Unlocking the potential of the human genome with RNA interference

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The discovery of RNA interference (RNAi) may well be one of the transforming events in biology in the past decade. RNAi can result in gene silencing or even in the expulsion of sequences from the genome. Harnessed as an experimental tool, RNAi has revolutionized approaches to decoding gene function. It also has the potential to be exploited therapeutically, and clinical trials to test this possibility are already being planned.

The formal description of RNAi as a biological response to double-stranded RNA (dsRNA) came about following experiments with dsRNA in the nematode *Caenorhabditis elegans*[1,2]. Injecting dsRNAs into the worm was found to silence genes whose sequences were complementary to those of the introduced dsRNAs[3]. It is now clear that an RNAi pathway is present in many, if not most, eukaryotes[4]. dsRNAs are processed into short interfering RNAs (siRNAs), about 22 nucleotides in length, by the RNase enzyme Dicer. These siRNAs are then incorporated into a silencing complex called RISC (RNA-induced silencing complex), which identifies and silences complementary messenger RNAs.

Before RNAi could be harnessed as an experimental tool for silencing specific genes in mammalian systems, a considerable hurdle had to be overcome. The problem lay in making exogenous dsRNA trigger silencing in a gene-specific manner without invoking nonspecific responses to foreign dsRNAs that are part of the cell's antiviral mechanism[5]. Work over the past two years has allowed investigators to meet this challenge, and RNAi has now been adopted as a standard methodology for silencing the expression of specific genes in mammalian cells. Here, we chronicle the development of RNAi as a genetic tool in mammals, focusing on recent advances and providing practical advice for its experimental application. We also make predictions about the potential future of RNAi as a potent and specific therapeutic tool that may escape some of the limitations of conventional medicinal chemistry.

Breaking the barrier to RNAi in mammals

For more than 30 years, it has been known that exposure of mammalian cells to long dsRNAs induces innate immune pathways, including interferon-regulated responses that serve as antiviral mechanisms. The enzyme dsRNA-dependent protein kinase (PKR) is activated on binding to dsRNA and localized, but sequence-independent destruction of RNAs and a generalized repression of protein synthesis results[5]. The existence of these innate immune pathways seemed incompatible with the use of dsRNA for silencing a particular target gene. However, evidence of an RNAi pathway in mammals came from the observation that key biochemical components of the RNAi pathway are conserved[6,7]. It was also shown that long dsRNAs can trigger gene-specific responses when they are introduced into mammalian embryos and embryonic cell lines in which nonspecific antiviral responses to dsRNAs are not prevalent[4]. This raised the problem of how to shift the response of a mammalian cell to foreign dsRNA from the non-specific sequence-independent defence pathways to the sequence-specific RNAi pathway. Attempts to meet this challenge have resulted in RNAi being established as a genetic tool in mammalian cells and animals.

Using siRNAs for RNAi

A biochemical understanding of the RNAi pathway (Fig. 1; see review in this issue by Meister and Tuschl, page 343) was crucial to realizing that dsRNAs shorter than 30 base pairs (bp) could be used to trigger an RNAi response in mammals. Tuschl and colleagues showed that transfection of mammalian cells with short RNAs could induce the sequence-specific RNAi pathway, and so overcame the barrier to the use of RNAi as a genetic tool in mammals[8]. The impetus to use siRNAs and other small RNAs in mammalian cells also came from the long-standing view that PKR activation and similar responses were not effectively triggered by short dsRNAs. Following the initial reports, it took a remarkably short period of time for siRNAs triggers to be adopted as a standard component of the molecular biology toolkit.

siRNAs can be introduced into mammalian cells using a variety of standard transfection methods. The strength and duration of the silencing response is determined by several factors: on a population basis, the silencing response is affected mainly by the overall efficiency of transfection, which can be addressed by optimizing conditions. In each cell, silencing depends on the amount of siRNA that is delivered and on the potential of each siRNA to suppress its target, or its potency. Even a relatively impotent siRNA can silence its target provided that sufficient quantities of the siRNA are delivered. However, essentially 'forcing' the system by delivering large amounts of reagent is likely to lead to numerous undesired effects (see section 'Intrinsic limits on the specificity of RNAi').

Using shRNAs for RNAi

The discovery of the endogenous triggers of the RNAi pathway in the form of small temporal RNAs — now termed microRNAs (miRNAs)[9–11] — suggested that RNAi might be triggered in mammalian cells by synthetic genes that express mimics of endogenous triggers. Several laboratories simultaneously used related approaches to test this idea. These involved expressing mimics of miRNAs in the form of short hairpin RNAs (shRNAs) from RNA polymerase II or III promoters[12]. The shRNAs themselves varied in size and design, with stems ranging from 19 to 29 nucleotides in length, and with various degrees of structural similarity to natural miRNAs. All these approaches were

