KILLING THE MESSENGER: SHORT RNAS THAT SILENCE GENE EXPRESSION

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Short interfering RNAs can be used to silence gene expression in a sequence-specific manner in a process that is known as RNA interference. The application of RNA interference in mammals has the potential to allow the systematic analysis of gene expression and holds the possibility of therapeutic gene silencing. Much of the promise of RNA interference will depend on the recent advances in short-RNA-based silencing technologies.

RIBOZYME TECHNOLOGY
This method uses an RNA molecule that binds the target messenger RNA in a sequence-specific manner and catalyses the cleavage of the mRNA. This ribozyme thereby prevents translation of the target mRNA into protein.

ANTISENSE TECHNOLOGY
This method uses either DNA or RNA molecules that are complementary to sequences on the target messenger RNA and inhibits protein production.

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In 1998, Fire and colleagues found that the injection of double-stranded (ds)RNA into *Caenorhabditis elegans* led to an efficient sequence-specific gene silencing[1], which is referred to as RNA interference (RNAi). RNAi has been linked to many previously described silencing phenomena such as post-transcriptional gene silencing (PTGS) in plants[2] and quelling in fungi[3,4]. Subsequent studies in *C. elegans* indicated that the first step in the RNAi pathway involved the generation of a sequence-specific effector molecule[5]. The first hint that the effector molecules that regulate PTGS might be short RNA species was the discovery of short RNA species — 21–25 nucleotides (nt) — in plants that were undergoing PTGS[6]. The RNAi reaction was recapitulated in *Drosophila melanogaster* embryo extracts, in which it was shown that long dsRNA substrates could be cleaved into short interfering dsRNA species (siRNAs) of ~22 nt[7] and that the introduction of chemically synthesized 21-nt and 22-nt siRNAs to these extracts facilitated the degradation of the homologous RNA[8]. Short RNA products were subsequently found in fly embryos[9] and worms[10] that were injected with dsRNA, as well as in *Drosophila* Schneider 2 (S2) cells that were transfected with long dsRNA[11]. These findings provided a new tool for studying gene function.

Gene targeting by homologous recombination is commonly used to determine gene function in mammals, but this is a costly and time-consuming process, and many organisms are not amenable to such gene-targeting methods. Furthermore, the function of targeted genes might not be determined by this approach owing to lethal or redundant phenotypes. Alternatively, the functions of many genes can be determined by RIBOZYME and ANTISENSE TECHNOLOGIES. Although successful in some situations, these technologies have been difficult to apply universally[12–14]. The advent of siRNA-directed 'knockdown' has sparked a revolution in somatic cell genetics, allowing the inexpensive and rapid analysis of gene function in mammals. Coupled with data from genome projects in various organisms, siRNA-directed gene silencing has the potential to allow for the determination of the function of each gene that is expressed in a cell-type- or pathway-specific manner. In addition, siRNA-directed gene silencing might allow the silencing of genes that are pathogenic to the host organism. This review focuses on the rapid advances that have been made in short-RNA-based silencing technologies and its application in deciphering gene function.

Mechanism of RNAi

Biochemical characterization showed that siRNAs are 21–23-nt dsRNA duplexes with symmetric 2–3-nt 3′ overhangs and 5′-phosphate and 3′-hydroxyl groups[8] (FIG. 1a). This structure is characteristic of an RNASE III-like enzymatic cleavage pattern, which led to the identification of the highly conserved Dicer family of RNase III enzymes as the mediators of the dsRNA cleavage[15–17].

