GENE SILENCING IN MAMMALS BY SMALL INTERFERING RNAs

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Among the 3 billion base pairs of the human genome, there are ~30,000–40,000 protein-coding genes, but the function of at least half of them remains unknown. A new tool — short interfering RNAs (siRNAs) — has now been developed for systematically deciphering the functions and interactions of these thousands of genes. siRNAs are an intermediate of RNA interference, the process by which double-stranded RNA silences homologous genes. Although the use of siRNAs to silence genes in vertebrate cells was only reported a year ago, the emerging literature indicates that most vertebrate genes can be studied with this technology.

FORWARD GENETICS
A genetic analysis that proceeds from phenotype to genotype by positional cloning or candidate-gene analysis.

REVERSE GENETICS
A genetic analysis that proceeds from genotype to phenotype by gene-manipulation techniques, such as homologous recombination in embryonic stem cells.

RIBOZYME
An RNA molecule with catalytic activity.

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In the early days of molecular biology, genes were first defined through the description of their mutant phenotype. FORWARD GENETICS has the advantage that the phenotype of the mutant gives a clue to the function of the gene. But with the advent of large-scale genome sequencing the situation is different — literally thousands of genes have been identified, but we know nothing of their function. REVERSE GENETICS is now the most effective way to assess the function of a gene, but so far there has been no general method for reverse genetics other than gene targeting by homologous recombination, which is slow and costly. Antisense approaches, such as antisense oligonucleotide and RIBOZYME technologies, have been useful in reverse genetics, but only to a limited degree. By contrast, the promise of small interfering RNA (siRNA) technology to 'knock down' the expression of any gene in vertebrate cells is set to revolutionize reverse genetic approaches.

In 1998, and at the time of the completion of the *Caenorhabditis elegans* genome project, Andrew Fire and Craig Mello described a new technology that was based on the silencing of specific genes by double-stranded RNA (dsRNA); a technology they called RNA interference (RNAi)[1]. This discovery related previous work on post-transcriptional gene silencing (PTGS) in plants (for recent reviews, see REFS 2,3) to the activity of dsRNA. Fire, Mello and colleagues showed that, in *C. elegans*, the presence of just a few molecules of dsRNA was sufficient to almost completely abolish the expression of a gene that was homologous to the dsRNA. This report was followed by a flood of papers that described RNAi silencing of *C. elegans* genes, which has irreversibly changed the field of *C. elegans* genetics. Since the recent development of RNAi technology in mammalian systems, a similar flood of papers has appeared. However, this time investigators are not only using RNAi to elucidate gene function, but also developing antiviral therapeutics. RNAi is evolving at a faster pace than ever, and this review provides a practical and theoretical update on the newest approaches to silencing mammalian genes in cell culture.

Introducing the short interfering RNAs

The discovery of RNAi was followed by studies of its mechanisms. Work in *C. elegans* indicated that RNAi involved at least two important steps, and that the first step probably involved the generation of a sequence-specific silencing agent[4]. A strong candidate for this agent was a special class of short RNAs that was originally reported by Andrew Hamilton and David Baulcombe[5]. They found that *Arabidopsis* plants undergoing transgene- or virus-induced PTGS contained 21–25-nucleotide (nt) long RNAs that were complementary to both strands of the silenced gene and that had been processed from a long dsRNA precursor. The processing of long dsRNAs to 21–23-nt RNAs was recapitulated *in vitro*, using extracts from *Drosophila*

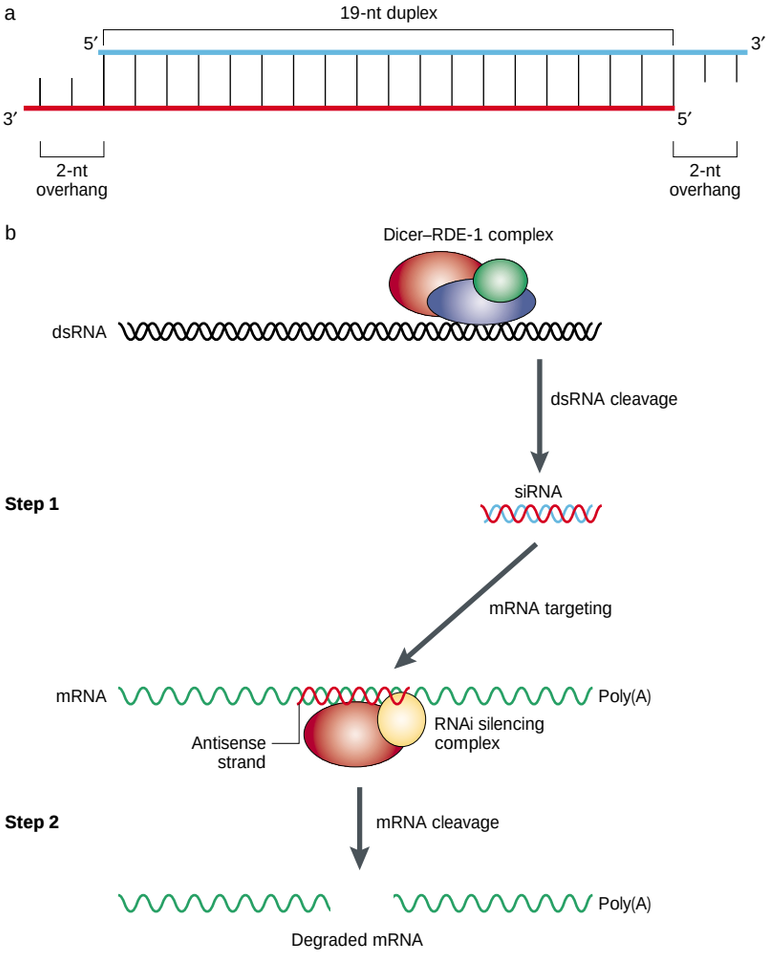

Figure 1 | **Short interfering RNAs are at the heart of RNA interference. a** | Short interfering RNAs (siRNAs) typically consist of two 21-nucleotide (nt) single-stranded RNAs that form a 19-bp duplex with 2-nt 3′ overhangs. **b** | Long double-stranded RNA (dsRNA) is processed by the Dicer–RDE-1 complex to form siRNAs. The antisense strand of the siRNA is used by an RNA interference (RNAi) silencing complex to guide mRNA cleavage, so promoting mRNA degradation. RDE-1 (RNAi deficient-1)[99].

