natl Acad. Sci. USA* **101**, 360–365 (2004).
53. Hutvágner, G. & Zamore, P. D. A microRNA in a multiple-turnover RNAi enzyme complex. *Science* **297**, 2056–2060 (2002).
54. Hemmings-Mieszczak, M., Dorn, G., Natt, F. J., Hall, J. & Wishart, W. L. Independent combinatorial effect of antisense oligonucleotides and RNAi-mediated specific inhibition of the recombinant rat $P2X_3$ receptor. *Nucleic Acids Res.* **31**, 2117–2126 (2003).
55. Miyagishi, M., Hayashi, M. & Taira, K. Comparison of the suppressive effects of antisense oligonucleotides and siRNAs directed against the same targets in mammalian cells. *Antisense Nucleic Acid Drug Dev.* **13**, 1–7 (2003).
56. Kretschmer-Kazemi Far, R. & Sczakiel, G. The activity of siRNA in mammalian cells is related to structural target accessibility: a comparison with antisense oligonucleotides. *Nucleic Acids Res.* **31**, 4417–4424 (2003).
57. Grunweller, A. *et al*. Comparison of different antisense strategies in mammalian cells using locked nucleic acids, 2′-*O*-methyl RNA, phosphorothioates and small interfering RNA. *Nucleic Acids Res.* **31**, 3185–3193 (2003).
58. Xu, Y. *et al*. Effective small interfering RNAs and phosphorothioate antisense DNAs have different preferences for target sites in the luciferase mRNAs. *Biochem. Biophys. Res. Commun.* **306**, 712–717 (2003).
59. Vickers, T. A. *et al*. Efficient reduction of target RNAs by small interfering RNA and RNase H-dependent antisense agents. A comparative analysis. *J. Biol. Chem.* **278**, 7108–7118 (2003).
60. Bertrand, J. R. *et al*. Comparison of antisense oligonucleotides and siRNAs in cell culture and *in vivo*. *Biochem. Biophys. Res. Commun.* **296**, 1000–1004 (2002).
61. Schwarz, D. S. *et al*. Asymmetry in the assembly of the RNAi enzyme complex. *Cell* **115**, 199–208 (2003).
62. Khvorova, A., Reynolds, A. & Jayasena, S. D. Functional siRNAs and miRNAs exhibit strand bias. *Cell* **115**, 209–216 (2003).
63. Harborth, J. *et al*. Sequence, chemical, and structural variation of small interfering RNAs and short hairpin RNAs and the effect on mammalian gene silencing. *Antisense Nucleic Acid Drug Dev.* **13**, 83–105 (2003).
64. Liu, Y., Braasch, D. A., Nulf, C. J. & Corey, D. R. Efficient and isoform-selective inhibition of cellular gene expression by peptide nucleic acids. *Biochemistry* **43**, 1921–1927 (2004).
65. Eckstein, F. Phosphorothioate oligodeoxynucleotides: what is their origin and what is unique about them? *Antisense Nucleic Acid Drug Dev.* **10**, 117–121 (2000).
Discusses the basic features and consequences of the most common chemical modification for ODNs.
66. Yokota, T. *et al*. siRNA-based inhibition specific for mutant SOD1 with single nucleotide alternation in familial ALS, compared with ribozyme and DNA enzyme. *Biochem. Biophys. Res. Commun.* **314**, 283–291 (2004).
67. Lee, N. S. *et al*. Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. *Nature Biotechnol.* **20**, 500–505 (2002).
68. Drew, H. R. *et al*. RNA hairpin loops repress protein synthesis more strongly than hammerhead ribozymes. *Eur. J. Biochem.* **266**, 260–273 (1999).
69. Lebedeva, I. & Stein, C. A. Antisense oligonucleotides: promise and reality. *Annu. Rev. Pharmacol. Toxicol.* **41**, 403–419 (2001).
70. Hafner, M. *et al*. Antimetastatic effect of CpG DNA mediated by type I IFN. *Cancer Res.* **61**, 5523–5528 (2001).
71. Roman, M. *et al*. Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. *Nature Med.* **3**, 849–854 (1997).
72. Rothenfusser, S., Tuma, E., Wagner, M., Endres, S. & Hartmann, G. Recent advances in immunostimulatory CpG oligonucleotides. *Curr. Opin. Mol. Ther.* **5**, 98–106 (2003).