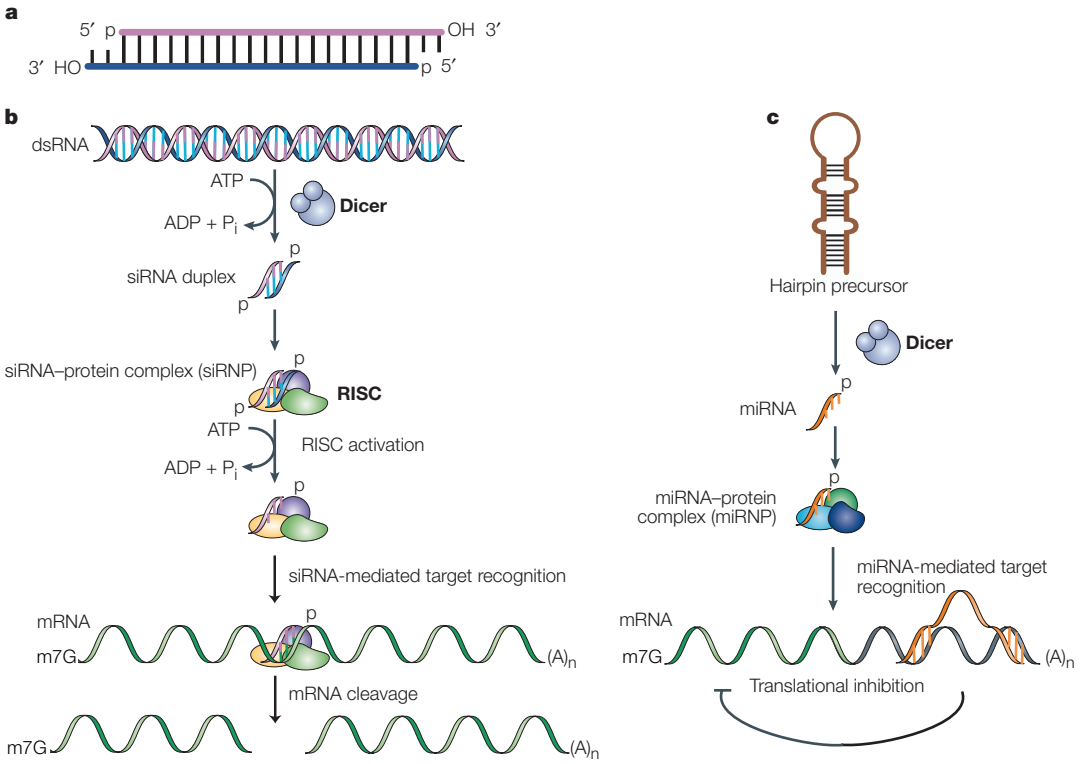

Figure 1 | **The RNA interference pathway. a** | Short interfering (si)RNAs. Molecular hallmarks of an siRNA include 5′ phosphorylated ends, a 19-nucleotide (nt) duplexed region and 2-nt unpaired and unphosphorylated 3′ ends that are characteristic of RNase III cleavage products[14]. **b** | The siRNA pathway. Long double-stranded (ds)RNA is cleaved by the RNase III family member, Dicer, into siRNAs in an ATP-dependent reaction[104]. These siRNAs are then incorporated into the RNA-inducing silencing complex (RISC). Although the uptake of siRNAs by RISC is independent of ATP, the unwinding of the siRNA duplex requires ATP. Once unwound, the single-stranded antisense strand guides RISC to messenger RNA that has a complementary sequence, which results in the endonucleolytic cleavage of the target mRNA. **c** | The micro (mi)RNA pathway. Although originally identified on the basis of its ability to process long dsRNA, Dicer can also cleave the ~70-nt hairpin miRNA precursor to produce ~22-nt miRNA. Unlike siRNAs, the miRNAs are single stranded and are incorporated into a miRNA–protein complex (miRNP)[20,21]. *Caenorhabditis elegans let-7* and *lin-4* miRNAs pair with partial sequence complementarity to target mRNA leading to translational repression[27,28]. In addition to Dicer, the two pathways require other PAZ/PIWI domain proteins (PPD), including eukaryotic translation initiation factor 2C 2 (eIF2C2)[22,29,30].

RNASE III
A double-stranded (ds)RNA-specific endoribonuclease that cleaves long dsRNA into short fragments that have a characteristic 3′ overhang and a recessed 5′ phosphate on each strand.

PAZ
(PIWI, argonaute and zwille). A putative protein interaction domain named after the founding members that contain this domain.

PIWI DOMAIN PROTEINS
Proteins that have a conserved protein domain of unknown function. In *Drosophila*, this family has been implicated in translational control and silencing of numerous copies of the alcohol dehydrogenase gene.

PPD PROTEIN
A protein that has a PAZ/PIWI domain.

INTERFERON
A small and highly potent molecule that functions in an autocrine and paracrine manner, and that induces cells to resist viral replication.

2′–5′ OLIGOADENYLATE SYNTHASE
A component of the interferon-response pathway that, when activated by long double-stranded RNA, catalyses the conversion of ATP to 2′–5′ A oligonucleotides.

RNASE L
An enzyme that is activated by 2′–5′ A oligonucleotides, leading to the cleavage of several RNA species including ribosomal RNA, resulting in an inhibition of messenger RNA translation.

PKR
A protein kinase that, when activated by long double-stranded RNA, phosphorylates and inactivates the translation initiation factor eIF2α, resulting in an inhibition of messenger RNA translation initiation.

Extensive biochemical and genetic evidence has provided a better understanding of how long dsRNAs could cause the degradation of the target messenger RNA (FIG. 1b; for recent reviews, see REFS 18–21). Several studies have shown that this process is restricted to the cytoplasm[22,23,24]. In the first step, Dicer cleaves long dsRNA to produce siRNAs. These siRNAs are incorporated into a multiprotein RNA-inducing silencing complex (RISC). There is a strict requirement for the siRNA to be 5′ phosphorylated to enter into RISC[25,26], and siRNAs that lack a 5′ phosphate are rapidly phosphorylated by an endogenous kinase[26]. The duplex siRNA is unwound, leaving the antisense strand to guide RISC to its homologous target mRNA for endonucleolytic cleavage. The target mRNA is cleaved at a single site in the centre of the duplex region between the guide siRNA and the target mRNA, 10 nt from the 5′ end of the siRNA[8].

Interestingly, endogenously expressed siRNAs have not been found in mammals. However, the related micro (mi)RNAs have been cloned from various organisms and cell types[27]. These short RNA species (~22 nt) are produced by Dicer cleavage of longer (~70 nt) endogenous precursors with imperfect hairpin RNA structures (FIG. 1c). The miRNAs are believed to bind to sites that have partial sequence complementarity in the 3′ untranslated region (UTR) of their target mRNA, causing repression of translation and inhibition of protein synthesis[28]. In addition to Dicer, other PAZ/PIWI DOMAIN PROTEINS (PPD), including eukaryotic translation initiation factor 2C 2 (eIF2C2), are likely to function in both pathways[22,29,30].

Silencing by siRNA

RNAi mediated by the introduction of long dsRNA has been used as a method to investigate gene function in various organisms including plants[31], planaria[32], Hydras[33], *Trypanosomes*[34], *Drosophila*[35,36], mosquitoes[37] and mouse oocytes[38,39] (FIG. 2A). Long dsRNA enables the effective silencing of gene expression by presenting various siRNA sequences to the target mRNA. The applicability of this approach is limited in mammals because the introduction of dsRNA longer than 30 nt induces a sequence-nonspecific INTERFERON response[40]. Interferon triggers the degradation of mRNA by inducing 2′-5′ OLIGOADENYLATE SYNTHASE, which in turn activates RNASE L. In addition, interferon