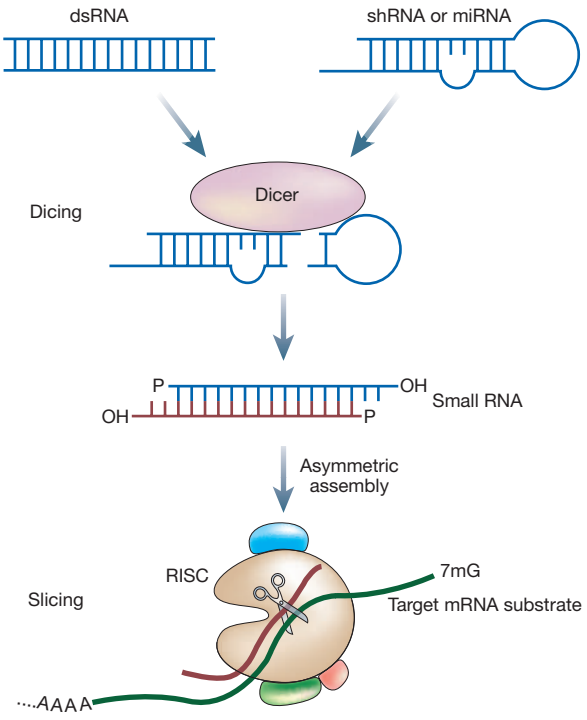

Figure 1 RNA silencing pathways in different organisms. Long dsRNA and miRNA precursors are processed to siRNA/miRNA duplexes by the RNase-III-like enzyme Dicer. These short dsRNAs are subsequently unwound and assembled into effector complexes, RISCs, which can direct RNA cleavage, mediate translational repression or induce chromatin modification. *S. pombe*, *C. elegans* and mammals carry only one Dicer gene. In *D. melanogaster* and *A. thaliana*, specialized Dicer or DLC proteins preferentially process long dsRNA or miRNA precursors. 7mG, 7-methyl guanine; AAAA, poly-adenosine tail; Me, methyl group; P, 5′ phosphate.

effective to varying degrees, and at present, no real consensus has developed on the most effective way to present synthetic miRNAs to the RNAi pathway.

Because these triggers are encoded by DNA vectors, they can be delivered to cells in any of the innumerable ways that have been devised for delivery of DNA constructs that allow ectopic mRNA expression. These include standard transient transfection, stable transfection and delivery using viruses ranging from retroviruses to adenoviruses. Expression can also be driven by either constitutive or inducible promoter systems[12].

Recent studies strongly indicate that each shRNA expression construct gives rise to a single siRNA (D. Siolas, G.J.H. and M. Cleary, unpublished work). Knowing precisely how this processing occurs for each shRNA cassette design has permitted the application of siRNA design criteria (see 'Features of effective siRNAs and shRNAs' below) to the design of effective shRNAs. The use of siRNAs and shRNAs are complementary approaches in the application of RNAi as a genetic tool in mammals, and the best approach depends on the type of study being performed.

Features of effective siRNAs and shRNAs

Observations of widely varying efficacy of individual siRNAs motivated a search for rules that might specify more effective siRNAs. Several groups took a 'black box' approach, which involved assaying large numbers of siRNAs, sorting them into classes depending on their potency, and then looking for characteristics that distinguished effective siRNAs from ineffective ones[13–15]. Some common rules have begun to emerge from these studies. siRNAs in which the helix at the 5′ end of the antisense strand has a lower stability than the 3′ end of the siRNA are generally more effective silencers than those with the opposite arrangement. A biochemical basis for the thermodynamic arrangement of effective siRNAs was provided by biochemical studies of the mRNA-cleavage complex, RISC (Fig. 1), in *Drosophila* embryo extracts. These studies showed unequal incorporation of the two strands of the siRNA into RISC[14]. Moreover, strand biases could be manipulated by altering the thermodynamic stability of the terminal nucleotides in a way that precisely matched the rules derived from empirical studies. Finally, an examination of miRNAs, most of which produce RISC-like complexes containing only one strand of the precursor (see review in this issue by Meister and Tuschl, page 343), showed the same pattern of thermodynamic asymmetry as that shown by effective siRNAs[13,14,16,17]. The rules for specifying effective siRNAs uncovered by these studies imply that the effectiveness of an RNAi response triggered by an siRNA is strongly dependent on siRNA structure and determined at the step of RISC assembly, during which the asymmetry in the dsRNA must be sensed and a single strand chosen for productive incorporation into the enzyme. Once the active RISC is formed, it is relatively insensitive to the placement or structure of the target site within the mRNA.

Intrinsic limits on the specificity of RNAi

Although RNAi silences gene expression in a sequence-specific manner, several recent studies have suggested that the specificity of silencing is not absolute. Off-target effects in mammals can come from several different sources. As discussed previously, transfection of cells with dsRNAs can activate innate immune pathways. PKR activation was thought to depend on the length of the dsRNA, with a minimal cut-off for PKR activation being roughly 30 bp of duplex. However, recent reports have suggested that both siRNAs and shRNAs can — under some circumstances and in certain cell types — activate a PKR response[18–20]. Furthermore, siRNAs transcribed *in vitro* using bacteriophage polymerases can be potent activators of an interferon response if the initiating triphosphate is not completely removed from the transcripts[21]. Further studies are required to investigate the frequency with which RNAi triggers provoke these antiviral response pathways, and the sequence or structural characteristics that might lead an siRNA or an shRNA to trigger such a response.

miRNAs recognize and regulate their targets despite a lack of perfect complementarity. This raises the possibility that siRNAs might also not require contiguous base pairing to suppress their targets effectively; several microarray studies suggested that siRNAs can provoke sequence-dependent, off-target effects and that these can be elicited by 14 base pairings, or possibly even fewer, between the siRNA and its target[22]. Notably, analysis of such interactions suggested that base pairing at the 5′ end of the siRNA contributed disproportionately to targeting, a conclusion also reached by analysis of interactions between miRNAs and their validated targets[23–26]. Although such information can aid the design of more specific siRNAs, we do not have sufficient understanding of target recognition by RISC to say with certainty that we can eliminate off-target effects. In fact, the intrinsic specificity of the RNAi pathway may be sufficiently low to prevent the design of a completely specific siRNA in mammals. Fewer studies have been carried out with shRNA expression cassettes, but similar caveats undoubtedly apply.