73. Dove, A. Antisense and sensibility. *Nature Biotechnol.* **20**, 121–124 (2002).
74. Lai, J. C. *et al*. G3139 (oblimersen) may inhibit prostate cancer cell growth in a partially bis-CpG-dependent non-antisense manner. *Mol. Cancer Ther.* **2**, 1031–1043 (2003).
75. Tsuchihashi, Z., Khosla, M. & Herschlag, D. Protein enhancement of hammerhead ribozyme catalysis. *Science* **262**, 99–102 (1993).
76. Heil, F. *et al*. Species-specific recognition of single-stranded RNA via Toll-like receptor 7 and 8. *Science* **303**, 1526–1529 (2004).
77. Diebold, S. S., Kaisho, T., Hemmi, H., Akira, S. & Reis e Sousa, C. Innate antiviral responses by means of TLR7-Mediated recognition of single-stranded RNA. *Science* **303**, 1529–1531 (2004).
78. Jackson, A. L. *et al*. Expression profiling reveals off-target gene regulation by RNAi. *Nature Biotechnol.* **21**, 635–637 (2003).
79. Chi, J. T. *et al*. Genomewide view of gene silencing by small interfering RNAs. *Proc. Natl Acad. Sci. USA* **100**, 6343–6346 (2003).
80. Semizarov, D. *et al*. Specificity of short interfering RNA determined through gene expression signatures. *Proc. Natl Acad. Sci. USA* **100**, 6347–6352 (2003).
81. Persengiev, S. P., Zhu, X. & Green, M. R. Nonspecific, concentration-dependent stimulation and repression of mammalian gene expression by small interfering RNAs (siRNAs). *RNA* **10**, 12–18 (2004).
82. Sledz, C. A., Holko, M., de Veer, M. J., Silverman, R. H. & Williams, B. R. Activation of the interferon system by short-interfering RNAs. *Nature Cell Biol.* **5**, 834–839 (2003).
83. Bridge, A. J., Pebernard, S., Ducraux, A., Nicoulaz, A. L. & Iggo, R. Induction of an interferon response by RNAi vectors in mammalian cells. *Nature Genet.* **34**, 263–264 (2003).
84. Giles, R. V. & Tidd, D. M. Increased specificity for antisense oligodeoxynucleotide targeting of RNA cleavage by RNase H using chimeric methylphosphonodiester/phosphodiester structures. *Nucleic Acids Res.* **20**, 763–770 (1992).
85. Monia, B. P. *et al*. Evaluation of 2′-modified oligonucleotides containing 2′-deoxy gaps as antisense inhibitors of gene expression. *J. Biol. Chem.* **268**, 14514–14522 (1993).
86. Saxena, S., Jonsson, Z. O. & Dutta, A. Small RNAs with imperfect match to endogenous mRNA repress translation. Implications for off-target activity of small inhibitory RNA in mammalian cells. *J. Biol. Chem.* **278**, 44312–44319 (2003).
87. Doench, J. G., Petersen, C. P. & Sharp, P. A. siRNAs can function as miRNAs. *Genes Dev.* **17**, 438–442 (2003).
88. Zeng, Y., Yi, R. & Cullen, B. R. MicroRNAs and small interfering RNAs can inhibit mRNA expression by similar mechanisms. *Proc. Natl Acad. Sci. USA* **100**, 9779–9784 (2003).
89. Editorial. Whither RNAi? *Nature Cell Biol.* **5**, 489–490 (2003).
90. Harborth, J., Elbashir, S. M., Bechert, K., Tuschl, T. & Weber, K. Identification of essential genes in cultured mammalian cells using small interfering RNAs. *J. Cell Sci.* **114**, 4557–4565 (2001).
91. Miller, V. M. *et al*. Allele-specific silencing of dominant disease genes. *Proc. Natl Acad. Sci. USA* **100**, 7195–7200 (2003).
92. Ding, H. *et al*. Selective silencing by RNAi of a dominant allele that causes amyotrophic lateral sclerosis. *Aging Cell* **2**, 209–217 (2003).
