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Stable RNA interference rules for silencing

Christof Fellmann and

Mirimus Inc., 1 Bungtown Road, Cold Spring Harbor, New York 11724, USA

Scott W. Lowe

Howard Hughes Medical Institute and Memorial Sloan-Kettering Cancer Center, 415 East 68th Street, New York, New York 10065, USA

Scott W. Lowe: lowes@mskcc.org

Abstract

RNA interference has become an indispensable tool for loss-of-function studies across eukaryotes. By enabling stable and reversible gene silencing, shRNAs provide a means to study long-term phenotypes, perform pool-based forward genetic screens and examine the consequences of temporary target inhibition *in vivo*. However, efficient implementation in vertebrate systems has been hindered by technical difficulties affecting potency and specificity. Focusing on these issues, we analyse current strategies to obtain maximal knockdown with minimal off-target effects.

In mammalian systems, RNA interference (RNAi) is an efficient alternative to insertional mutagenesis or gene deletion by homologous recombination for studying loss-of-function phenotypes, as well as being an avenue for high-throughput genetics that has proven crucial in interpreting gene function on a genome-wide scale (Fig. 1). RNAi is also being harnessed as a counterpart to small-molecule inhibitors of protein function and, in principle, can inhibit ‘undruggable’ targets¹ by suppressing the product of any gene. Experimental RNAi takes advantage of conserved cellular machinery that processes exogenous long double-stranded RNAs or endogenous microRNAs (miRNAs) to suppress the expression of homologous genes (reviewed in refs 2,3). Primary miRNAs are expressed like protein-coding genes from promoters dependent on the activity of RNA polymerase II (Pol II), either as ‘stand-alone’ units or embedded in protein-coding transcripts. These are then processed by the microprocessor complex into short stem-loop structures defined as precursor (pre)-miRNAs, which are further cleaved by Dicer to yield mature miRNAs (see Box 1 for details). One strand of a mature miRNA is then loaded into the RNA-induced silencing complex (RISC) to guide the cleaving or translational inhibition of homologous mRNAs (reviewed in refs 4,5).

COMPETING FINANCIAL INTERESTS

C.F. and S.W.L. are founders of Mirimus Inc., and C.F. is an employee of Mirimus Inc., a company that has licensed some of the shRNA technology discussed in this work.

BOX 1**Harnessing endogenous RNAi pathways**

Observations on gene regulation in model organisms have led to the realization that double-stranded RNA (dsRNA) can be used as a tool to control endogenous gene expression^{94–97,103}. In metazoans, various forms of small non-coding RNAs are involved in cellular functions ranging from genome defence to regulation of chromatin structures (reviewed in ref. 105). miRNAs form the largest class of small non-coding RNAs, and guide the RNAi-mediated control of endogenous gene activity, primarily through post-transcriptional regulation of gene expression^{100–102}.

miRNAs are transcribed as long pri-miRNAs, predominantly by RNA Pol II (refs 104,106), and processed into pre-miRNAs through a microprocessor complex involving the class II RNase III endonuclease Drosha^{107,108} (Fig. 2). These pre-miRNAs are then exported to the cytoplasm by the Exportin-5 transporter^{109–111} and cleaved into small RNA duplexes of approximately 22 nucleotides (nt) by the class III RNase III endonuclease Dicer^{112–115}. Mature duplexes are loaded into RISC and ultimately serve as specificity determinants for the recognition of complementary targets^{116–119}.

As part of RISC, Argonaute (Ago) proteins recognize small RNA duplexes and select one strand as the guide for target identification^{120–123}, a process that relies on the ‘seed sequence’ (nt 2–8) as the key specificity determinant (reviewed in ref. 4). Although strand selection has been proposed to depend on the thermodynamic traits of the duplex termini^{26,27}, competitive binding of both 5 nucleotides of small RNA duplexes to the MID domain of Ago2 may also be a crucial factor²⁴. Guide strands with partial target hybridization promote translational repression, whereas those with perfect complementarity mainly induce mRNA degradation through cleavage^{124–126}. Target hydrolysis is catalysed by Ago2, the ‘slicer’ component of RISC, which cleaves the phosphodiester bond coupling the target nt paired to guide nt 10 and 11 (refs 127,128). Cleaved substrates are then released in an ATP-dependent process, while RISC — including the guide RNA — is recycled for further rounds of silencing¹²⁹.