Off-target effects can also occur at the level of protein synthesis. miRNAs in animals often regulate protein expression without having corresponding effects on mRNA levels. Several studies have indicated that siRNAs also do this, provided that mRNA cleavage is blocked by altering the geometry of the target–substrate interaction[24–26]. However, suppression of a reporter gene in this manner was only effective when several siRNA binding sites were present. Although these findings might provide some degree of comfort to those using siRNAs experimentally, a recent study suggested that changes in the expression levels of a large number of proteins occurred in cells treated

with siRNAs. However, it should be noted that the siRNAs in question were relatively impotent[27]. Therefore, we must view unwanted changes in protein expression levels as the 'monster under the bed' for RNAi-based studies of gene function. Ultimately, caveats in the specificity of the RNAi response make it essential to follow relatively simple guidelines for good experimental practice. These are outlined in Box 1.

RNAi as a solution for mammalian genetics

One of the first choices to make in any RNAi-based genetic experiment is whether to trigger suppression through the use of siRNAs or shRNAs. The advantages of using siRNAs are relative ease of availability and high efficiency of delivery. In addition, pre-validated siRNAs are becoming increasingly available as commercial suppliers and the scientific community acquire more experience. Overall, siRNA delivery is likely to result in the highest intracellular concentration of the gene silencer. But limitations to the use of siRNAs are that their effects are transient and restricted by the rate of cell division: mammalian cells do not have mechanisms to amplify and propagate RNAi (unlike *C. elegans* and plants). In addition, some cell types are notoriously difficult to transfect, and the procedure of transfection itself can alter the physiology of the cell. However, despite these drawbacks, transfection of siRNAs is probably the fastest and easiest method currently available for producing a knockdown of gene expression in cell culture by means of RNAi.

With shRNAs, the up-front investment is greater. First, DNA oligonucleotides must be cloned and sequenced so that a construct can be produced. Second, the shRNA must be designed effectively, and consensus on the most effective design, with respect to either the structure of the shRNA itself or the structure of the expression vector, is only just beginning to emerge. However, shRNAs are capable of producing sustained repression, and allow for delivery by conventional transfection or by several advanced viral vectors that also permit stable integration into the genome. In addition, shRNA expression vectors can be propagated indefinitely. As with siRNAs, design algorithms can be applied to shRNAs to maximize the probability of success in a suppression experiment. However, the application of such algorithms requires a detailed understanding of the vector system being used.

Both siRNAs and shRNAs have been used for studies of gene function *in vivo*, primarily in mice. Both types of trigger can be

Box 1
Rules of the road for effective RNAi experiments

Given the significant concerns about the specificity of RNAi-mediated repression, how can investigators maximize confidence in the results of studies that use these tools? It is important to note that no approach used to inactivate gene function is free from potential problems. Even conventional gene knockouts are known to be subject to compensation during development. Thus, the enthusiasm for the use of RNAi as a genetic tool should be tempered by a recognition of the potential problems and good practices should be applied to avoid misinterpreting results. Four guidelines for good practice in RNAi experiments in mammals are presented below.

1. Get the right strand into RISC by using good design

RNAi-based experiments will be more informative and go more smoothly if effective and highly specific RNA triggers are used. Many algorithms now exist for choosing effective sequences. In addition, homology to other sequences in the genome should be minimized, with particular attention to the 5′ end of the antisense strand. Use of design algorithms based on thermodynamic criteria can aid biased incorporation of the antisense strand of the siRNA into RISC. Several public websites provide support for such designs (see for example http://web.mit.edu/mmcmanus/www/home1.2files/siRNAs.htm; http://hydra1.wistar.upenn.edu/Projects/siRNA/siRNAindex.htm; http://www.cshl.edu/public/SCIENCE/hannon.html).

2. Several alleles are better than one

Several siRNAs or shRNAs should give the same phenotypic outcome, as it is extremely unlikely that different triggers will have the same off-target effects[22]. It is critical to correlate this phenotypic outcome with the effectiveness of suppression. Only effective siRNAs against a given target, but not inefffective siRNAs, should yield similar phenotypes. Importantly, discrimination between effective and ineffective siRNAs can only be accomplished by examining target protein levels. There are numerous anecdotal reports of siRNAs effectively suppressing protein production without changing mRNA levels. In addition, siRNAs or shRNAs that do not affect the target protein should be used as negative controls. Arguably, one could use a 'scrambled' siRNA or shRNA for this purpose. However, such scrambled siRNAs may not have any biological activity, and it is undoubtedly better to use an RNA that is known to enter the RNAi pathway effectively. For example, an RNA targeting luciferase, green fluorescent protein or another marker gene (that is validated against its target) would be expected to enter RISC but would not be expected to affect the expression of proteins in a mammalian cell. Other possible controls include an RNA with flipped asymmetry. This could be achieved by creating an siRNA with a more stable helix at the 5′ end of the antisense strand.

3. Work at the lowest possible concentrations

RISC is a conventional enzyme, and working at high enzyme to substrate ratios is likely to affect its specificity. Therefore, it is important to identify RNAi triggers that work at very low effective concentrations. With siRNAs, this can be achieved by titrating siRNA concentrations and by correlating their effects on phenotypic outcome with both the concentration of the siRNA used and with the degree of suppression obtained. For example, if the siRNA shows maximal suppression at 5 nM but the phenotype is not observed until the concentration reaches 100 nM, off-target effects must be suspected. In fact, some siRNAs in HeLa cells have shown IC_{50} values (the amount of siRNA required to suppress the target to 50% of its original level) of as little as 500 pM. Similarly, titration of shRNA-expression vectors should also be performed.