93. Abdelgany, A., Wood, M. & Beeson, D. Allele-specific silencing of a pathogenic mutant acetylcholine receptor subunit by RNA interference. *Hum. Mol. Genet.* **12**, 2637–2644 (2003).
94. Gonzalez-Alegre, P., Miller, V. M., Davidson, B. L. & Paulson, H. L. Toward therapy for DYT1 dystonia: allele-specific silencing of mutant TorsinA. *Ann. Neurol.* **53**, 781–787 (2003).
95. Tuschl, T. Expanding small RNA interference. *Nature Biotechnol.* **20**, 446–448 (2002).
96. Xia, H., Mao, Q., Paulson, H. L. & Davidson, B. L. siRNA-mediated gene silencing *in vitro* and *in vivo*. *Nature Biotechnol.* **20**, 1006–1010 (2002).
97. Arts, G. J. *et al*. Adenoviral vectors expressing siRNAs for discovery and validation of gene function. *Genome. Res.* **13**, 2325–2332 (2003).
98. Shen, C., Buck, A. K., Liu, X., Winkler, M. & Reske, S. N. Gene silencing by adenovirus-delivered siRNA. *FEBS Lett.* **539**, 111–114 (2003).
99. Zhao, L. J., Jian, H. & Zhu, H. Specific gene inhibition by adenovirus-mediated expression of small interfering RNA. *Gene* **316**, 137–141 (2003).
100. Tomar, R. S., Matta, H. & Chaudhary, P. M. Use of adeno-associated viral vector for delivery of small interfering RNA. *Oncogene* **22**, 5712–5715 (2003).
101. Hommel, J. D., Sears, R. M., Georgescu, D., Simmons, D. L. & DiLeone, R. J. Local gene knockdown in the brain using viral-mediated RNA interference. *Nature Med.* **9**, 1539–1544 (2003).
102. Brummelkamp, T. R., Bernards, R. & Agami, R. Stable suppression of tumorigenicity by virus-mediated RNA interference. *Cancer Cell* **2**, 243–247 (2002).
103. Wiznerowicz, M. & Trono, D. Conditional suppression of cellular genes: lentivirus vector-mediated drug-inducible RNA interference. *J. Virol.* **77**, 8957–8961 (2003).
104. Rubinson, D. A. *et al*. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nature Genet.* **33**, 401–406 (2003).
105. An, D. S. *et al*. Efficient lentiviral vectors for short hairpin RNA delivery into human cells. *Hum. Gene Ther.* **14**, 1207–1212 (2003).
106. van de Wetering, M. *et al*. Specific inhibition of gene expression using a stably integrated, inducible small-interfering-RNA vector. *EMBO Rep.* **4**, 609–615 (2003).
107. Czauderna, F. *et al*. Inducible shRNA expression for application in a prostate cancer mouse model. *Nucleic Acids Res.* **31**, e127 (2003).
108. Matsukura, S., Jones, P. A. & Takai, D. Establishment of conditional vectors for hairpin siRNA knockdowns. *Nucleic Acids Res.* **31**, e77 (2003).
109. Shinagawa, T. & Ishii, S. Generation of Ski-knockdown mice by expressing a long double-strand RNA from an RNA polymerase II promoter. *Genes Dev.* **17**, 1340–1345 (2003).
110. Matsuda, T. & Cepko, C. L. Electroporation and RNA interference in the rodent retina *in vivo* and *in vitro*. *Proc. Natl Acad. Sci. USA* **101**, 16–22 (2003).
111. Mellitzer, G., Hallonet, M., Chen, L. & Ang, S. Spatial and temporal 'knock down' of gene expression by electroporation of double-stranded RNA and morpholinos into early postimplantation mouse embryos. *Mech. Dev.* **118**, 57 (2002).
112. Bai, J. *et al*. RNAi reveals doublecortin is required for radial migration in rat neocortex. *Nature Neurosci.* **6**, 1277–1283 (2003).
113. Calegari, F., Haubensak, W., Yang, D., Huttner, W. B. & Buchholz, F. Tissue-specific RNA interference in postimplantation mouse embryos with endoribonuclease-prepared short interfering RNA. *Proc. Natl Acad. Sci. USA* **99**, 14236–14240 (2002).