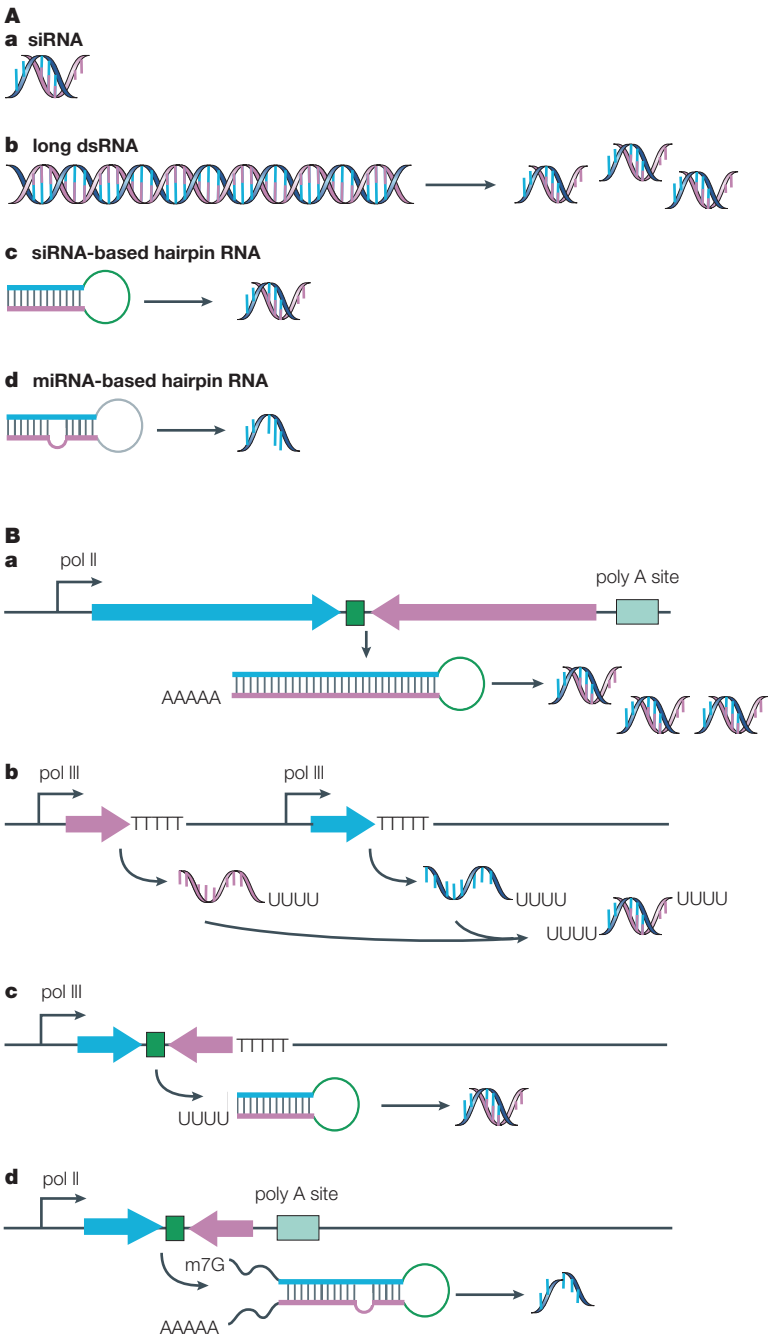

Figure 2 | **Methods to generate short RNAs that silence gene expression. A** | Silencing by RNAs that are generated *in vitro*. **Aa** | Chemically synthesized short interfering (si)RNAs that are introduced into cells bypass the 'dicing' step and are incorporated into the RNA-inducing silencing complex (RISC) for targeted messenger RNA degradation[40,99]. **Ab** | Long double-stranded (ds)RNAs that are introduced into cells can be processed by Dicer into siRNAs that silence gene expression[7–9,31–39]. **Ac** | Perfect duplex hairpin RNA can be cleaved by Dicer into siRNAs[65]. **Ad** | Imperfect duplex hairpin RNA, based on pre-micro (mi)RNA structures, can be cleaved by Dicer into miRNAs and direct gene silencing[65]. **B** | Silencing by short RNAs that are generated *in vivo*. **Ba** | Long hairpin RNA expressed from an RNA polymerase (pol) II promoter yields a population of siRNAs with several sequence specificities. siRNAs with a single sequence specificity can be expressed either by **Bb** | tandem pol III promoters that express individual sense and antisense strands of the siRNA that associate in *trans*[46,53] or by **Bc** | a single pol III promoter that expresses short hairpin (sh)RNA with the sense and antisense strands of the siRNA that associate in *cis*[48,57,63–69,75–78]. **Bd** | Incorporation of an imperfect duplex hairpin structure that is based on pre-miRNA structures can be expressed from a pol II promoter and processed by Dicer into a mature miRNA, which can direct gene silencing[102].

activates the protein kinase PKR, which phosphorylates the translation initiation factor eIF2α leading to a global inhibition of mRNA translation[41].

To test whether siRNAs could mediate effective silencing of gene expression without inducing the interferon response, Tuschl and colleagues[40] introduced chemically synthesized siRNA into mammalian cells (FIG. 2A). First, they showed that the synthetic siRNAs were functional *in vivo* by co-transfecting *Drosophila* S2 cells with *luciferase* siRNA and a *luciferase* reporter construct. This resulted in a loss of luciferase activity comparable to that obtained with long dsRNA[42–44]. More importantly, they showed that siRNA transfection resulted in the sequence-specific silencing of luciferase expression, as well as the endogenous nuclear envelope proteins lamin A/C, in several mammalian cell lines without activating nonspecific effects. These findings have led to the widespread use of this technology to study gene function including the targeted disruption of clinically relevant genes (TABLE 1), alluding to the potential therapeutic applications of RNAi-based technologies.

To promote efficient gene silencing using an siRNA to a single site in the target mRNA, consideration of the siRNA sequence is crucial. Although the rules that govern efficient siRNA-directed gene silencing remain undefined (BOX 1), it is known that siRNAs that target different regions of the same gene vary markedly in their effectiveness[45–48]. The base composition of the siRNA sequence is probably not the only determinant of how efficiently it will silence a gene. Other factors that are likely to have a role include the secondary structure of the mRNA target and the binding of RNA-binding proteins (BOX 2). In an attempt to optimize the siRNA sequences, several groups have used a SYNTHETIC OLIGODEOXYRIBONUCLEOTIDE/RNASE H METHOD to determine sites on the mRNA that are in a conformation that is susceptible to siRNA-directed silencing[47,49]. These studies found that there was a significant correlation between the RNase-H-sensitive sites and sites that promote efficient siRNA-directed mRNA degradation. Vickers *et al.* found that placing the mRNA recognition site of a usually active siRNA into a highly structured RNA region abrogated its ability to inhibit gene expression[47]. Although this work indicates that there is an interplay between the effectiveness of the siRNA and the mRNA structure of the target region, more work is necessary to define this relationship precisely.

Recently, several groups have used either *Escherichia coli* RNase III (REFS 50,51) or recombinant human Dicer[52,53] to cleave *in vitro* transcribed long dsRNA into siRNAs that can be transfected into mammalian cells. This approach allows for the presentation of siRNAs with multiple specificities to the target without activating an interferon response.