4. Rescue to the rescue

Ultimately, the best experiments demonstrate that expression of a version of the targeted gene that cannot be recognized by the siRNA reverts the phenotype. This can be achieved in several ways. First, mutations can be introduced into a cDNA encoding the targeted gene that destroy complementarity with the siRNA or shRNA while maintaining the wild-type protein sequence. Alternatively, the phenotype can be validated by using siRNAs or shRNAs that target untranslated regions, and then by rescuing the phenotype with an expression construct containing only the coding sequence. Although rescue experiments provide the ultimate test of the specificity of a given effect, these can be problematic. For example, it may be difficult to achieve appropriate expression levels of a particular protein. Overexpression could cause artefactual effects (for example, a pathway could be rescued by bypassing its requirements, rather than truly reverting a specific effect).

Ultimately, as our understanding of the RNAi pathway deepens, we will be able to predict with good accuracy all the on- and off-target effects of siRNAs. This will allow not only the generation of RNAi triggers with maximal specificity, but also the design of triggers that are directed against the most likely off-target genes for each siRNA or shRNA.

delivered transiently. The first demonstration of RNAi-mediated repression in an adult animal showed effective repression of a luciferase reporter gene following hydrodynamic transfection of siRNAs or shRNA expression plasmids into mouse liver[28]. Subsequent studies have delivered siRNAs or shRNAs by various methods, including lipid-based delivery and naked RNA or DNA injection[29–32].

Long-term gene silencing has been demonstrated *in vivo* using both genetic mosaics and germline modification. For example, the growth of a tumour cell line in a xenograft model can be attenuated by engineering that cell line with an shRNA cassette that targets the activated *ras* oncogene before the tumour cells are subcutaneously injected into the host animal[33]. Genetically mosaic animals have been created by engineering stem cells with shRNA vectors and then by using those stem cells to repopulate an organ system[34,35]. Strains of mice have been engineered to heritably suppress a targeted gene based on inheritance of a dominantly acting shRNA expression cassette[36–39]. Several approaches have been used to create such strains, including standard nuclear injection, creation of chimaeras with engineered embryonic stem cells, and transgenesis mediated by subzonal injection of fertilized eggs with recombinant lentiviruses. Ultimately these developments will rapidly lead to the creation of animals with inducible, tissue-specific silencing of almost any gene. RNAi is therefore likely to complement existing large-scale efforts to functionally map the mouse genome by chemical or insertional mutagenesis. RNAi is certainly complementary to such approaches, because each approach can generate different types of allele. However, unlike mutational approaches RNAi has the potential to be extended beyond mice into animals where recombinant organisms cannot be generated using embryonic stem cells.

RNAi as a tool for genome-wide studies

The success in using RNAi for analysing single genes has led inevitably to efforts to apply this approach on a large scale for forward genetics (whereby mutant genes are isolated from organisms showing abnormal physical and behavioural characteristics). Indeed, given the recent completion of the human, mouse and rat genomes, RNAi provides a ready mechanism by which this enormous wealth of sequence information can be translated into functional definitions for every gene.

Genome-wide libraries of siRNAs can be constructed in fundamentally different ways, including chemical synthesis or enzymatic digestion of long dsRNAs. An example of progress towards this goal can be found in a small-scale effort[40] to target genes in the phosphatidylinositol 3-OH kinase (PI(3)K) pathway in which a mini-library (148 siRNAs) was searched for genes that affected phosphorylation of Akt, a downstream substrate for PI(3)K.

Alternatively, libraries can be produced by constructing shRNA expression vectors that target each gene. As with siRNAs, proof of principle came initially from small-scale efforts. Using a library directed against the family of de-ubiquinating enzymes, the tumour suppressor CYLD (encoded by the familial cylindromatosis susceptibility gene) was identified as a suppressor of NF-κB activity[41]. This result led to proposals for treating cylindromatosis with existing drugs and provided powerful confirmation that unbiased, genetic approaches can lead not only to new insights in biology but also to practical advances in the treatment of disease. Two groups have recently reported the production of arrayed libraries from chemically synthesized oligonucleotides that cover about 10,000 different human genes each[42,43]. Another group has generated a library of polymerase chain reaction (PCR) products that encode shRNAs[44], and several groups have reported methods for constructing random shRNA libraries based on manipulation of complementary DNA or genomic DNA[45–47]. Each approach has specific advantages. Random libraries are relatively inexpensive to produce and can cover an individual gene with many different shRNAs. However, they suffer from a lack of normal representation of all genes. Libraries produced from chemically synthesized oligonucleotides are expensive. However, they permit the use of powerful informatic tools to aid shRNA design and allow flexibility in optimizing the structure of the shRNA for entry into the RNAi pathway. In addition, synthetic libraries can be used either as mixtures or as individual arrays, in a similar way to siRNA libraries.

Large-scale screening using siRNA libraries must be carried out by individual transfection and phenotypic characterization of target cells (Fig. 2). As such, siRNA libraries can be applied to the wide range of screening methods that are being developed by the pharmaceutical industry in the form of cell-based assays for drug development. These include fluorescent reporter screens, assays for various activities in cell lysates and screening by means of automated microscopy. Alternatively, RNAi triggers can be printed on microarrays and tested for their effects following transfection *in situ*[48,49]. Arrayed libraries of shRNAs can be used in a similar fashion: this was demonstrated by applying an arrayed library to a search for genes that affect proteasome function[43]. shRNA libraries can also be assayed, following their integration into the genomes of target cells, in pools using protocols that filter populations based on phenotypic criteria, such as a growth selection (Fig. 2). Such a test of one shRNA library yielded new links between several genes and the p53 tumour-suppressor pathway[42]. A conceptually more complicated application of pooled screens involves using molecular 'barcodes' to track how individual shRNAs behave as members of complex populations (Fig. 2).

Clearly, large-scale library efforts will evolve with our advancing understanding of the RNAi pathway. As the quality of resources improves, there will be opportunities to progress from relatively straightforward screening protocols in cultured cells to more complex genetics in whole animals.