melanogaster embryos[6] and extracts from *Drosophila* S2 cells (a standard *Drosophila* cell line)[7]. Other investigations revealed that small 21–23-nt RNAs were associated with RNAi silencing in *C. elegans* and *Drosophila*[8–10]. The cloning and sequencing of these RNAs revealed that they had a very specific structure: 21–23-nt dsRNAs with 2-nt 3′-end overhangs[11] (FIG. 1a). The evidence that these short RNAs determined RNAi specificity came from studies in *Drosophila*, in which small RNAs that were isolated from cells undergoing silencing were shown to be sufficient to induce specific silencing in naive *Drosophila* embryo lysates and S2 cells[6,9,11]. In addition, when synthetic 21- and 22-nt RNA duplexes were added to the lysate they were able to guide efficient sequence-specific mRNA degradation (FIG. 1b). These small RNAs were named short interfering RNAs (siRNAs). Since these reports, RNA synthesizers around the world have been working overtime to meet the demand for synthetic siRNAs, which seem to be as active as the ones produced enzymatically *in vitro*. Because chemically synthesized siRNAs can be expensive, several groups have devised strategies to synthesize short RNAs *in vitro* that rely on T7 phage polymerase. In one scheme, the T7 polymerase is used to transcribe individual siRNA sense and antisense strands, which are then annealed to produce a siRNA[12,13]. In another scheme, the T7 polymerase is used to transcribe siRNA strands that are linked *in cis*, so forming a hairpin structure[13,14]. Notably, in both cases, the transcribed RNAs contain 5′ triphosphate termini, as opposed to a 5′ monophosphate that is required for siRNA functionality in *Drosophila*[15]. So, either the 5′ triphosphate is converted to a 5′ monophosphate in mammalian cells, or their 5′ structure requirement is not as stringent. Another group has developed an approach to produce siRNAs from *in vitro* nucleolytic processing of longer dsRNAs. In this strategy, long *in vitro*-transcribed dsRNA is processed to siRNAs by a purified bacterial RNase III enzyme[16].

Introducing the small temporal RNA

A new strategy for regulating gene expression *in vivo* was discovered when *lin-4* (lineage-abnormal-4) and *let-7* (*lethal-7*) RNAs were identified in *C. elegans*[17,18]. The *lin-4* and *let-7* RNAs are unusual because they are expressed as 22-nt RNAs, having been processed from ~70-nt precursor hairpins. Both genes affect the timing and sequence of postembryonic development in *C. elegans*[18–22], and worms that are mutant at either of these loci are said to have a heterochronic phenotype — a defect in developmental timing. Worms that are mutant for *lin-4* reiterate an early larval developmental stage instead of progressing to later stages of development, whereas those that are mutant for *let-7* do not progress through the last larval moult into adulthood.

The *lin-4* RNA is a negative regulator of the developmentally important protein-coding *lin-14* and *lin-28* genes[17,19–21]. The 22-nt *lin-4* RNA contains homology to specific regions (target sequences) of the 3′ untranslated regions (UTRs) of *lin-14* and *lin-28* and, perhaps, to other developmentally important genes. Genetic data indicate that the deletion of the *lin-4* target sequences in the 3′ UTR causes an unregulated gain-of-function phenotype. Similarly, fusion of the *lin-4* 3′ UTR to a reporter renders it susceptible to developmental regulation[17,21]. Northern blot analysis of the target mRNAs indicates that the message might remain stably associated with POLYSOMES, and the current models suggest that *lin-4*, and probably *let-7*, brings about the translational repression of its targets[19].

let-7 is a translational negative regulator that targets the 3′ UTR of *lin-41* (FIG. 2a), another developmentally important gene. The sequence of *let-7* is remarkably conserved among bilaterally symmetrical animals, and Northern blots confirmed that *let-7* was indeed expressed across phyla[23]. On the basis of these data, Amy Pasquinelli *et al.* proposed the name small temporal RNAs (stRNAs) for genes such as *lin-4* and *let-7*, and have suggested that other similar RNAs might be found[23].

POLYSOME
A functional unit of protein synthesis that consists of several ribosomes that are attached along the length of a single molecule of RNA.