DNA-vector-mediated RNAi

Unlike fungi[54], plants[55] and worms[56], which can replicate siRNAs, there is no indication of siRNA replication in mammals[23,57–59] (for a review, see REF. 21). Therefore, siRNA-directed silencing by transfection is limited in

Table 1 | **Examples of disease-related genes that have been targeted in mammals using siRNA**

Gene/mRNA targeted	Type of gene	Method	Phenotype	References
HIV-1				
p24	HIV-1 capsid protein	siRNA transfection; siRNA transfection of *in vitro* transcribed RNA	Decreased viral protein expression, decreased virus production; inhibition of HIV replication after fusion and before reverse transcription and transcription from integrated provirus	104,105
Rev	HIV-1 regulatory protein	siRNA transfection; plasmid-vector-mediated siRNA expression (tandem U6 promoters)	Decreased viral protein expression, decreased virus production	53,106
Vif	HIV-1 regulatory protein	siRNA transfection; plasmid-vector-mediated siRNA expression	Inhibition of HIV replication, degradation of preintegrated genomic HIV RNA	107
Tat	HIV-1 regulatory protein	siRNA transfection	Decreased viral protein expression, decreased virus production	106
LTR mRNA	HIV-1 long terminal repeat	siRNA transfection, *in vitro* transcribed siRNA	Inhibition of HIV replication after fusion and before reverse transription and transcription from integrated provirus	105
Other viruses				
Poliovirus capsid	Capsid structural protein	siRNA transfection	Reduced viral titer, clearance of virus from infected cells	108
Poliovirus RNAP	RNAP	siRNA transfection	Reduced viral titer, clearance of virus from infected cells	108
HPV E6 mRNA	Viral transcript E6	siRNA transfection	Selective degradation of *E6* mRNA, accumulation of cellular p53, reduced cell growth	109
HPV E7 mRNA	Viral transcript E7	siRNA transfection	Selective degradation of *E7* mRNA, induced apoptotic cell death	109
RSV P protein	Phosphoprotein, smaller subunit of the RNA-dependent RNAP	siRNA transfection	Inhibition of P protein expression, reduced amounts of all viral proteins, no syncytia formation	110
RSV F protein	Fusion protein	siRNA transfection	No detectable F protein, no effect on other viral proteins, no syncytia formation	110
Hepatitis C virus NS5B	Non-structural protein 5B, viral polymerase mRNA	'Hydrodynamic' siRNA injection	Decreased levels of the NS5B–luciferase fusion protein in mouse hepatocytes	92
Oncogenes				
Ras(V12)	Constitutively active oncogenic ras mutant	Moloney-based retroviral-vector-mediated siRNA expression	CAPAN-1 cells failed to form colonies in soft agar and failed to form tumours in nude mice when injected subcutaneously	75
bcr–abl	Oncogene, fusion of abl and bcr	siRNA transfection	Specifically decreased the *bcr–abl* mRNA without targeting either the *c-abl* or *c-bcr* mRNA, inhibited bcr–abl-dependent cellular proliferation	111
Tumour suppressors				
p53	Tumour suppressor gene	Plasmid-vector-mediated siRNA expression, Moloney-based retroviral-vector-mediated siRNA expression	Selection of cells stably knocked down in p53 expression; different p53 shRNAs produced different degrees of silencing, which was directly correlated with the severity of Myc-induced lymphomagenesis; loss of ras-induced senescence, growth in soft agar	63,48,75
53bp1	p53-binding-protein-1, mediator of DNA damage checkpoint	siRNA transfection	Decreased p53 accumulation, disruption of G2–M checkpoint arrest, intra-S-phase checkpoint in response to ionizing radiation	112
p73Dn	Tumour suppressor gene	siRNA transfection	Increased activity of p53-responsive promoter	113
Cell-surface receptors				
Fas receptor	Proapototic Fas receptor	'Hydrodynamic' siRNA injection	Decreased levels of Fas receptor in murine hepatocytes *in vivo*, increased resistance to Fas-mediated apoptosis	88
CD4	Cell surface receptor, HIV-1 coreceptor	siRNA transfection	Decreased HIV-1 infection, decreased free viral titers	104
CCR5	Cell surface receptor; HIV-1 coreceptor	siRNA transfection; lentiviral-vector-mediated siRNA expression	Decreased cell surface expression of receptors, inhibition of CCR5 tropic HIV-1 virus replication	114,78
CXCR4	Cell surface receptors, HIV-1 coreceptors	siRNA transfection	Decreased cell surface expression of receptors, inhibition of CXCR4 tropic HIV-1 virus replication	114
CD25	IL2 receptor α	Lentiviral-vector-mediated siRNA expression	Reduced cell surface expression of CD25, decreased proliferation of T cells when challenged with IL-2	77

HPV, human papilloma virus; mRNA, messenger RNA; siRNA, short interfering RNA; shRNA, short hairpin RNA; RNAP, RNA polymerase; RSV, respiratory syncytial virus.

Box 1 | Designing the perfect siRNA

Choosing short interfering (si)RNAs is an empirical process, as the rules that govern efficient siRNA-directed silencing are still unknown. On the basis of the analyses of a small number of target genes, several groups have proposed a set of guidelines that seek to narrow the choices of siRNAs that could potentially silence gene expression (REFS 57,99; C.D.N. and P.A.S., unpublished observations).

Several sequence motifs are consistent with effective siRNA-directed silencing, including $AAN_{19}TT$, $NAN_{19}NN$, $NARN_{17}YNN$ and $NANN_{17}YNN$ (where N is any nucleotide, R is a purine and Y is a pyrimidine). When choosing siRNAs, regions of complementary DNA are selected that have non-repetitive sequences. Intronic sequences are avoided as mammalian RNA interference is a cytoplasmic process[100]. Some groups suggest choosing siRNAs with ~50% GC content (30–70%). Our own observations indicate that sequences with an even representation of all nucleotides on the antisense strand are favoured and that regions with stretches of a single nucleotide, especially G, should be avoided (C.D.N. and P.A.S., unpublished observations). Elbashir *et al.*[99] have suggested that the use of 2′-deoxythymidines for the 2-nt 3′ overhangs might protect siRNAs from exonuclease activity. However, many groups have found that siRNAs that have ribonucleotides in the overhangs show no obvious impairment in silencing activity when compared with the same siRNA sequence with 2′-deoxythymidine overhangs.