RNAi in drug discovery and disease therapy

RNAi has begun to produce a paradigm shift in the process of drug discovery. With the large-scale screening approaches described above, RNAi can winnow lists of potential drug targets so that efforts can be focused on the most promising candidates. Moreover, since the first description of RNAi in mammalian cells, there have been numerous studies aimed towards using RNAi to treat disease. The strong appeal of RNAi in therapeutics is the potency and specificity with which gene expression can be inhibited. The possible targets for various diseases range from oncogenes to growth factors and single nucleotide polymorphisms (SNP). There is also potential for using RNAi for the treatment of viral diseases such as those caused by the hepatitis C virus (HCV) and the human immunodeficiency virus (HIV). Despite the excitement and some early proofs of principle in the literature, there are important issues and concerns about the therapeutic application of this technology, including difficulties with delivery and uncertainty about potential toxicity. However, proposals for clinical trials using either synthetic siRNAs or viral-vector-delivered shRNAs have been put forward — although none has yet been approved.

RNAi as a treatment for HIV

The development and use of double and triple drug combinations for the treatment of HIV infection has led to dramatic improvements in the lives of HIV-infected individuals. But despite the apparent successes of the new anti-retroviral drugs there are the emerging problems of drug-resistant viral variants and toxicities of the combination drugs now in use. Therefore, there is still great interest in exploring new antiviral therapeutic approaches. HIV was the first infectious agent targeted by RNAi, perhaps because the lifecycle and pattern of gene expression of HIV is well understood. Synthetic siRNAs and expressed shRNAs have been used to target several early and late HIV-encoded RNAs in cell lines and in primary haematopoietic cells including the TAR element[50], tat[51–53], rev[51,52], gag[54,55], env[55], vif[50], nef[50], and reverse transcriptase[53].

Despite the success of RNAi-mediated inhibition of HIV-encoded RNAs in cell culture, targeting the virus directly represents a substantial challenge for clinical applications because the high viral mutation rate

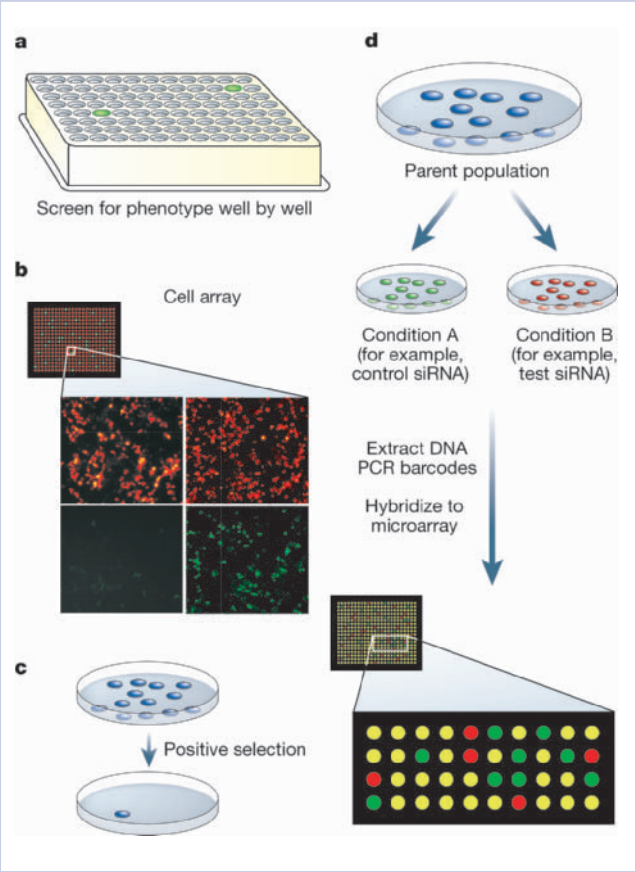

Figure 2 Genome-wide screens using RNAi. **a**, Standard methodologies can be used to screen siRNAs or shRNAs individually in 96-well plates using morphological readouts, reporters or biochemical assays. **b**, Similar approaches can be taken using siRNAs or shRNAs that have been printed on microarrays for reverse transfection[49]. Reverse transfection involves the deposition of lipid–nucleic-acid complexes on a solid surface, often a glass microarray slide. Cells plated on top of the slide take up the encapsulated DNA or RNA, and this can direct mRNA expression or gene silencing. The enlarged image shows cell populations, which would be observed within individual spots of the array, expressing fluorescent proteins, red and green. In the left panels, expression of GFP has been ablated by co-deposition of a GFP siRNA. **c**, Complex (mixed) populations of shRNA-expression vectors can be filtered through positive selections in cultured cells. Selection can be for drug resistance, cytokines, genetic alterations, or — in the case of fluorescence activated cell sorter (FACS)-based selection — for cells that activate a particular marker. **d**, Complex populations can be monitored using molecular barcodes that track individual shRNA-expressing cells and their responses to various stimuli. The use of molecular barcodes combines the advantages of well-by-well screens with the advantages of carrying out pooled selections, thereby allowing the identification of phenotypes in complex populations, which do not necessarily confer a growth advantage (for example, a synthetic lethal phenotype). Each vector is tagged with a unique sequence, which can comprise the shRNA itself or a separate random or selected barcode. The frequency (representation) of each vector in a mixed population can be measured by hybridizing barcodes to an oligonucleotide microarray. If the population is subjected to selective pressure, the representation of individual shRNA constructs is expected to change as a result. This change can be detected by comparing hybridization signals for the starting population with those of the population exposed to selection. The relative signal of shRNAs that increase resistance to the selection will increase, whereas the relative signal of those that sensitize to the selection will decrease.