114. Reich, S. J. *et al*. Small interfering RNA (siRNA) targeting VEGF effectively inhibits ocular neovascularization in a mouse model. *Mol. Vis.* **9**, 210–216 (2003).
115. Sorensen, D. R., Leirdal, M. & Sioud, M. Gene silencing by systemic delivery of synthetic siRNAs in adult mice. *J. Mol. Biol.* **327**, 761–766 (2003).
116. Kong, X. C., Barzaghi, P. & Ruegg, M. A. Inhibition of synapse assembly in mammalian muscle *in vivo* by RNA interference. *EMBO Rep.* **5**, 183–188 (2004).
117. McCaffrey, A. P. *et al*. RNA interference in adult mice. *Nature* **418**, 38–39 (2002).
118. Lewis, D. L., Hagstrom, J. E., Loomis, A. G., Wolff, J. A. & Herweijer, H. Efficient delivery of siRNA for inhibition of gene expression in postnatal mice. *Nature Genet.* **32**, 107–108 (2002).
Paper describing the *in vivo* delivery of siRNAs through high-pressure tail vein injection.

119. Giladi, H. *et al*. Small interfering RNA inhibits hepatitis B virus replication in mice. *Mol. Ther.* **8**, 769–776 (2003).
120. Klein, C. *et al*. Inhibition of hepatitis B virus replication *in vivo* by nucleoside analogues and siRNA. *Gastroenterology* **125**, 9–18 (2003).
121. Zender, L. *et al*. Caspase 8 small interfering RNA prevents acute liver failure in mice. *Proc. Natl Acad. Sci. USA* **100**, 7797–7802 (2003).
122. Song, E. *et al*. RNA interference targeting Fas protects mice from fulminant hepatitis. *Nature Med.* **9**, 347–351 (2003). **Paper establishing proof of principle for siRNA-based therapeutics.**
123. McCaffrey, A. P. *et al*. Inhibition of hepatitis B virus in mice by RNA interference. *Nature Biotechnol.* **21**, 639–644 (2003).
124. Sullenger, B. A. & Gilboa, E. Emerging clinical applications of RNA. *Nature* **418**, 252–258 (2002).
125. Stevenson, M. Dissecting HIV-1 through RNA interference. *Nature Rev. Immunol.* **3**, 851–858 (2003).
126. Opalinska, J. B. & Gewirtz, A. M. Nucleic-acid therapeutics: basic principles and recent applications. *Nature Rev. Drug Discov.* **1**, 503–514 (2002).
127. Brenner, T. *et al*. The role of readthrough acetylcholinesterase in the pathophysiology of myasthenia gravis. *FASEB J.* **17**, 214–222 (2003).
128. Geary, R. S. *et al*. Absolute bioavailability of 2′-*O*-(2-methoxyethyl)-modified antisense oligonucleotides following intraduodenal instillation in rats. *J. Pharmacol. Exp. Ther.* **296**, 898–904 (2001).
129. Sussman, H. E. Success for oral antisense therapy. *Drug. Discov. Today* **8**, 516–517 (2003).
130. Karande, P., Jain, A. & Mitragotri, S. Discovery of transdermal penetration enhancers by high-throughput screening. *Nature Biotechnol.* **22**, 192–197 (2004).
131. Gautam, A., Densmore, C. L., Xu, B. & Waldrep, J. C. Enhanced gene expression in mouse lung after PEI-DNA aerosol delivery. *Mol. Ther.* **2**, 63–70 (2000).
132. Carmell, M. A., Zhang, L., Conklin, D. S., Hannon, G. J. & Rosenquist, T. A. Germline transmission of RNAi in mice. *Nature Struct. Biol.* **10**, 91–92 (2003).
133. Hemann, M. T. *et al*. An epi-allelic series of p53 hypomorphs created by stable RNAi produces distinct tumor phenotypes *in vivo*. *Nature Genet.* **33**, 396–400 (2003).
134. Davies, J. A. *et al*. Development of an siRNA-based method for repressing specific genes in renal organ culture and its use to show that the Wt1 tumour suppressor is required for nephron differentiation. *Hum. Mol. Genet.* **13**, 235–246 (2003).