There are several other parameters, in addition to the sequence considerations, that might affect the efficiency of siRNA-directed messenger RNA cleavage (BOX 2). Any region of mRNA can be targeted, however, sequences that are known sites for mRNA-binding proteins in the 5′ untranslated region (UTR), 3′ UTR, start codon or exon–exon boundaries should be avoided. Although Elbashir *et al.*[99] suggest selecting sequences that are 50–100 nt downstream of the start codon, our observations indicate that there is a predilection for effective siRNA-directed silencing towards the 3′ portion of the gene (C.D.N. and P.A.S., unpublished observations). The choice of siRNA is dictated by the sequence of the target gene and, sometimes, siRNAs must be chosen that do not have many of the parameters for efficient gene silencing. These potential parameters require systematic testing before they are codified into a set of rules that unequivocally promote efficient target-gene silencing. As these rules have not been tested systematically, researchers seeking to silence gene expression should synthesize several siRNAs to a gene and validate the efficiency of each.

To ensure that the chosen siRNA sequence targets a single gene, a BLAST search of the selected sequence should be carried out against sequence databases such as EST or Unigene libraries using the National Center for Biotechnology Information (NCBI) website (see Online links). Sequences in these databases that share partial homology to siRNAs might be targeted for silencing by the siRNA. Potential off-target effects of the siRNA might be minimized by choosing an siRNA with maximum sequence divergence from the list of genes with partial sequence identity to the intended mRNA target. For selected websites that are designed to pick siRNAs, please see the Online links.

Drosophila and mammals by its transient nature (BOX 3). To overcome some of the shortcomings of the transfection of chemically synthesized siRNA into cells, several groups have developed DNA-vector-mediated mechanisms to express substrates that can be converted into siRNA *in vivo*[24,46,53,60–69].

Expression systems mediated by RNA pol II. In organisms and cell types with weak or absent interferon responses, constructs that express long hairpins have been used. These constructs make use of RNA POLYMERASE II (pol II) promoters to drive the expression of long hairpin RNA, which can be cleaved by Dicer into siRNAs (FIG. 2B). These long-hairpin expression systems have effectively silenced target-gene expression in several different organisms, including mouse oocytes and preimplantation embryos[60], *C. elegans*[61] and *Drosophila*[62]. Pol II promoters allow inducible, tissue- or cell-type-specific RNA expression. For example, Kennerdell and Carthew[62] used a Gal4-inducible system to express a hairpin RNA to target β-galactosidase in *Drosophila*. By placing the expression of the Gal4 transactivator under the control of the heat shock protein 70 (*hsp70*) promoter, the expression of the hairpin was controlled by simply changing the temperature at which the flies were grown. Although these expression systems have been effective at mediating RNAi, the expression of long hairpin RNA in many mammalian cells induces the interferon response, thereby limiting how useful they are.

Expression of hairpin RNA mediated by RNA pol III. Plasmid-based expression systems using RNA POLYMERASE III (pol III) promoters that produce short RNA species and do not trigger significant interferon responses have been developed by several groups[24,46,53,63–69]. Two pol III promoters have been used predominately — the U6 promoter and the H1 promoter. Both of these promoters are members of the type III class of pol III promoters.

Although most RNA pol III promoters have sequences downstream of the transcription start site (+1) that are essential for transcription (class I and class II), several class III promoters lack downstream transcriptional elements. In fact, deletion of the sequences downstream of the +1 transcription start site in the mouse and human U6 promoters has no effect on the level of transcription[70]. Although the U6 and H1 promoters contain the same set of *CIS*-ACTING ELEMENTS (octamer motif, Staf-binding site, proximal sequence element (PSE) and TATA motif), the H1 promoter has a more compact organization[71]. The U6 promoter has a requirement for a guanosine in the +1 position, whereas the H1 promoter is much more permissive. In addition, RNA pol III recognizes a simple cluster of four or more T residues as a termination signal that accurately and

SYNTHETIC OLIGODEOXYRIBONUCLEOTIDE/RNASE H METHOD
A method that is used for mapping endonuclease-sensitive sites and for inhibiting gene expression. Synthetic single-stranded oligodeoxy-ribonucleotide and a complementary sequence to a target messenger RNA are transfected into cells, leading to the formation of an RNA–DNA hybrid. Endogenous RNase H cleaves the RNA molecule of an RNA–DNA hybrid and prevents protein synthesis.

RNA POLYMERASE II
(pol II). The enzyme that transcribes messenger RNA and most of the small nuclear RNAs of eukaryotes, in conjunction with various transcription factors.

RNA POLYMERASE III
(pol III). The enzyme that transcribes stable RNA products that are not translated into proteins, particularly transfer RNAs. However, pol III also transcribes the 5S ribosomal RNA, 7SL RNA and U6 small nuclear RNA.

CIS-ACTING ELEMENT
An arrangement of sequences on a contiguous piece of DNA.

Box 2 | **Potential determinants of efficient siRNA-directed gene silencing**

Sequence determinants intrinsic to the short interfering (si)RNA, the messenger RNA or both might affect the efficiency of each step of the siRNA-directed mRNA cleavage that results in efficient gene silencing.

siRNA

- **Incorporation into the RNA-inducing silencing complex (RISC) and stability in RISC.**
- **Basepairing with mRNA.**
- **Cleavage of mRNA.**
- **Turnover of mRNA after cleavage.**

mRNA

- **The position of the siRNA-binding target region.**
- **Secondary and tertiary structures in mRNA.**
- **Binding of mRNA-associated proteins.**
- **Basepairing with siRNA.**
- **The rate of mRNA translation.**
- **The number of polysomes that are associated with translating mRNA.**
- **The abundance and half-life of mRNA.**
- **The subcellular location of mRNA.**

efficiently terminates transcription in the absence of other factors[70,71].

Two approaches have been used for the expression of siRNA species by constructs that are driven by RNA pol III. In the first approach, the sense and antisense strands of the siRNA are expressed from different, usually TANDEM, promoters. Alternatively, short hairpin (sh)RNAs are expressed and processed by Dicer into siRNAs.

Expression of short RNA from tandem promoters. Several groups have recently described tandem U6 promoters that express the sense and the antisense strands from separate transcription units (FIG. 2B). *In vivo*, these strands come together to form a 19-nt duplex with 4-nt overhangs from the pol III termination signal. Miyagashi and Taira[46] used this technology successfully to target the green fluorescent protein (*GFP*) and *luciferase* genes as well as endogenous β-catenin expression. Lee *et al.*[53] applied this technology to target the HIV-1 *rev* gene and showed that it efficiently decreased the expression of a rev–GFP fusion protein. They also found that the co-transfection of the *rev* siRNA expression construct with the HIV-1 genomic DNA (NL43) in 293T cells caused a marked decrease in virus production.