will lead to mutants that can escape being targeted[56]. Therefore RNAi-mediated downregulation of the cellular cofactors required for HIV infection is an attractive alternative or complementary approach. Cellular cofactors such as NF-κB[53], the HIV receptor CD4[54], and the co-receptors CXCR4 and CCR5[57] have been successfully downregulated by RNAi, resulting in the inhibition of HIV replication in numerous human cell lines and in primary cells including T lymphocytes and haematopoietic-stem-cell-derived macrophages[50–52,54,57–60]. Although targeting NF-κB is not appropriate as a therapy owing to the important role NF-κB has in the cell (for example, it mediates interferon-induced gene expression) the macrophage-tropic CCR5 co-receptor holds particular promise as a target. This receptor is not essential for normal immune function, and individuals homozygous for a 32-bp deletion in this gene are resistant to HIV infection, whereas individuals who are heterozygous for this deletion show delayed progression to AIDS[61,62]. Qin *et al.*[63] used a lentiviral vector to transduce an anti-CCR5 shRNA in human lymphocytes. Downregulation of CCR5 resulted in a modest, but nevertheless significant three- to sevenfold reduction in viral infectivity relative to controls. Despite this downregulation, the CCR5-shRNA-treated cells were still susceptible to infection by the T-tropic virus that uses CXCR4. However, because CXCR4 is essential for the normal function of haematopoietic stem cells[64], targeting this receptor is not a good choice for an anti-HIV therapy, nor is targeting the essential CD4 receptor. So there are drawbacks in targeting cellular HIV cofactors because non-infected cells will inevitably be targeted as well, leading to toxicities that are similar to those observed with the current anti-retroviral drugs. Viral targets will need to be included in any successful strategy using RNAi. These targets should be sequences that are highly conserved throughout the various clades to ensure efficacy against all viral strains.

The delivery of siRNAs or shRNAs to HIV-infected cells is also a challenge. The target cells are primarily T lymphocytes, monocytes and macrophages. As synthetic siRNAs do not persist for long periods in cells, they would have to be delivered repeatedly for years to effectively treat the infection. Systemic delivery of siRNAs to T lymphocytes is probably not feasible owing to the immense number of these cells. Using viral vectors to deliver anti-HIV-encoding shRNA genes is also problematic, and systemic delivery is not yet practicable because the immunogenicity of the vectors themselves precludes performing multiple injections. Therefore the preferred method is to isolate T cells from patients; these T cells are then transduced, expanded and re-infused into the same patients. In a continuing clinical trial, T lymphocytes from HIV-infected individuals are transduced *ex vivo* with a lentiviral vector that encodes an anti-HIV antisense RNA. The transduced cells are subsequently expanded and reinfused into patients[65,66]. This type of therapeutic approach would also be applicable to vectors harbouring genes that encode siRNAs. A different approach is to transduce isolated haematopoietic progenitor or stem cells with vectors harbouring the therapeutic genes. These cells give rise to all the haematopoietic cells capable of being infected by the virus. Haematopoietic stem cells are mobilized from the patient and transduced *ex vivo* before reinfusion (Fig. 3). Two clinical trials in which retroviral vectors expressing ribozymes were transduced into haematopoietic stem cells have demonstrated the feasibility of this approach[67,68]. Because RNAi is more potent than ribozyme or antisense approaches, movement of this technology to a human clinical trial for HIV treatment is expected to take place in the next year or two.

RNAi to treat viral hepatitis

Hepatitis induced by the hepatitis B virus (HBV) and by HCV is a major health problem. At present hundreds of millions of individuals are infected worldwide. There is an effective vaccine against HBV, but this treatment is only useful for the prevention of viral infection and there is no vaccine for HCV. Therefore, hepatitis caused by these two viruses has been an important target for potential RNAi therapy. The first demonstration of RNAi efficacy against a virus *in vivo* involved hydrodynamic co-delivery of an HBV replicon and an expression unit encoding an anti-HBV shRNA in mice[69]. This study demonstrated that a significant knockdown (99%) of the HBV core antigen in liver hepatocytes could be achieved by the shRNA, providing an important proof of principle for future antiviral applications of RNAi in the liver.