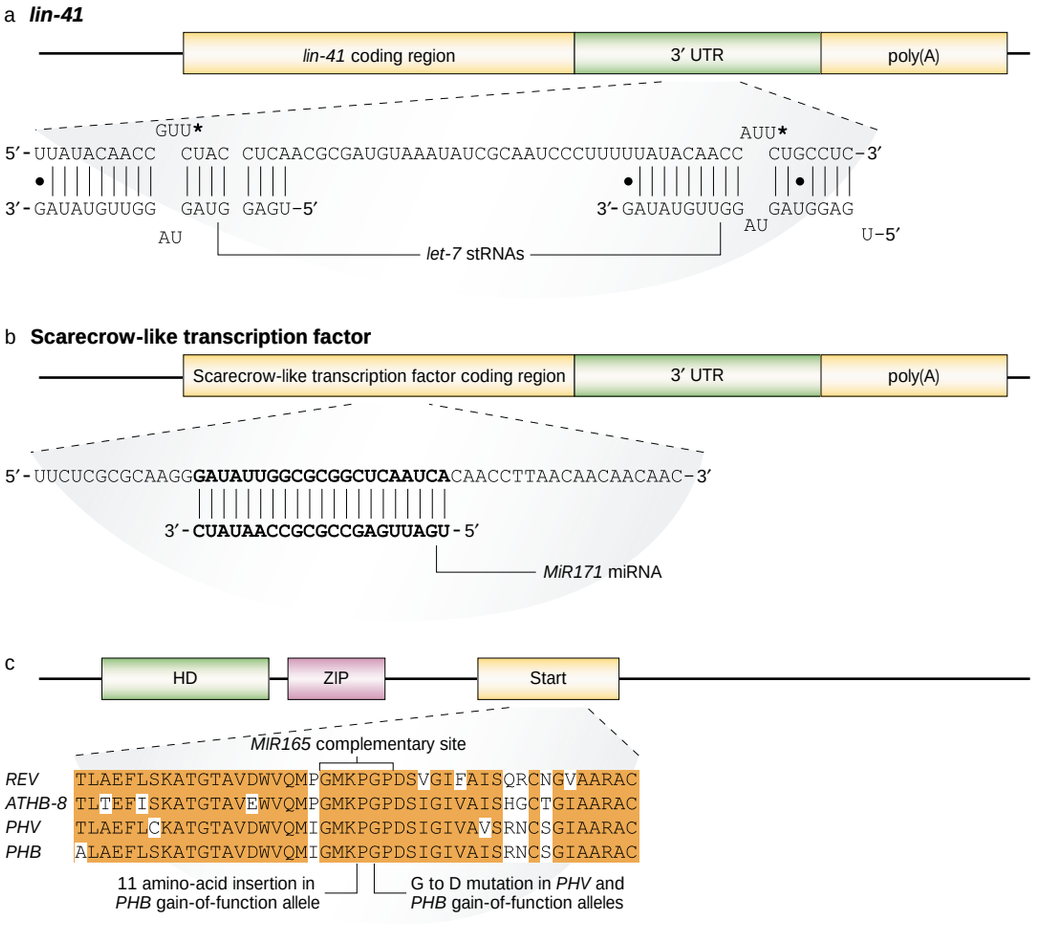

Figure 2 | **Small RNAs in development. a** | An example of a short temporal (st)RNA–mRNA interaction in *Caenorhabditis elegans*. *let-7* (*lethal-7*) encodes a non-coding hairpin RNA that is processed to form a 22-nucleotide (nt) stRNA, which regulates *lin-41* (lineage-abnormal-41) expression. *lin-41* in turn regulates the timing of the transition from the last larval stage to adulthood. The *let-7* stRNA is shown annealed to two target sites in the 3′ untranslated region (UTR) of *lin-41* (a line indicates complementary base pairing, whereas a dot indicates a mismatch). The *lin-41* UTR contains other presumptive interaction sites for *let-7* and perhaps *lin-4* stRNA, another micro (mi)RNA that controls developmental timing. The asterisks indicate a mismatch. **b** | Plant miRNAs are nearly perfectly complementary to their putative targets. So far, 15 identified plant miRNAs contain perfect or near-perfect complementarity to sequences in plant mRNAs[41,43]. The *MIR171* miRNA is perfectly complementary to sequences in mRNAs from three developmentally important Scarecrow-like transcription factor family[41,42]. **c** | The *MIR165* miRNA potentially targets the PHABULOSA (*PHB*) and PHAVOLUTA (*PHV*) mRNAs[43]. Its predicted target sequence lies in a region to which previously identified gain-of-function mutations map. If *MIR165* miRNA can no longer bind to these mutated sequences, then the ectopic expression of *PHB* and *PHV* in these mutants might result from a loss of *MIR165*-mediated repression[43,95]. The *Arabidopsis* ATHB proteins are characterized by the presence of a homeodomain (HD) with a closely linked leucine zipper motif (ZIP) and are able to bind DYAD-SYMMETRIC DNA sequences in a different way to the classic HD proteins. Moreover, they contain a star-related lipid-transfer (START) domain, which is a property of proteins that are involved in lipid transport, signal transduction and transcriptional regulation. PHB, PHV, REV (REVOLUTA) and Scarecrow-like proteins have been implicated in cell patterning during development[95,96]. *ATHB-8* (Athiln-8).

A common pathway for stRNAs and siRNAs

The size similarity between the siRNAs and stRNAs indicated a potential link between RNAi and stRNA-mediated silencing[23–25]. Genetic data on silencing through siRNAs and stRNAs revealed that the enzyme Dicer links these two processes[26,27]. Dicer contains two RNase III motifs, an RNA helicase domain and a dsRNA-binding domain, and as initially predicted by Brenda Bass[28], this enzyme catalyses the cleavage of long dsRNA to 21–23-nt siRNA products[7]. In addition to being involved in siRNA production[7], Dicer is also involved in the processing of the 21–23-nt stRNAs from ~70-nt stable hairpin (or stem–loop) precursors[26,27,29]. When Dicer was knocked down in human HeLa cells using siRNAs, 72-nt unprocessed human *let-7* precursor accumulated in these cells[29]. Analogously, knocking down Dicer in worms resulted in heterochronic phenotypes, as well as in the accumulation of unprocessed *let-7* and *lin-4* (REFS 26,27,30).

The EIF2C (elongation initiation factor 2c)/Argonaute class of proteins provides another link between RNAi and stRNAs. This class of proteins is

DYAD SYMMETRY
A sequence is said to be dyad symmetric (or pallindromic) when it is symmetric about a central axis. **[Au: ok?]**

characterized by a conserved PAZ (Piwi-argonaute-zwille) domain, which is also found in Dicer. Although the function of this domain is unknown, it was shown that proteins of this family have a crucial role in RNAi and PTGS in different organisms[31–33]. The original descriptions of the EIF2C class characterized its association with the ribosome and its role in protein translation[34,35], and there is evidence that the RNAi machinery might be associated with the ribosomal machinery[10]. It is disappointing that this family does not contain any obvious motifs or domains that could indicate how its members might function in the cell. Worms that are deficient for two proteins in this class, ALG-1 (Argonaute-like gene 1) and ALG-2, have a heterochronic phenotype[26] and accumulate unprocessed *lin-4* precursor hairpins. Similarly, two Argonaute homologues are required for RNAi in *Drosophila*[31,33]. In fact, the human Argonaute protein, EIF2C, purifies as a component of the RNAi-induced silencing complex (RISC)[36,51].

Recently, many more 21–23-nt RNAs have been found in different organisms. Endogenous 21- and 22-mer RNAs of *Drosophila* were identified in the course of cloning and analysing 21–23-nt RNAs that had been produced in a *Drosophila* RNAi *in vitro* assay[11]. Soon after, three groups embarked on specifically identifying endogenous small RNA regulators in worms, flies and humans[37–39]. These early efforts have resulted in the identification of more than 90 potential hairpin structures, the flanking sequences of which could generate 21–23-nt regulating RNAs. Because not all of these RNAs seemed to be temporally expressed like *lin-4* and *let-7*, the term micro-RNAs (miRNAs) was given to this large class of 21–23-nt RNAs. So, the *lin-4* and *let-7* RNAs are considered to be miRNAs as well, but because of their temporal expression and function, they satisfy the definition of stRNAs. Similarly, it is possible that other functional groupings will emerge, such as miRNAs that mediate spatial development (sdRNAs), the stress response (srRNAs) or even the cell cycle (ccRNAs). Analysis of the miRNA distribution in mouse tissues has indicated that many of them are tissue specific[40]. The same can be said for plants, in which some miRNAs are enriched in specific plant organs, such as flowers[41,42]. Recent data from David Bartel and colleagues indicate that miRNAs in plants might target open reading frames (ORFs) and possibly silence genes through mRNA-degradative siRNA-like mechanisms[43]. It seems that, in plants, most miRNAs are perfectly, or near-perfectly, complementary to sequences in the mRNAs of several transcription factors[41,43] (FIG. 2b).