135. Carpenter, A. E. & Sabatini, D. M. Systematic genome-wide screens of gene function. *Nature Rev. Genet.* **5**, 11–22 (2004).
136. Kamath, R. S. *et al*. Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature* **421**, 231–237 (2003).
137. Ashrafi, K. *et al*. Genome-wide RNAi analysis of *Caenorhabditis elegans* fat regulatory genes. *Nature* **421**, 268–272 (2003).
138. Fraser, A. G. *et al*. Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference. *Nature* **408**, 325–330 (2000).
139. Gonczy, P. *et al*. Functional genomic analysis of cell division in *C. elegans* using RNAi of genes on chromosome III. *Nature* **408**, 331–336 (2000).
140. Maeda, I., Kohara, Y., Yamamoto, M. & Sugimoto, A. Large-scale analysis of gene function in *Caenorhabditis elegans* by high-throughput RNAi. *Curr. Biol.* **11**, 171–176 (2001).
141. Piano, F. *et al*. Gene clustering based on RNAi phenotypes of ovary-enriched genes in *C. elegans*. *Curr. Biol.* **12**, 1959–19564 (2002).
142. Piano, F., Schetter, A. J., Mangone, M., Stein, L. & Kemphues, K. J. RNAi analysis of genes expressed in the ovary of *Caenorhabditis elegans*. *Curr. Biol.* **10**, 1619–1622 (2000).
143. Simmer, F. *et al*. Genome-wide RNAi of *C. elegans* using the hypersensitive rrf-3 strain reveals novel gene functions. *PLoS Biol.* **1**, E12 (2003).
144. Vastenhouw, N. L. *et al*. A genome-wide screen identifies 27 genes involved in transposon silencing in *C. elegans*. *Curr. Biol.* **13**, 1311–1316 (2003).
145. Pothof, J. *et al*. Identification of genes that protect the *C. elegans* genome against mutations by genome-wide RNAi. *Genes Dev.* **17**, 443–448 (2003).
146. Lee, S. S. *et al*. A systematic RNAi screen identifies a critical role for mitochondria in *C. elegans* longevity. *Nature Genet.* **33**, 40–48 (2003).
147. Lum, L. *et al*. Identification of Hedgehog pathway components by RNAi in *Drosophila* cultured cells. *Science* **299**, 2039–2045 (2003).
148. Kiger, A. *et al*. A functional genomic analysis of cell morphology using RNA interference. *J. Biol.* **2**, 27 (2003).
149. Boutros, M. *et al*. Genome-wide RNAi analysis of growth and viability in *Drosophila* cells. *Science* **303**, 832–835 (2004).
150. Nolan, P. M., Kapfhamer, D. & Bucan, M. Random mutagenesis screen for dominant behavioral mutations in mice. *Methods* **13**, 379–395 (1997).
151. Rinchik, E. M. & Carpenter, D. A. *N*-ethyl-*N*-nitrosourea mutagenesis of a 6- to 11-cM subregion of the Fah-Hbb interval of mouse chromosome 7: completed testing of 4557 gametes and deletion mapping and complementation analysis of 31 mutations. *Genetics* **152**, 373–383 (1999).
152. Nolan, P. M. *et al*. A systematic, genome-wide, phenotype-driven mutagenesis programme for gene function studies in the mouse. *Nature Genet.* **25**, 440–443 (2000).
153. Hrabe de Angelis, M. H. *et al*. Genome-wide, large-scale production of mutant mice by ENU mutagenesis. *Nature Genet.* **25**, 444–447 (2000).
154. Kile, B. T. *et al*. Functional genetic analysis of mouse chromosome 11. *Nature* **425**, 81–86 (2003).
155. Oates, A. C., Bruce, A. E. & Ho, R. K. Too much interference: injection of double-stranded RNA has nonspecific effects in the zebrafish embryo. *Dev. Biol.* **224**, 20–28 (2000).
156. Lim, L. P., Glasner, M. E., Yekta, S., Burge, C. B. & Bartel, D. P. Vertebrate microRNA genes. *Science* **299**, 1540 (2003).
157. Zhou, Y., Ching, Y.-P., Kok, K. H., Kung, H. & Jin, D.-J. Post-transcriptional suppression of gene expression in *Xenopus* embryos by small interfering RNAs. *Nucleic Acids Res.* **30**, 1664–1669 (2002).