Expression of short hairpin RNA. Although originally identified for its ability to cleave long dsRNA, *in vitro* and *in vivo* data have shown that Dicer can process hairpin RNA structures. Dicer is required for the processing of pre-*let7* RNA, which is a structured ~70-nt hairpin, into the mature 22-nt active species miRNA[22,29,72–74]. Brummelkamp and colleagues[63] designed an H1 RNA-pol-III-based shRNA expression vector (known as pSuper) to produce hairpin RNA with a 19-nt stem and a short loop. This system was used to inhibit the expression of CDH1 (E-cadherin) and p53 with an efficiency that was comparable to siRNA transfection. Using RNA structures based on the *let7* precursor, Paddison *et al.*[65] targeted *luciferase* mRNA for degradation by including a 32-nt sequence that was complementary to *luciferase* in the stem of the hairpin. When transfected into *Drosophila* S2 cells, they found that, although the *let7*-based structures could target the *luciferase* mRNA, the most effective inhibitors had a simple hairpin structure with full complementarity in the stem. To express hairpin RNA in mammalian cells, they developed a U6 RNA-pol-III-based expression system (known as pSsh), which used a 29-nt sequence that was complementary to the *luciferase* gene and an 8-nt loop.

Several other groups have developed similar plasmid-based shRNA expression systems that differ in their stem length and loop length and composition. BOX 4 summarizes some of the important issues to consider when designing effective shRNA-based silencing systems.

Although most expression systems use either the U6 or H1 promoter, Kawasaki and Taira[24] recently described an expression system that uses the transfer (t)RNA^{Val} promoter. shRNAs that have been generated from this expression system show a strong cytoplasmic localization and are efficiently processed by Dicer into siRNAs.

Separate strands versus hairpin RNA. The main difference between the expression of the siRNAs as two different strands (sense and antisense) and the expression of the siRNAs from hairpin RNA is the dependency of the shRNA on Dicer processing. It is difficult to say which of these technologies is more efficient as a tool for the inhibition of gene expression. However, Hutvagner and Zamore[22] found that the introduction of 100 nM of the hairpin-structured pre-*let7* RNA into HeLa cytoplasmic extracts resulted in ~5 nM of Dicer-processed product (*let7* miRNA), which was able to target mRNA containing the complementary sequence as efficiently as 100 nM *let7* siRNA. This may imply that the RNA molecules that are produced by Dicer cleavage enter the RISC-mediated 'slicing' step of the pathway more efficiently than RNA molecules that are given directly as siRNAs.

Virus-vector-mediated RNAi

Although plasmid vectors have been effective at delivering siRNAs they have several limitations (BOX 5). To overcome some of these limitations, several groups have reported the use of retrovirus vectors to deliver siRNAs into cells[48,57,75–78]. Two types of retrovirus vectors have been used as gene delivery systems: oncoretrovirus vectors that are based on the Moloney murine leukemia virus (MoMuLV) or the murine stem cell virus (MSCV), and lentivirus vectors that are derived from human immunodeficiency virus-1 (HIV-1).

Oncoretrovirus vectors. Paddison and Hannon[57] incorporated a U6 expression cassette into the LONG TERMINAL REPEAT (LTR) of the MoMuLV-based vector, pBabe-puro. Owing to the activity of the REVERSE TRANSCRIPTASE, which duplicates the LTR, the proviral (integrated) form contains two copies of the LTR and therefore two copies of

TANDEM PROMOTERS
Promoters that are arranged in the same orientation in close proximity on a contiguous piece of DNA.

LONG TERMINAL REPEAT (LTR). A sequence that is repeated at both ends of a retroviral DNA that is required for retroviral insertion into its target genomic DNA.

REVERSE TRANSCRIPTASE
An enzyme that is used by retroviruses and retrotransposons to synthesize DNA.

Box 3 | **Limitations of gene silencing by transfected siRNA**

Although short interfering (si)RNAs have proven to be very potent inhibitors of gene expression and have allowed for the elucidation and better understanding of gene functions in many different cell lines and organisms, there are several limitations to siRNA-knockdown technology.

Transient nature of the response

The transduction of siRNA into cells leads to only a transient knockdown of the gene of interest. As siRNAs seem to be relatively resistant to degradation, the transient nature of the knockdown is determined by the rate of cell growth and the dilution of the siRNAs below a crucial threshold level that is necessary to maintain the inhibition of gene expression.

In actively dividing cells, the duration of silencing is directly related to the number of cell doublings. For example, in HeLa cells, which double approximately every 24 hours, the maximum amount of silencing is usually seen ~72 hours post-transfection, depending on the gene targeted[99]. However, we have targeted a gene the knockdown of which leads to a decrease in the doubling time. In these cells the maximum level of silencing was observed at 96 hours and the length of the silencing was extended by several days (D.M.D. and P.A.S., unpublished observations).

Another factor that could limit siRNA-mediated silencing is the half-life of the protein. It might be difficult to effectively silence genes that encode proteins with long half-lives by transient transfection of siRNA.

Transduction problems

The introduction of siRNAs to mammalian cells has been accomplished by the transfection of the siRNAs using lipid-based reagents[20,99]. Each cell type must be optimized with respect to the number of cells plated and the cells:siRNA:lipid-carrier ratio for efficient transfection. There are many cell lines that are refractory to transfection including many primary cells, which might require electroporation for the delivery of siRNAs[63,101]. Although this technique increases the number of cells that have taken up siRNAs, many cells die during electroporation.

Non-renewable nature of siRNAs

Unlike plasmid DNA, which can be grown in bacteria for the production of large amounts of plasmid DNA vectors, siRNAs must be chemically or enzymatically synthesized, which remains a costly process.

the U6 expression cassette. Expression of shRNA against the tumour suppressor p53 silenced p53 stably, and resulted in a bypassing of senescence and a transformed morphology that showed little or no apparent growth arrest. shRNAs targeted against different sites on the p53 gene resulted in different levels of silencing in retrovirally infected haematopoetic stem cells derived from Eμ-myc mice that aberrantly express the *myc* oncogene in lymphocytes[48]. When the different cell lines were used to reconstitute the immune system of lethally irradiated mice, the mice developed Myc-induced lymphomagenesis whose severity correlated directly with the degree of p53 silencing.