Experimental RNAi relies on programming the conserved cellular RNAi machinery with exogenously provided triggers that can enter the pathway at different points to downregulate homologous genes. While the design of potent shRNAs is hampered by our incomplete understanding of the sequence determinants dictating gene silencing efficiency, further knowledge of the underlying biology continues to improve RNAi tools. For example, a recent study looking at miRNA factors affecting microprocessor function identified a conserved sequence motif as critically required for efficient processing of many vertebrate miRNAs¹³⁰. Importantly, a similar motif drastically enhances the potency of experimental miRNA-based shRNAs¹³¹.

From a practical point of view, the first step in any gene silencing experiment is the choice of an RNAi expression system. One option is to use a stem-loop design implemented in shRNA collections such as the Broad Institute TRC library¹³² and the NKI-AvL library¹³³. Cellecta (www.cellecta.com) also provides libraries based on a stem-loop structure. Another option is to use a miRNA-embedded design put into effect

using the human miRNA (miR)-30 scaffold in the genome-wide libraries produced at Cold Spring Harbor Laboratory¹¹ and various focused libraries recently built for *in vivo* screening^{66,76}. Similarly, Life Technologies (www.lifetechnologies.com) distributes libraries based on a minimal human miR-155 backbone that are also effective. In theory, exploiting non-canonical miRNA pathways provides a third option; for example, the class of miRNAs defined by miR-451 bypasses Dicer and instead relies on the catalytic activity of Ago2 for passenger strand cleavage and opening of the stem-loop structure^{134–136}. Hence, miR-451-based shRNAs could be used in tumours with impaired miRNA processing owing to Dicer deficiencies¹³⁷.

A recent trend in miRNA-based RNAi directly couples shRNA expression to a fluorescent reporter, enabling investigators to track shRNAs indirectly through reporter expression and allowing analysis or purification of only those cells that productively express an shRNA⁸⁵.

The endogenous RNAi machinery can be engaged by providing exogenous triggers that enter the pathway at different points (Fig. 2). One approach relies on transfection of chemically synthesized short interfering RNAs (siRNAs) that can suppress endogenous or heterologous gene expression in cultured cells⁶. Although the effects can last for days, they are transient and limited to cells amenable to transfection. Genomic integration of vectors stably expressing stem-loop short hairpin RNAs (shRNAs) that mimic pre-miRNAs overcomes this restriction by providing a continuous and heritable source of RNAi triggers^{7–9}. However, such stem-loop shRNAs are expressed from RNA polymerase III (Pol III) promoters and skip the early steps of miRNA biogenesis. Further embedding shRNA sequences into an endogenous miRNA backbone creates a configuration recognized as a natural substrate of the RNAi pathway^{10–15}. This ensures efficient production of mature small RNA duplexes and reduces toxicity^{16–19}. The use of an miRNA backbone also enables stable and regulated expression from Pol II promoters²⁰, as well as the construction of polycistronic ‘tandem’ shRNA vectors and linking to fluorescent reporters²¹.

By exploiting these tools, RNAi can, in principle, be used to suppress the expression of any gene. However, owing to our incomplete understanding of the mechanisms behind miRNA biogenesis and target inhibition, this process is somewhat unpredictable and often not as efficient as desired. As the seed sequence that ultimately drives homology-dependent knockdown is relatively short, not all designed sequences are target-specific^{22,23}. Furthermore, highly potent shRNA sequences are rare and need to be identified among hundreds to thousands of possibilities within a given transcript. Although less efficient sequences can be effective when expressed at high copy or transfected at high concentration, expression of the same shRNAs from a single genomic integration (‘single-copy’) often results in insufficient target knockdown²⁴. However, many key applications such as pooled shRNA screens and RNAi transgenic animals inherently require single-copy conditions to enable deconvolution of screening results and for site-directed integration of shRNAs, respectively. Here we review recent developments in the field, concentrating on the optimization of stable RNAi for vertebrate systems.