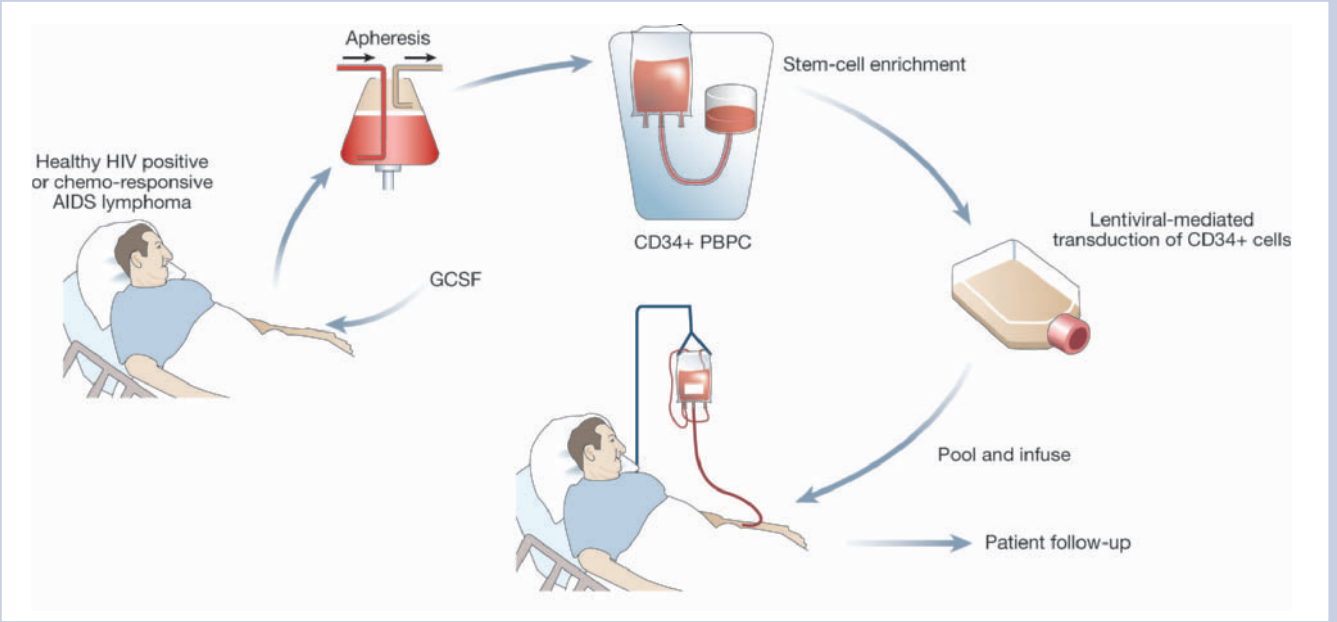

Figure 3 Proposed scheme for the treatment of HIV patients using lentiviral vectors to transduce anti-HIV shRNA genes into the patient's haematopoietic stem cells. Patients are given several injections of granulocyte colony stimulation factor (GCSF), which mobilizes haematopoietic stem cells into the patients' peripheral circulation. Haematopoietic stem cells expressing the CD34 antigen are collected by affinity columns (apheresis) and transduced with a lentiviral vector harbouring the anti-HIV shRNA genes. The cells are then re-infused into patients. Depending on the population, the patient will have been pretreated with no, or with one, or with more than one marrow-chemoablative agent. Following stem-cell engraftment, patients are monitored for a period of several years for HIV loads, CD4+ T-lymphocytes and shRNA gene expression. This overall scheme follows that described by Michienzi *et al.*[68]. PBPC, peripheral blood progenitor cells.

More advanced studies have been carried out for RNAi therapies against HCV, a virus that now infects an estimated 3% of the world's population. HCV is a major cause of chronic liver disease, which can lead to liver cirrhosis and hepatocellular carcinoma. The HCV genome is a positive-strand RNA molecule with a single open reading frame encoding a polyprotein that is processed post-translationally to produce at least ten proteins. The only therapy currently available is a combination of interferon and ribavirin, but response to this therapy is often poor, particularly with certain HCV subtypes.

Subgenomic and full-length HCV replicons that replicate and express HCV proteins in stably transfected human hepatoma-derived Huh-7 cells have been used to study the effects of various antiviral drugs[70–73]. Several groups have now tested the efficacy of siRNA mediated inhibition of replicon function using these systems[74–76]. siRNAs targeting the internal ribosome entry site (IRES) or mRNAs encoding the viral non-structural proteins NS3 and NS5B inhibited HCV replicon function in cell culture[75]. Furthermore, anti-HCV siRNAs depleted Huh-7 cells of persistently replicating HCV replicons[74]. McCaffrey *et al.* performed hydrodynamic tail-vein injections of siRNAs or anti-HCV shRNAs to direct efficient cleavage of HCV sequences in an HCV-luciferase fusion construct in mouse hepatocytes *in vivo*[28].

In another *in vivo* study, siRNAs were used to treat fulminant hepatitis induced by an agonistic Fas-specific antibody in mice. Anti-Fas siRNAs were hydrodynamically injected into the antibody-treated mice: 82% of the mice survived for 10 days of observation whereas all control mice died within 3 days[77]. Importantly, mice already suffering from auto-immune hepatitis also improved after the Fas siRNA treatment. So it may be feasible to use siRNAs to ameliorate the severity of certain diseases by targeting the inflammatory response pathways rather than the infectious agent.

As with HIV therapeutics, delivery of the siRNAs or shRNA vectors is the main challenge for successful treatment of HCV. The method of delivery used in several *in vivo* studies — hydrodynamic intravenous injection — is not feasible for the treatment of human hepatitis. In mice, genetic material can be introduced into hepatocytes using catheters or even localized hydrodynamic procedures[78], but it is yet to be determined whether such procedures can be used to deliver siRNAs in larger mammals.

RNAi and cancer

Many studies have used siRNAs as an experimental tool to dissect the cellular pathways that lead to uncontrolled cell proliferation and to cancer. Moreover, RNAi has been proposed as a potential treatment for cancer[79–81]. Although no clinical trials are yet underway, a precedent might be set by ongoing clinical trials that use antisense reagents. The first systemically delivered antisense oligonucleotide for the treatment of cancer, Genasense (Genta, Inc.), which targets the anti-apoptotic gene *BCL2*, has shown promise in clinical trials for metastatic melanoma when used in combination with conventional chemotherapeutics[82]. However, its use as a US Food and Drug Administration (FDA)-approved drug has recently been put on hold. The potential for using RNAi to treat metastatic cancers will of course depend on finding good cellular targets.

Highly efficient mechanisms for the delivery of siRNA to the relevant cells will also be particularly important for successful treatment of metastatic cancer. Several groups have developed backbone modifications to synthetic siRNAs that provide them with resistance to serum nucleases and should therefore increase the half-life of circulating siRNAs in animal models[83,84]. However, enhancing siRNA stability is not enough unless the siRNAs can penetrate cells and tissue *in vivo* in concentrations sufficient to be therapeutically functional. As siRNAs are double-stranded molecules, delivery and cellular uptake is more of a challenge than for single-stranded antisense agents, which bind to serum proteins and are taken up by cells and tissues *in vivo*[85]. There are a few reports of functional RNAi being obtained by systemic delivery of liposome-encapsulated siRNAs, but the use of cationic or anionic lipids for *in vivo* delivery of antisense agents has never reached a clinical trial. Therefore, we still need to understand better which backbone modifications might be useful for enhancing cellular and tissue uptake of naked RNAs, or we need to develop alternative carriers for systemic delivery of siRNAs — a feat that will be essential in treating metastatic cancers.