Brummelkamp and colleagues[75] incorporated a H1 expression cassette into a self-inactivating MSCV vector and successfully targeted a constitutively active form of the *ras* oncogene (*ras-V12*) that differed by a single nucleotide from wild-type *ras*. This construct, which was used to infect human bladder cancer EJ cells, greatly decreased the expression of Ras-V12 without altering the levels of wild-type Ras. Similarly, human pancreatic carcinoma CAPAN-1 cells that were infected with this oncoretroviral vector silenced Ras-V12, leading to the loss of their oncogenic potential as shown by their inability to form colonies in soft agar and tumours in nude mice.

Lentivirus vectors. Lentiviruses are a class of retrovirus, but they have two distinct characteristics that make them more effective gene delivery vectors as compared with the oncoretrovirus vectors. Unlike oncoretrovirus vectors, HIV-1-based lentivirus vectors can infect both actively dividing and non-dividing, post-mitotic cells[79]. In addition, oncoretroviruses undergo proviral silencing during development, which leads to decreased or abrogated gene expression[80]. Lentivirus-based vectors are resistant to this silencing and therefore can be used to generate transgenic animals.

Lentivirus-delivered hairpin RNAs have been used to infect primary dendritic cells *ex vivo*[76,77]. Dendritic cells are important in the modulation of immune responses but have been difficult to study because they are refractory to transfection. Lentivirus vectors that were used to target either endogenously expressed GFP[76] or the proapoptotic Bim1 (Bcl2 interacting mediator of cell death)[77] led to a significant reduction in the level of gene expression. Primary T cells that were infected with a lentivirus targeting CD25 (the IL-2 receptor chain α) showed the functional consequences of silencing of gene expression. IL-2 is required for T-cell proliferation, and the lentivirus-infected cells showed a marked reduction (75–80%) in their ability to proliferate in the presence of IL-2 (REF. 77).

Human peripheral blood T lymphocytes that were infected with a lentivirus vector expressing a shRNA against the HIV-1 coreceptor CCR5 showed a 10-fold decrease in CCR5 expression, and when challenged with a CCR5-tropic HIV-1 virus resulted in a 3–7-fold reduction in HIV-1-infected cells[78]. Although lentivirus vectors hold promise as vehicles for gene therapy, the development of leukaemias in two patients that were undergoing retroviral-based therapy for X-linked severe combined immunodeficiency indicate that better control must be achieved before

Box 4 | **Designing shRNA-expressing vectors**

In general, chemically synthesized short interfering (si)RNA sequences that are effective at silencing gene expression are also effective when generated from short hairpin (sh)RNAs (D.M.D. and P.A.S., unpublished observations). However, the length of the stem and the size and composition of the loop might be important for the efficiency of silencing. Stem lengths of 19–29 nucleotides (nt) have been shown to silence genes effectively[63–69], which indicates that stem length is not the main parameter governing effective target-gene silencing. Loops that vary from 4–23 nt have been described[63–69], which indicates that loop lengths are also not the main parameter governing efficient gene silencing. In a direct comparison of 5-, 7- and 9-nt loops using a constant 19-nt duplex, the 9-nt loop (5′-UUCAAGAGA-3′)[63] was the most efficient silencer. It should be noted that the 9-nt loop might actually form a 5-nt loop because of U:A and U:G base pairs at the ends. As 21–22-nt short RNA were generated from a 19-nt duplexed region, processing of the 19-nt stem would require Dicer cleavage in the loop sequence[63]. In this case, the sequence and potentially the length of the loop might be more crucial for processing. In constructs that have a longer stem, Dicer could choose numerous cleavage sites without having to cleave in the loop. So, choosing hairpin structures with duplexed regions that are longer than 21 nt, regardless of loop sequences and lengths, might promote the most effective siRNA-directed silencing. More experiments are needed to establish the contribution of the stem and loop to the effectiveness of Dicer processing and to gene silencing.

There is increasing evidence that long regions of single-stranded (ss)RNA 5′ and 3′ of the hairpin RNA affect the ability to target messenger RNA cleavage[65,102,103]. It seems that shorter duplex RNAs are more sensitive to the surrounding RNA sequence than longer duplex RNAs. The incorporation of a ~70-nt pre-miR30 micro (mi)RNA sequence in a larger transcript was processed and silenced gene expression, whereas the shorter (22-nt) miR30 sequence was unable to silence gene expression, presumably because it was not processed by Dicer[102]. Xia *et al.*[103] produced similar results with a RNA-polymerase-II-driven shRNA expression construct. A U6 expression cassette containing the first 27 nt of the endogenous transcript had no detrimental effect on gene silencing[67]. However, unlike a random sequence, the first 27 nt of the U6 transcript encodes a stable hairpin structure, which might not inhibit, but actually augment production of the short RNA, thereby increasing Dicer processing near the hairpin construct.

retroviruses can be used to deliver hairpin RNAs for therapeutic purposes[81–83].

Transgene-based RNAi

With the advent of vector-mediated siRNA delivery methods it is now possible to make transgenic animals that can silence gene expression stably. This can be done by standard transgene technology[84] or by the infection of embryonic stem (ES) cells or blastocysts with lentivirus vectors.

For example, mouse ES cells have been transduced with a plasmid expressing a shRNA that targets the DNA *N*-glycosylase, Neil-1, producing several stably integrated ES cell lines with varying levels of silencing[85]. The ES cell lines were used to obtain mice that had undergone germ-line transmission of the shRNA expression cassette. The shRNA-positive F1 mice showed approximately the same level of reduction of Neil-1 as the ES cell line from which it was established, demonstrating the stability of the silencing phenotype from the ES cell lines to the mice.

Using mice and rats that endogenously express GFP, Hasuwa *et al.*[86] injected a pol III expression vector targeting GFP into the pronuclei of mice or rat single-cell embryos to produce silenced blastocysts. The resulting mice were crossed to produce F1 progeny that showed virtually complete silencing in all of the tissues that were examined. The success of transgene-based RNAi in rats means that this technique should allow the targeted silencing of genes in animals that are not amenable to homologous-recombination-based gene targeting due to the lack of ES cell lines.