158. Pekarik, V. *et al*. Screening for gene function in chicken embryo using RNAi and electroporation. *Nature Biotechnol.* **21**, 93–96 (2003).
159. Svoboda, P., Stein, P., Hayashi, H. & Schultz, R. M. Selective reduction of dormant maternal mRNAs in mouse oocytes by RNA interference. *Development* **127**, 4147–4156 (2000).
160. Stein, P., Svoboda, P. & Schultz, R. M. Transgenic RNAi in mouse oocytes: a simple and fast approach to study gene function. *Dev. Biol.* **256**, 187–193 (2003).
161. Aza-Blanc, P. *et al*. Identification of modulators of TRAIL-induced apoptosis via RNAi-based phenotypic screening. *Mol. Cell* **12**, 627–637 (2003).
162. Zheng, L. *et al*. An approach to genomewide screens of expressed small interfering RNAs in mammalian cells. *Proc. Natl Acad. Sci. USA* **101**, 135–140 (2003).
163. Brummelkamp, T. R., Nijman, S. M., Dirac, A. M. & Bernards, R. Loss of the cylindromatosis tumour suppressor inhibits apoptosis by activating NF-κB. *Nature* **424**, 797–801 (2003).
164. Berns, K. *et al*. A large-scale RNAi screen in human cells identifies novel components of the p53 pathway. *Nature* (in the press). **Paper describing a high-throughput siRNA-based screen in mammalian cells.**
165. Walters, D. K. & Jelinek, D. F. The effectiveness of double-stranded short inhibitory RNAs (siRNAs) may depend on the method of transfection. *Antisense Nucleic Acid Drug Dev.* **12**, 411–418 (2002).
166. Weil, D. *et al*. Targeting the kinesin Eg5 to monitor siRNA transfection in mammalian cells. *Biotechniques* **33**, 1244–1248 (2002).
167. Zhou, A., Scoggin, S., Gaynor, R. B. & Williams, N. S. Identification of NF-κB-regulated genes induced by TNFα utilizing expression profiling and RNA interference. *Oncogene* **22**, 2054–2064 (2003).
168. Williams, N. S. *et al*. Identification and validation of genes involved in the pathogenesis of colorectal cancer using cDNA microarrays and RNA interference. *Clin. Cancer Res.* **9**, 931–946 (2003).
169. Ziauddin, J. & Sabatini, D. M. Microarrays of cells expressing defined cDNAs. *Nature* **411**, 107–110 (2001).
170. Bailey, S. N., Wu, R. Z. & Sabatini, D. M. Applications of transfected cell microarrays in high-throughput drug discovery. *Drug Discov. Today* **7**, S113–S118 (2002).
171. Mousses, S. *et al*. RNAi microarray analysis in cultured mammalian cells. *Genome Res* **13**, 2341–2347 (2003).
172. Reynolds, A. *et al*. Rational siRNA design for RNA interference. *Nature Biotechnol.* **22**, 326–330 (2004).
173. Hohjoh, H. Enhancement of RNAi activity by improved siRNA duplexes. *FEBS Lett.* **557**, 193–198 (2004).
174. Silva, J. M., Sachidanandam, R. & Hannon, G. J. Free energy lights the path toward more effective RNAi. *Nature Genet.* **35**, 303–305 (2003).
175. 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).
176. Shirane, D. *et al*. Enzymatic production of RNAi libraries from cDNAs. *Nature Genet.* **36**, 190–196 (2004).
177. Sen, G., Wehrman, T. S., Myers, J. W. & Blau, H. M. Restriction enzyme-generated siRNA (REGS) vectors and libraries. *Nature Genet.* **36**, 183–189 (2004).
178. Kawasaki, H., Suyama, E., Iyo, M. & Taira, K. siRNAs generated by recombinant human Dicer induce specific and significant but target site-independent gene silencing in human cells. *Nucleic Acids Res.* **31**, 981–987 (2003).
179. Howard, K. Unlocking the money-making potential of RNAi. *Nature Biotechnol.* **21**, 1441–1446 (2003).
180. Song, E. *et al*. Sustained small interfering RNA-mediated human immunodeficiency virus type 1 inhibition in primary macrophages. *J. Virol.* **77**, 7174–7181 (2003).