In mammals, many of the miRNAs are produced in a stage- and/or tissue-specific manner, potentially implicating them in the stRNA pathways. Many of the vertebrate miRNAs were identified just once, indicating that the sequencing screens that lead to their identification have not been saturating. So, the number of miRNAs might be far greater than the ~150 sequences so far identified. The group of Gideon Dreyfuss described ~40 miRNAs, a few of which were identical to those previously reported[44]. This new batch was identified from EIF2C immunoprecipitations of GEMIN3 (also known as DDX20, DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 20) and GEMIN4 (Gem (nuclear organelle)-associated protein 4) proteins, two proteins that are known to associate with the survival of motor neurons (SMN) complex. This complex is known to have an important role in the assembly, restructuring and function of diverse ribonucleoprotein (RNP) complexes[45–50]. Importantly, instead of SMN, the complex of miRNAs and GEMIN3/4 contains the Argonaute homologue EIF2C and the enzyme DICER (Z. Mourelatos, personal communication). It is not clear whether the GEMIN3/4 complex has a role in RNAi, but GEMIN3 contains helicase motifs, suggesting that it might have a role in the processing of miRNAs[44]. Data from György Hutvágner and Phillip Zamore support the finding that miRNAs and siRNAs might reside in the same complex[51]. They found that the endogenous human homologue of *let-7* was present in a complex that can direct targeted degradation of an exogenous mRNA that contains a site of perfect complementarity to *let-7* (FIG. 3). Together, these studies provide extra links between RNAi and the endogenous miRNAs.

Using siRNAs in mammalian cells

When RNAi was first discovered in the worm, researchers immediately began using this technology to analyse the functions of genes. A similarly rapid adoption of siRNA technology followed the definition of the structure of the siRNAs and their ability to silence genes in mammalian cells[11,52]. More and more groups are seizing siRNA technology to elucidate mammalian gene function.

TABLE 1 summarizes the work of several groups who have recently reported successful siRNA-mediated knockdown of mammalian genes. A brief inspection shows that the types of gene that are being knocked down vary widely, from structural to catalytic proteins. HeLa cells, well known for their ease of transfection, are now the cell line of choice for siRNA knockdowns. Essentially, 100% of HeLa cells can be transfected with siRNAs using lipophilic agents that will transport the nucleic acids across the membranes. The efficiency of transfection is typically higher for siRNAs than for plasmid DNA, for any given transfection agent or method[53] (M.T.M. and P.A.S., unpublished data). As TABLE 1 indicates, various cell lines from different species have also been shown to be good recipients for siRNAs. At present, the transfectability of cells is the limiting step in siRNA-mediated gene silencing. PRIMARY CELLS are notorious for having low transfection efficiencies (less than a few per cent) when plasmid DNA is used. However, a few groups have carried out RNAi in cells that are difficult to transform, such as in T cells and primary cells[53–55].

The efficiency of transfection depends not only on the cell type, but also on the passage number and the CONFLUENCY of the cells. Most groups use Oligofectamine™ as a cationic lipid carrier for nucleic acids because of its low toxicity and its ability to transfect at low cell confluency. Depending on the cell type used, non-cationic lipid-based carriers (such as Transit-TKO™) might be preferable. Many other transfection

PRIMARY CELL
A cell that is taken from a tissue source and whose progeny are grown in culture, before subdivision and transfer to a subculture.

CONFLUENCE
The degree at which a monolayer of tissue culture cells occupies the growth dish.

DEAD BOX
A highly conserved motif in a family of putative RNA helicases; it takes its name from the single letter code for its amino-acid sequence.

reagents are available, and as different reagents can be cell specific, it is always prudent to test which one works best for a given cell line. Several groups have used multiple lipid transfections for siRNA-mediated knockdowns (TABLE 1). These knockdowns have to be carried out over a period of a week, and might be useful for proteins that are unusually stable or when cells need to be grown for a long time to observe a phenotype. siRNA can also be electroporated into cells that are difficult to transfect[53].