Recently, Baltimore and colleagues[87] produced transgenic mice and rats that expressed endogenous GFP by infecting mouse ES cells or mouse and rat single-cell embryos with a lentivirus vector that contained the GFP gene. Unlike oncoretrovirus vectors, the transgene expression of which is silenced during development, the lentivirus-delivered transgene continued to be expressed. To show that lentivirus vectors can be used for transgene-based RNAi, fertilized eggs from GFP-positive mice were infected with a lentivirus vector that expressed siRNA that targeted GFP. The resulting blastocysts and mice had significantly reduced levels of green fluorescence[88]. Similarly, ES cells were infected with a lentivirus vector that silenced CD8 expression and then injected into RAG-DEFICIENT BLASTOCYSTS. The immune system of the resulting chimeric mice would have to come from the infected ES cells because RAG-deficient mice are not able to produce B or T cells. The transgenic mice had a greatly reduced amount of CD8-positive T cells in the thymus and spleen. The same vector was used to infect single-cell embryos, producing mice that were deficient in CD8-positive T cells[77].

The results of these transgenic experiments show that siRNA-mediated gene silencing is heritable, stable and can potentially be applied to various organisms. In addition, these results show that RNAi functions in all the cell and tissue types tested, from early embryos and blastocysts to adult animals. Methods that allow inducible and cell- and tissue-specific expression are being developed, and these will increase the versatility and applicability of these technologies.

siRNA silencing in somatic tissues

Originally described for the delivery of plasmid DNA to various organs[89,90], by the rapid injection of large volumes of physiological solution into the tail vein of postnatal mice, hydrodynamic 'high pressure' delivery of siRNAs has been used to silence gene expression in various mouse tissues[91,92]. Co-injection of a siRNA against the *luciferase* gene and a *luciferase* expression plasmid led to *luciferase* gene silencing in several tissues including liver[91,92], kidney, spleen, lung and pancreas[91]. In the case of the liver, the silencing persisted for several days. Lieberman and colleagues[93] delivered siRNAs by hydrodynamic injection into mice, silencing the proapototic Fas receptor. Fas-receptor silencing protected mice from Fas-mediated apoptosis in hepatocytes for up to 10 days after injection, despite the lack of siRNA replication mechanisms. These results show that injected siRNAs are stable and not rapidly diluted *in vivo*, and that they remain sufficiently concentrated to produce a physiological outcome, even for proteins with a long half-life, which indicates that there might be a direct application for siRNAs in the analysis of gene expression in organisms.

RAG-DEFICIENT BLASTOCYSTS
Blastocysts derived from mice that lack the recombinase-activating gene. Mice that are RAG deficient are unable to produce mature B and T cells and are therefore immunocompromised.

Box 5 | **Comparison of plasmid-based versus siRNA silencing**

There are two principal advantages of short interfering (si)RNA transfection over plasmid-based gene silencing. First, siRNA transfection is more efficient than plasmid DNA transfection. More cells will silence gene expression after siRNA transfection. Second, the initiation of siRNA-transfected silencing is immediate. Plasmid-based strategies require transcription and in the case of hairpin RNA, Dicer processing.

There are two principal advantages of plasmid-based RNA interference (RNAi) expression systems over siRNA transfection. First, plasmid DNA can be readily regenerated. Second, the duration of silencing can be extended. Transfection of siRNAs leads to transient silencing and might not work for genes that encode proteins with long half-lives (BOX 3). Cell lines can be created that stably express the short hairpin (sh)RNA and a drug-resistance marker (either on the same plasmid or from a co-transfected plasmid). Stably silenced clones can be maintained indefinitely. After plasmid transfection and drug selection for cells expressing the resistance marker, populations of cells are derived that have heterogeneous levels of silencing. To derive a homogenous population of cells that can efficiently silence gene expression, single-cell clones must be obtained and screened, which can be a laborious process. However, the utility of plasmids will be limited in cell lines that are difficult to transfect and that can not be grown for long periods of time in culture, such as primary cells.

siRNA and functional genomics

Several reverse-genetic approaches have been successfully used to inhibit gene expression, including the use of antisense and gene targeting by homologous recombination methods. As RNAi can be applied to many cell types and because the genomic sequences of many organisms are available, it is now possible to harness the technology of RNAi to look for the function of virtually all of the genes in an organism's genome.

It is fitting that the organism *C. elegans*, which has provided so much of the understanding of RNAi and small-RNA biology, has also led the way in the use of RNAi for the large-scale functional analysis of virtually all of its ~19,000 genes. *C. elegans* is a highly genetically tractable system, a large bank of mutant worm lines has been established using traditional genetic techniques, and these mutant worm lines can function as a reference point for large-scale RNAi screens.

Although functional-genomic studies using dsRNA injection have been carried out[94], the most promising approach for large-scale RNAi studies has been the development of feeding libraries. Several groups have used RNAi libraries that express dsRNA in *E. coli* to screen for genes that are involved in various traits, including abnormal anatomy and motility, altered sex ratios, sterility[95], longevity[96] and fat-regulatory genes[97]. In the most comprehensive genome-wide studies so far, Ahringer and colleagues created an RNAi feeding library that represents ~86% of the *C. elegans* genes (16,757) and identified mutant phenotypes for 1,722 genes[98]. Similar strategies are undoubtedly being pursued in other organisms[19]. Although siRNAs have to be chosen and validated for functional-genomic approaches to work in mammals, it is conceivable that groups of genes can be targeted for silencing in a cell-type-, tissue-type- or pathway-specific fashion.

Conclusions

Since its discovery in *C. elegans*, RNAi has become an effective method for the analysis of gene function. Retrovirus delivery and hydrodynamic infusion of siRNAs into primary tissues allows the analysis of gene function in a physiological context without the production of knockout mice through homologous recombination. Lentiviral delivery of hairpin RNA to ES cells or blastocysts for the production of knockdown mice allows the rapid analysis of gene function through stable and heritable gene silencing. Each of these advances has brought a functional-genomic approach to gene expression in mammals closer to reality. Not only does siRNA-based gene silencing offer the potential for gene-function determination, it holds promise for the development of therapeutic gene silencing.

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The following terms in this article are linked online to:
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