181. Filleur, S. *et al*. SiRNA-mediated inhibition of vascular endothelial growth factor severely limits tumor resistance to antiangiogenic thrombospondin-1 and slows tumor vascularization and growth. *Cancer Res.* **63**, 3919–3922 (2003).
182. Yang, G., Thompson, J. A., Fang, B. & Liu, J. Silencing of *H-ras* gene expression by retrovirus-mediated siRNA decreases transformation efficiency and tumor growth in a model of human ovarian cancer. *Oncogene* **22**, 5694–5701 (2003).
183. Li, K., Lin, S. Y., Brunicardi, F. C. & Seu, P. Use of RNA interference to target cyclin E-overexpressing hepatocellular carcinoma. *Cancer Res.* **63**, 3593–3597 (2003).
184. Verma, U. N., Surabhi, R. M., Schmaltieg, A., Becerra, C. & Gaynor, R. B. Small interfering RNAs directed against β-catenin inhibit the *in vitro* and *in vivo* growth of colon cancer cells. *Clin. Cancer. Res.* **9**, 1291–1300 (2003).
185. Yoshinouchi, M. *et al*. *In vitro* and *in vivo* growth suppression of human papillomavirus 16-positive cervical cancer cells by E6 siRNA. *Mol. Ther.* **8**, 762–768 (2003).
186. Pieken, W. A., Olsen, D. B., Benseler, F., Aurup, H. & Eckstein, F. Kinetic characterization of ribonuclease-resistant 2′-modified hammerhead ribozymes. *Science* **253**, 314–317 (1991).
187. Beigelman, L. *et al*. Chemical modification of hammerhead ribozymes. Catalytic activity and nuclease resistance. *J. Biol. Chem.* **270**, 25702–25708 (1995).
188. Levin, A. A. A review of the issues in the pharmacokinetics and toxicology of phosphorothioate antisense oligonucleotides. *Biochim. Biophys. Acta* **1489**, 69–84 (1999).
189. Czauderna, F. *et al*. Structural variations and stabilising modifications of synthetic siRNAs in mammalian cells. *Nucleic Acids Res.* **31**, 2705–2716 (2003).
190. Chiu, Y. L. & Rana, T. M. siRNA function in RNAi: a chemical modification analysis. *RNA* **9**, 1034–1048 (2003).
191. Amarzguioui, M., Holen, T., Babaie, E. & Prydz, H. Tolerance for mutations and chemical modifications in a siRNA. *Nucleic Acids Res.* **31**, 589–595 (2003).
192. Braasch, D. A. *et al*. RNA interference in mammalian cells by chemically-modified RNA. *Biochemistry* **42**, 7967–7975 (2003).
193. Chiu, Y. L. & Rana, T. M. RNAi in human cells. Basic structural and functional features of small interfering RNA. *Mol. Cell* **10**, 549–561 (2002).
194. Holen, T., Amarzguioui, M., Wiiger, M. T., Babaie, E. & Prydz, H. Positional effects of short interfering RNAs targeting the human coagulation trigger tissue factor. *Nucleic Acids Res.* **30**, 1757–1766 (2002).
195. Holen, T., Amarzguioui, M., Babaie, E. & Prydz, H. Similar behaviour of single-strand and double-strand siRNAs suggests they act through a common RNAi pathway. *Nucleic Acids Res.* **31**, 2401–2407 (2003).
196. Capodici, J., Kariko, K. & Weissman, D. Inhibition of HIV-1 infection by small interfering RNA-mediated RNA interference. *J. Immunol.* **169**, 5196–5201 (2002).

Acknowledgments

We would like to thank F. Eckstein and M. Manoharan for their thoughtful comments on the manuscript. We would also like to thank A. Patkaniowska for the Table of siRNA chemical modifications, and the members of the Tuschl laboratory for critical comments.

Competing interests statement

The authors declare that they have competing financial interests; see Web version for details.

Online links

FURTHER INFORMATION
RNA interference web focus:
http://www.nature.com/focus/rnai/
Access to this interactive links box is free online.