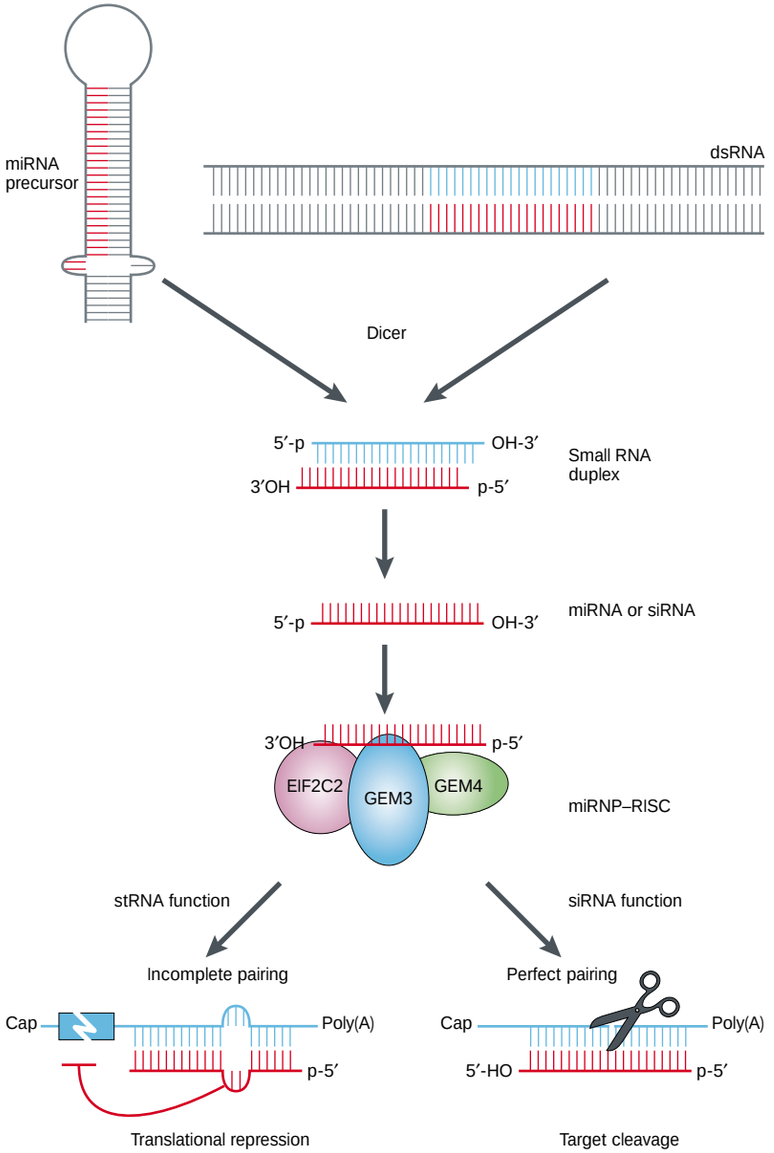

Figure 3 | **MicroRNAs and short interfering RNAs might use the same RNA-processing complex to direct silencing**. Accumulating data indicate that microRNAs (miRNAs) and short interfering (siRNAs) might reside in the same silencing complex that could also contain EIF2C2 (a eukaryotic translation initiation factor), GEMIN3 and GEMIN4 (GEM3, GEM4, DEAD BOX containing, putative RNA helicases)[33,36,44,51]. Processing of the miRNA precursor hairpin or long double-stranded RNA (dsRNA) would lead to a single-stranded ~21–23-nucleotide (nt) RNA that is associated with the miRNP–RISC complex. This complex might direct either mRNA translational repression or mRNA target cleavage, depending on the degree of complementarity between the ~21–23-nt RNA and the mRNA (the blue box represents the coding region). RISC, RNAi-silencing complex; miRNP, microribonucleoprotein; stRNA, small temporal RNA.

Although this method gives high siRNA transfection efficiency (>95%), 50% or more cells can die during the electroporation. If the cells are adherent, dead cells can simply be washed off the plate on the following day.

In several lower eukaryotes, an RNA-dependent polymerase is thought to amplify the introduced long dsRNA, possibly leading to higher levels of siRNAs[56–59]. In *C. elegans*, data support the idea that siRNA pairing with mRNA results in the extension of the siRNA along the mRNA template to produce a long dsRNA, which could then be processed by Dicer to produce even more siRNAs[59]. Mammalian cells probably lack the siRNA amplification mechanisms that confer RNAi potency and longevity in organisms such as worms or plants. This being the case, it seems intuitive that a highly expressed mammalian transcript might be more difficult to knock down than a rare one. Although this question has not been systematically studied, moderately to highly expressed genes, such as the nuclear lamin genes, can be knocked down to undetectable levels[52,60]. Also, the abundant transcripts of actin and vimentin can be knocked down to levels that are sufficient to confer phenotypes[60]. This is probably because each RNAi silencing complex in the cell can carry out several rounds of mRNA cleavage[51]. Differences in siRNA effectiveness, transfection efficiencies, cell type and protein stability might all contribute to the efficiency of siRNA-mediated RNAi, making it difficult to compare the results from studies of different genes. A scan of TABLE 1 yields numerous examples of genes that are expressed at low levels and that can be efficiently silenced.

But what if we wanted to knock down two or more highly expressed genes simultaneously? One report indicates that a double knockdown of the nuclear mitotic apparatus protein (NUMA1) and lamin can be accomplished in HeLa cells[61]. But M.T.M. and colleagues reported that the simultaneous knockdown of the highly abundant T-cell CD4 and CD8 cell-surface markers was not trivial, and that CD4 siRNAs could compete with CD8 siRNAs in silencing experiments[53]. These results support the idea that the RNAi machinery might be titratable or limiting in mammalian cells and in *Drosophila*[9,62]. So, the use of several siRNAs to study different genes in a given pathway or network is likely to require careful control of siRNA concentrations.

It is important to establish a time course for the knockdown of protein levels in siRNA experiments. Generally, the siRNA directs rapid reduction in mRNA levels that is readily observable in 18 hours or less[53]. However, different proteins have different turnover rates, and stable proteins require a longer period of exposure to siRNAs to be knocked down than do less stable ones. In most cases, siRNA-mediated RNAi lasts for ~3–5 cell doublings, that is, 3–5 days for most cell lines, and normal gene expression resumes in ~7–10 cell doublings. If the cells are serum starved or their growth is otherwise arrested, this window might be extended, indicating that the loss of siRNA effectiveness is probably due to dilution below an effective level, rather than degradation of the siRNAs.

Table 1 | Use of small interfering RNAs in mammalian cells

Targeted genes	Cell type	Location on mRNA*	Effective/ tested siRNAs	Time (hours)	%KD	Verification‡	No. of tfxs	Tfx method§	Phenotype	References
CEP135	CHO	8–26	1/1	48	85–90	W, IF	2	Lfm	Cell growth, microtubule organization	100
CP110	HeLa	397–418, 896–917	2/2	48	97–99	W, IF	3	Ofm	Centrosome amplification	101
Multiple (21)	HeLa, 3T3, rat FB	See reference	21/22	40–48	See reference	W, IF	1	Ofm	Apoptosis, arrest, loss of stress fibres, aberrant spindles, blebbing; 13 out of 21 genes were essential for growth	60
RalA, *RASSF1*	HeLa, Caco2	NR	2/2	72	97–99	W	1	Ofm	Exocytosis defect	102
ShcA	HeLa	677–697, 236–256	2/2	48	97–99	W	1	Ofm	ND	103
HMps1 kinase	HeLa	135–155	1/1	48–72	NR	W, IF	1	Ofm	Checkpoint-arrest defect	104
PRSC (corin)	HL-5, 293	NR	1/1	24–28	NR	NR	1	Ofm	Inhibition of pro-ANP processing	105
GAGII	HeLa	65–87, 461–482	2/2	96	NR	NR	3	Ofm	Inhibition of GAG synthesis	106
CD4, *CD8*	T cells, HeLa	See reference	1/5	36	85–90	N, F	1	E, Ofm	Inhibition of CD4, CD8; primary T cells	53
HTF, *PSKH1*	HeLa, Cos-1, 293, HaCaT	See reference	6/16	96–120	90	N	1	Lpf	Inhibition of TF activity	63
Tsg101	293T	413–433	1/1	72	NR	N, W	1	Ofm	Arrest of HIV budding	107
Cdc14A	HeLa	89–109	1/1	48–72	NR	IF, W	1	Ofm	Aberrant chromosome partioning	108
Rad17	HeLa	NR	1/1	48	NR	W, IF	2	Ofm	Defective DNA–damage response	109
EGFP	HeLa, 293, MEF	NR	1/1	24	>85	N, F	1	Lfm	GFP silencing	54
Luc, *LMNA*	HeLa, cos, 293, 3T3	Distributed	6/7	40–48	>85	W, IF	1–3	Ofm, Lfm	Luciferase, lamin silencing	52