Using RNAi to target genes expressing oncogenic fusion proteins, such as the Bcr-Abl oncoprotein p210 that is characteristic of chronic myelogenous leukaemia (CML), has provided excellent proof of principle for RNAi as an anti-cancer therapeutic agent. For CML, the main treatment options have been chemotherapy, allogeneic bone-marrow transplant and most recently, the use of a small molecule, the tyrosine kinase inhibitor, imatinib. Despite initial excitement about the potential of imatinib, a growing number of patients have developed resistance to it[86–89], necessitating alternative forms of therapy. Bcr-Abl p210 has been selectively downregulated by both synthetic siRNAs and lentiviral-vector-transduced shRNAs in cell lines[90–92]. Importantly, the downregulation is selective for only the p210 oncoprotein and its mRNA, which results in inhibition of cell proliferation as a direct consequence of RNAi. Haematologic malignancies are often treated by bone-marrow transplantation. Therefore, a possible therapeutic application would be to transduce haematopoietic stem cells with vectors harbouring a gene that targets the mRNA encoding the oncogenic p210 protein, thereby protecting patients from relapse caused by proliferation of latent leukaemic stem cells. Again, delivery is the key issue; 100% transduction of the stem cells reinfused in a bone-marrow transplant setting will be required to make this therapeutically effective. The improvements in viral vector titres and transduction efficiencies may make this possible.

RNAi for genetic diseases

A promising lead towards using RNAi for the treatment of genetic diseases has been provided by preliminary studies that demonstrate how SNPs in mutant allele transcripts can be used as selective targets for RNAi[93,94]. Finding an siRNA that is highly selective for a particular SNP is a challenge, but has been accomplished by systematic analyses of siRNAs in which the polymorphic nucleotide is complementary to the mid-region of the siRNA. In certain examples, the siRNAs direct selective degradation of only the mutant transcripts, leaving the wild-type transcripts intact despite only a single mismatch[93,94]. Another example of siRNAs targeting an SNP was recently reported in studies of amyotrophic lateral sclerosis (ALS) caused by mutations in the Cu, Zn superoxide dismutase (*SOD1*) gene[95]. Because the wild-type SOD1 performs important functions, it is important to selectively eliminate expression of only the mutant allelic transcript. Many SOD1 mutations are single-nucleotide changes. Ding *et al.*[95] achieved selective degradation of a mutant *SOD1* allele, thereby providing a potential therapeutic application for the treatment of ALS.

Disease-causing polyglutamine proteins encoded by CAG-repeat-containing transcripts are found in several neurological diseases such as Huntington's disease. These proteins are especially challenging targets for RNAi because CAG repeats are common to many normal transcripts as well, and the repeats themselves cannot be selectively targeted by siRNAs. But with the recent finding that delivery of siRNAs and viral vectors expressing siRNAs to diseased regions of the brain is technically feasible[96], coupled with selective targeting of SNPs in the mutant transcripts, the promise of clinical use of RNAi for the treatment of degenerative, neurological diseases should be realised.

Challenges for RNAi as a therapy

Two key challenges in developing RNAi as a therapy are avoiding off-target effects and ensuring efficient delivery. One potential risk for side effects emerges from the feature that distinguishes RNAi from other antisense technologies — the use of cellular machinery for directing sequence-specific silencing. This machinery has specific purposes, such as miRNA-mediated gene regulation[97,98]. Using siRNAs to target specific cellular or viral transcripts in essence hijacks the endogenous RNAi machinery, and we know little about the potential for saturating the RNAi pathway in primary cells, although saturation of RISC is demonstrable in cultured cells[99]. So endogenous RNAi pathways could potentially be affected by siRNAs. It will be important to pay close attention to basic research studies on off-target effects of siRNAs and on the design of effective siRNAs[22,27,100]. A better understanding of the mechanisms that lead to nonspecific effects of short dsRNAs is essential before the use of siRNAs or shRNAs can be tested in patient trials.

The issue of delivery has restricted the antisense field for almost two decades. It is feasible to infuse backbone-modified oligonucleotides *in vivo*, but achieving intracellular delivery at therapeutically effective concentrations is a major challenge. Targeted delivery to specific cell or tissue types is still not a practical reality for oligonucleotide-based therapeutics. The alternative approach is viral-vector-mediated delivery of therapeutic shRNA genes. Because this type of delivery results in gene therapy, there are several associated safety concerns, and systemic delivery of viral vectors is still a major hurdle. Nevertheless, the potency and potential general therapeutic utility of RNAi is prompting renewed vigour into delivery-related research. It remains to be determined whether backbone-modified, nuclease-resistant siRNAs will move to the clinic more quickly than synthetic deoxyoligonucleotides.

Perspective

In a remarkably short time since its discovery in model organisms, the RNAi pathway has emerged as a powerful tool for the study of gene function in mammals. As our understanding of the under-lying biology and biochemistry of this conserved gene-regulatory mechanism improves, so does our ability to exploit RNAi as an experimental tool. With the use of RNAi in whole animals increasing, we anticipate growing enthusiasm for the use of RNAi triggers in therapy. Despite considerable hurdles to overcome, it seems likely that RNAi will find a place alongside more conventional approaches in the treatment of diseases, although it is unclear how long we will have to wait to witness the first RNAi-based drug. The big question is whether RNAi can revolutionize the treatment of human disease in the same way that it has revolutionized basic research into gene function. □

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