*Location of the siRNA target site along the mRNA is expressed as the number of nucleotides downstream from the start codon. ‡Knockdown (KD) verification was carried out by western blot (W), northern blot (N), immunofluorescence (IF) or flow cytometry (F). Transfection (Tfx) method§ included Oligofectamine (Ofm), Lipofectamine (Lfm), Lipofectin (Lpf) and electroporation (E). ANP, atrial natriuretic peptide; *Cdc14A*, cell division cycle 14 homologue A; *CEP135*, centrosomal protein of 135 kDa; *CP110*, centriolar protein of 110 kDa; EGFP, enhanced green fluorescent protein; GAG, glycosaminoglycan; *GAGII*, beta 3GalT6; *HMps1* kinase, human mono polar spindle-kinase; *HTF*, human tissue factor; *LMNA*, lamin A/C; *Luc*, luciferase; ND, not determined; NR, not reported; *PSKH1*, protein serine kinase H1; *Rad17*, *RAD17* homologue; *RalA*, v-ral simian leukaemia viral oncogene homologue A; *RASSF1*, Ras association (RalGDS/AF-6) domain family 1; *ShcA*, src homology 2 domain-containing transforming protein C1; *Tsg101*, tumour susceptibility gene 101.

mRNA targeting by siRNAs

Typically, the objective of using siRNAs is to silence a specific gene in a mammalian cell, therefore the base-pairing region for an siRNA must be selected carefully to avoid chance complementarity to an unrelated mRNA. BLAST sequence analysis programs should be used to screen candidate siRNA sequences for specificity, although this recommendation is based on the assumption that the sequence database is complete. To a certain degree, RNAi can tolerate siRNA:mRNA mismatches, with 1- and 2-bp mismatches only partially reducing the rate and extent of cleavage[63], particularly if the mismatches occur near the end of the duplex[64]. It has been suggested that the first 50–100 nt of a cDNA sequence, downstream of the translation start site, should be used to target a gene, and that 5′ or 3′ UTRs, as well as the regions around the start site, should be avoided, as they might be rich in regulatory protein binding sites[65]. However, we do not yet know if any mRNA region is the most optimal for siRNA targeting. In fact, the data in TABLE 1 indicate that successful RNAi can be attained by targeting regions that are distributed throughout the mRNA, including the 3′ UTR. However, targeting different regions of a given mRNA might give different results. For example, only about one in five siRNAs that are specific for the mouse *CD4* and *CD8* genes tested were effective[53]. In this case, only those that targeted the 3′ UTR or the vicinity of the mouse *CD4* and *CD8* genes were active. By contrast, siRNAs that target the ORF of human *CD4* are effective[66,67]. One advantage of targeting the 3′ UTR is that the knockdown phenotype can be verified by transfecting a plasmid that contains the gene sequence with a different 3′ UTR.

So far, there has not yet been a systematic study of relative efficiencies of different siRNAs tiled across an entire length of mRNA. Torgeir Holen *et al.* carried out limited tiling across a region of the human coagulation trigger factor gene (*TF*)[63]. These experiments indicated positional effects at the level of codon resolution; that is, shifting the siRNA target site by only three nucleotides at a time resulted in different degrees of silencing. These studies indicate that mRNA structure might govern accessibility to the siRNA. Alternatively, the sequence composition of siRNAs could influence its activity *in vivo*. Michaela Scherr and colleagues have developed a method for selecting regions of mRNA that are most effective for antisense targeting, and this method has already been used to target HIV with siRNAs[67–69]. Further studies will show whether this method is generally applicable. In practical terms, if knockdown of a gene is not seen, it is easiest to simply try another siRNA sequence that targets a different region of the gene.

Inhibiting viral infection in mammalian cells

RNAi and related phenomena, such as PTGS, protect plant tissues from viral infection (for a review, see REF. 70). This is probably also the case in other organisms, but so far there has been no evidence for endogenous RNAi activity that limits virus infection in vertebrates. Initial studies to test the potential application of synthetic siRNAs to inhibit virus infection have been promising[55,66,67,71–73,74].

Vira Bitko and Sailen Barik recently described an approach to silence mRNAs that are expressed by the RESPIRATORY SYNCYTIAL VIRUS (RSV), a negative-strand virus that is the causative agent of a severe respiratory disease[71]. In these studies, siRNAs were directed against specific RSV mRNAs, resulting in decreased mRNA expression. However, a knockdown of the full-length viral genomic strand was not seen, despite the effects on the viral mRNAs. The authors reasoned that the viral genome might be inaccessible to siRNAs, perhaps due to being enclosed in a condensed protein–RNA complex. In other studies, siRNA-mediated silencing of HIV was seen in cultured cell lines and in human primary T cells[55,66,67,73,74]. In these experiments, siRNAs were used to target the HIV long-terminal-repeat sequence, as well as five genes that are encoded by the HIV virus. From these studies, it is clear that the silencing of HIV by siRNAs can reduce viral levels by as much as 30–50-fold. Some reduction is also seen with siRNAs that contain a single nucleotide mismatch, but not with four nucleotide mismatches[55]. siRNAs can also confer resistance to poliovirus[72]. In this study, the protective effect of siRNAs over time was evaluated, and siRNA-resistant virus was detected. However, resistant virus molecules were already present in the original viral population and were given a selective advantage on treatment with siRNA. These data indicate that several siRNA sequences should be used to avoid selection pressure for the replication of viruses that render them immune to siRNA-mediated silencing. Although these data should be considered as preliminary in terms of medical applications, they provide strong justification for further investigation of the use of siRNAs as antiviral agents in therapeutic settings.

Recent data indicate that the *in vivo* delivery of siRNAs in mice is possible. In two studies, siRNAs were injected under hydrostatic pressure into the mouse tail vein, and silencing of reporter transgenes was observed in different tissues[75,76]. Not all tissues responded equally to the siRNA treatment, but 5–10-fold downregulation of transgene expression was seen in the liver, spleen, lung, pancreas and kidney. One study showed effective silencing of a hepatitis C viral transgene that was co-transfected with siRNAs[75]. The transfection method used in these studies is unlikely to be easy to use in humans because of the detrimental physiological side effects. Therefore, finding appropriate delivery methods for siRNAs might be a major barrier for therapeutics in humans. However, the fact that siRNAs can silence transgene and pathogenic gene expression *in vivo* fuels the optimism that siRNAs might one day be used to treat human diseases.

In plants, RNAi mediates an antiviral response, and viruses have responded by evolving potent RNAi inhibitors. The sequences of these RNAi inhibitors are quite diverse, and different proteins can inhibit different steps in the RNAi pathway. Although the RNAi inhibitors described so far are all proteins, it is conceivable that some RNAi inhibitors might be RNA based. For example, viruses might encode their own miRNAs and use them to target cellular machinery, including components of the RNAi pathway. It is not clear whether mammalian viruses encode either protein- or RNA-based RNAi inhibitors, but a recent report indicates that a virus shared by insects and plants contains a protein that inhibits RNAi in both plants and *Drosophila* cells[77].

The structure of endogenous silencing RNAs

Most miRNAs seem to be processed asymmetrically from a precursor hairpin stem; that is, only one 21–24-nt strand of the precursor stem accumulates in cells. Sometimes the 21–23-nt miRNA forms a perfect duplex in the hairpin stem, but more often, multiple bulges disrupt the 21–23-nt duplex. Similar to other RNase III enzymes, Dicer can process complex hairpin structures that contain several mismatches in the helical stem[29]. Little is known about the structural determinants that are necessary for the processing of stRNAs or miRNAs into ~21-nt RNAs. Studies of RNase III enzymes from bacteria have shown that dsRNA cleavage can be directed by the sequences and structural features of the precursor RNA[78–80]. However, a comparison carried out in a large class of miRNA hairpins that are likely to be processed by Dicer has yet to reveal any obvious determinants that might guide Dicer recognition and processing.

Curiously, when the sequences of *lin-4* and *let-7* were tested for complementarity to their targets, it turned out that only 50–85% of the stRNA residues

RESPIRATORY SYNCYTIAL VIRUS
A virus that is responsible for many respiratory tract infections, including the common cold.

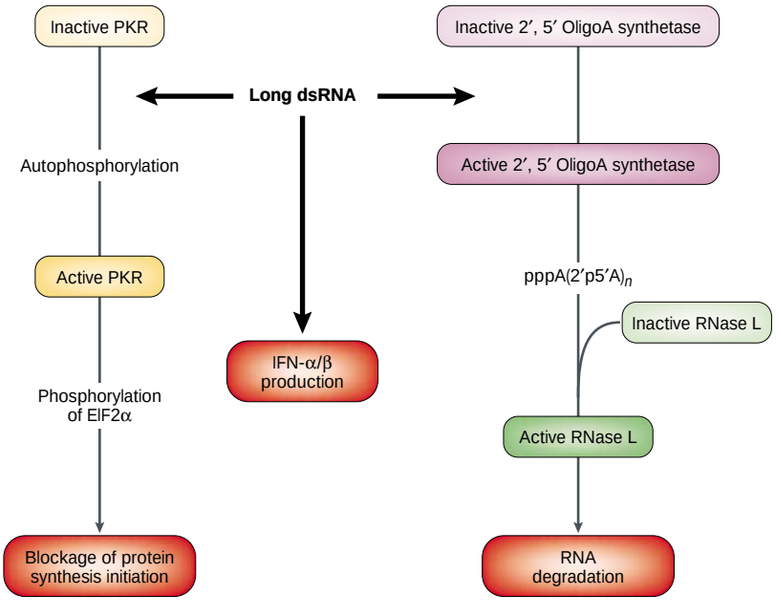

Figure 4 | **Long cytoplasmic double-stranded RNA activates many cellular pathways.** Even though long double-stranded RNA (dsRNA) can mediate gene-specific interference in early mouse embryos and in mouse oocytes, in somatic mammalian cells it also activates many cellular pathways, some of which can lead to apoptosis. The dsRNA-dependent protein kinase (PKR) is an important antiviral 'detector protein', and is activated by binding to long dsRNAs, such as those produced by viruses. Once PKR is activated, it inhibits protein translation by phosphorylating the α-subunit of the translation initiation factor (EIF2α). EIF2α phosphorylation is followed by a general suppression of protein synthesis that directs the cell towards apoptosis. PKR also activates the 2′, 5′-OligoA synthetase/RNase L enzymes, which are central components of an important anti-viral pathway. On binding of the dsRNAs, 2′, 5′-OligoA synthetase is activated, generating oligoadenylates that activate RNase L, which in turn degrades RNA. dsRNA can also induce interferon (IFN)-α and -β expression, which, in conjunction with the other signals, can stimulate apoptosis (for reviews, see REFS 97,98). Because siRNAs are <30 bp long, they fail to activate the PKR and interferon pathways efficiently. pppA(2′p5′A) is an oligoadenylate involved in the PKR pathway.

base pair with the mRNA 3′ UTR (FIG. 3a). By contrast, Sayda Elbashir and colleagues have shown that nearly perfect duplex formation between the target mRNA and the siRNA antisense strand was required for mRNA cleavage in RNAi[64]. One possibility is that the stRNAs cannot activate the degradation pathway because they do not form complete duplexes with the target mRNA. Because the homology between the stRNAs and the target mRNA typically comprises only 5–14 nt, it is difficult to find target genes using a bioinformatics approach. In *Drosophila*, two 3′-UTR sequence motifs, the K box (cUGUGAUa) and the Brd box (AGCUUUA) have been suggested as potential miRNA target sites[81,82]. These boxes are complementary to the 5′ end of several miRNAs, and their removal from the 3′ UTR of a gene relieves the post-transcriptional repression that they mediate[81,82]. The 5′ ends of many miRNAs are perfectly complementary to these 3′-UTR repressor sequence motifs, indicating that potentially many miRNAs could regulate a given target.

Making hairpin RNAs that silence genes

RNAi analysis of gene function in lower organisms has been greatly assisted by the development of *in vivo* systems that express long dsRNA hairpins (typically, of 500–1,000 nt)[83–86]. In mammals, this has been accomplished in mouse embryonic stem (ES) cells[87,88]. However, attempts to develop such long dsRNA hairpin-based gene-silencing systems in differentiated somatic mammalian cells have so far been unsuccessful, possibly due to the non-specific effects on gene expression that result from long (>50 bp) dsRNA activation of the interferon (IFN)-related pathways[87,88] (FIG. 4). The discovery of the stRNAs and miRNAs with hairpin precursors has prompted many researchers to investigate whether silencing could be achieved using small hairpins, thereby avoiding the potent IFN response.

A method for stable gene silencing in mammalian cells through a siRNA-related process has recently been developed. During the course of three months, nine papers reported that the expression of siRNAs from a DNA template can silence gene expression as effectively as do synthetic siRNAs[13,14,67,89–94] (TABLE 2). These reports indicate how two concerns about siRNA technology — the efficiency of transfection and the longevity of the silenced state — can be solved. The first of these papers reported the generation of a stable culture of cells, passaged for more than two months, in which significant knockdown of endogenous *TP53* expression was achieved through an integrated copy of a synthetic gene that expressed a specifically designed hairpin RNA[89]. Polymerase III (pol III), which normally transcribes small RNAs such as tRNAs, was used in this and other reports to transcribe the siRNAs or RNA hairpins (FIG. 5). One key advantage of the pol III system is that transcription terminates at a defined stretch of thymidine residues (uridines in RNA), leaving 1–4 uridines at the 3′-terminus of the nascent RNA, thereby making it similar to many siRNAs.

Among these reports are attempts to make hairpins that are directly modelled on stRNAs/miRNAs, including bulges and mismatches along the hairpin stem and even loop sequences that are directly derived from stRNAs/miRNAs. In other experiments, the two strands of an active siRNA were simply linked at one end by a few nucleotides. It seems that both approaches can work, although certain structural and sequence configurations have failed to do so. Many of these early reports indicated that even minor base changes in the hairpin orientation, hairpin loop sequences or stem structure were sufficient to affect silencing. Thyn Brummelkamp *et al.*[89] suggested that two uridines placed at the base of the hairpin loop were key features for silencing. M.T.M. and colleagues[94] found large differences in silencing activity when just a single nucleotide was changed in a bulge at the base of the 21-nt stem. Patrick Paddison *et al.*[14] suggested that longer duplexes, up to 29 nt, were markedly more efficient at silencing than were the shorter ones. At the moment, it is not clear what makes the 'best' RNA-silencing hairpin.

Table 2 | DNA silencing constructs

Genes	Cell type	Construct type*	Loop size (nt)	Maximum % knockdown	Verification‡	Comment	References
Luc, *p53*	HeLa, 293, Cos-1, NIH3T3, IMR90	HP	4–23	85–90	N, IF,	P53 null phenotype	14
HIV rev	293	SIT	NA	90	N, IF	HIV inhibition	67
Neuronal tubulin, *GFP*	P19	SIT, HP	3	NR	IF	Experiments performed in differentiating cells	13
Luc, *CD4*, *CD8*	T cell, HeLa	HP	4–9	80–85	N, F	Silencing in T cells	53
GFP, *Dnmt1*, *LMNA*, *cdk-2*	HeLa, U2OS, H1299, C-33A	HP	6	>90	W, IF	NR	92
GFP, *LMNA*	HeLa	HP	4	>90	IF	*N* + 27§	90
P53, *CDH1*, *CDC20*	HeLa, MCF7	HP	2–9	>90	N, IF, W	P53 null phenotype; experiments performed in stable cells	89
Hyg/*GFP*, *Luc*, *CTNNB1*	HeLa	SIT	NA	95	IF, N	EBNA-ori in plasmids	91
Tat, *CAT*	293T	HP	15	70–80	N, IF, W	Pol II transcription TL silencing	93

*HP, DNA constructs that produce hairpin RNAs; SIT, separate short interfering RNA strands are transcribed in *trans*.; F, flow cytometry; IF, immunofluorescence; N, northern blot; W, western blot; §*N* + 27, first 27 nucleotides of the *U6* gene were present in the hairpin; EBNA-ori, Epstein Barr Virus origin of replication; TL silencing, translational silencing observed; *CAT*, chloramphenicol acetyltransferase; *CDC20*, CDC20 cell division cycle 20 homologue; *CDH1*, cadherin 1, type 1, E-cadherin; *cdk-2*, cyclin-dependent kinase 2; *CTNNB1*, β-catenin; *DNMT1*, DNA methyltransferase (cytosine-5) 1; *GFP*, green fluorescent protein; *HIV rev*, human immunodeficiency virus regulator of virion expression; *Hyg*, hygromycin, *LMNA*, lamin A/C; *Luc*, luciferase; *Tat*, transactivator of transcription. NR, not reported.

The data in TABLE 2 indicate that, similar to siRNAs, RNAi from DNA templates are effective in a wide number of cell types and against a wide variety of genes. This is not surprising, as the active silencing molecule is probably siRNA. Several of these experiments were followed up with northern blots that confirmed that the transcribed hairpin RNAs are processed to smaller ~21–23-nt RNAs, indicating that siRNA-mediated mRNA cleavage resulted in silencing. In fact, in most cases, reduced target mRNA levels are seen, a result that is consistent with RNAi-mediated mechanisms (TABLE 2).

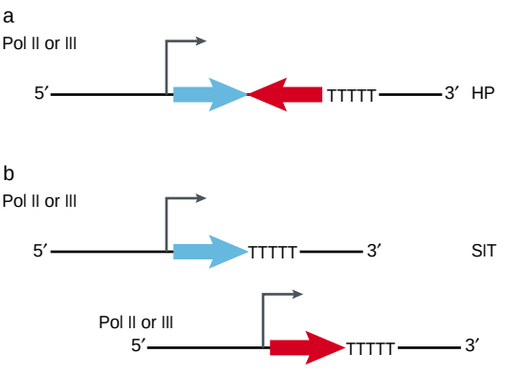

Figure 5 | **Two new approaches for stable RNA interference silencing in mammalian cells.** To achieve stable RNA interference (RNAi) silencing in mammalian cells, DNA constructs, from which silencing RNAs can be made *in vivo*, can be transfected into the cells. Polymerase II or III promoters (for example, CMV or H1 and U6, respectively) can be used to drive the expression of silencing RNAs. Polymerase III transcription terminates at a run of thymidine (T) residues, and therefore the newly transcribed RNA will contain several uridines at the 3′-end. Depending on construct design, **a** | silencing RNAs might adopt an inverted hairpin RNA (HP) structure, or **b** | the ~21-nucleotide sense and antisense RNAs can be transcribed separately (*in trans*), and only subsequent annealing would lead to siRNA formation (siRNA-*in-trans*, SIT). Black arrows indicate transcription.

Yan Zeng *et al.*[93] showed that silencing at the level of translation could be achieved by adding artificial miRNA target sites to the 3′ end of a mRNA. This is the first demonstration of an artificial mRNA–miRNA interaction that mimics translational repression as opposed to mRNA degradation. The mechanisms behind these activities need further investigation, and translational repression should be considered as a separate and potentially powerful avenue for targeted gene silencing. Zeng *et al.* also report[93] that polymerase II (pol II), which normally transcribes mRNAs, can be used to transcribe a silencing hairpin RNA — in their study, the miRNA and miRNA-containing hairpin were expressed from sequences downstream of a CMV (cytomegalovirus) promoter. Which polymerase transcribes precursor miRNAs *in vivo* remains unknown.

Conclusion

It is likely that gene vectors that encode siRNAs or miRNAs will become widely accepted as gene-knockout tools. It is also foreseeable that these new RNAi constructs could be coupled to lentivirus, adenovirus or other delivery vectors that are used in gene therapy, which paves the way for a new wave of therapeutic molecules in the years to come. It is certain that the ability of siRNAs to silence specific genes, either when transfected directly as siRNAs or when generated from DNA vectors, will transform future studies of cellular systems and biology in mammalian cells.

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