Identifying the right shRNA

Efforts to identify effective RNAi triggers have led to design rules and algorithms based on empirical and systematic analysis of siRNAs using conventional^{25–27} and machine learning-based approaches^{28,29}. Such studies have advanced our ability to predict efficient siRNAs (reviewed in refs 30,31), but when used to design single-copy shRNAs, the output typically contains a mixture of functional and non-functional sequences that require further validation³². This may be a consequence of the limited expression strength from a single-copy genomic integration, or be due to the additional processing requirements of shRNA precursors compared to siRNAs (see Table 1 for details). Consequently, single-gene studies still depend on laborious testing of many candidates, and pooled shRNA screens contain non-functional sequences that make the interpretation of negative results inconclusive³³.

Prior evaluation of shRNAs through reporter assays can overcome this limitation by placing cognate target sites in the 3′ untranslated region (UTR) of a marker gene and quantifying its RNAi-mediated repression following exposure to the candidate RNAi triggers^{34,35}. For example, we established a high-throughput assay for testing tens of thousands of shRNA candidates in parallel and showed that it robustly identifies potent single-copy shRNAs²⁴. Although other strategies to assess target knockdown, such as immunoblotting, might be easier to implement on a gene-by-gene level, this assay can identify potent sequences through the tiling of entire transcripts or assessment of preselected candidates. To build such a candidate list, several design criteria must be considered to maximize the number of potent shRNAs recovered (Fig. 3). Starting with NCBI’s Reference Sequence (RefSeq) collection, we use either the unique or common part of multiple RefSeq transcripts per gene to design shRNA molecules. Further, since alternative cleavage and polyadenylation (APA) can result in widespread shortening of 3′ UTRs in proliferating and cancer cells^{36,37}, the most broadly applicable shRNAs should target sequences preceding the first alternative poly (A) site.

Unlike the apparent restriction of miRNA target sites to the 3′ UTRs of genes (reviewed in ref. 4), potent shRNA target sites seem to be uniformly distributed throughout the 5′ UTR, coding sequence and 3′ UTR (ref. 24). However, potent sites are less frequent in GC-rich regions³⁸. A ranking of candidate RNAi triggers can be obtained using a series of different tools (reviewed in ref. 39). Although most of these were built for siRNAs, shRNA-specific predictors are starting to emerge⁴⁰. Time will prove the practical value of these tools; however, additional functional data sets may be required to better probe the biological sequence space and boost predictive power. Meanwhile, one practical way to minimize the effort for identifying potent shRNAs is to select candidates based on overlap between siRNA predictions and shRNA-specific ‘rules’ that further eliminate non-functional sequences²⁴.

The origin and circumvention of off-target effects

Potential off-target effects remain a primary concern when interpreting RNAi results (reviewed in ref. 41). It is thus vital to understand how they arise and can be mitigated. Off-target effects fall into three categories, those arising from: (1) sequence-based homology with non-target transcripts; (2) effects related to aberrant processing of endogenous miRNAs

or undesired editing of experimental RNAi triggers; and (3) general cell perturbations owing to the copious presence of exogenous vectors and/or RNA species. Most of these effects can be minimized with an appropriate shRNA design and certain experimental precautions (Fig. 4).

Sequence-based off-target effects^{22,23} can be limited by excluding shRNAs with a high likelihood of targeting non-target transcripts using bioinformatics approaches (reviewed in refs 31,42,43) or through the use of multiple shRNAs targeting the same gene to experimentally reject phenotypes associated with off-target silencing. Furthermore, since knockdown potency probably depends on target hybridization efficiency in a nonlinear manner, a reduction of guide-strand concentration should decrease off-target effects more significantly than the on-target response. This implies that shRNAs capable of potentially suppressing gene expression at low concentrations should intrinsically produce fewer off-target effects, a prediction that has been experimentally confirmed⁴⁴. Still, ‘rescue’ experiments using overexpressed cDNAs devoid of targeted 3′ UTRs or with mutagenized targeting sites help conclusively assign a phenotype to the inhibition of a given target, although in some cases the results are confounded by artefacts produced by overexpressing the non-repressible transcript.

Sequence-based off-target effects can be caused by both guide- and passenger-strand-mediated cross-hybridization with unintended transcripts. The ratio at which the RNAi effector protein Argonaute (Ago) chooses one strand of a mature small RNA duplex as the guide over the other strand varies greatly based on the shRNA sequence composition^{24,26,27}. As incorporation of the designed passenger strand into RISC can only lead to off-target effects, while concomitantly decreasing the on-target response, it is crucial to shift the strand selection bias as much as possible to the intended guide. For vertebrate Ago2, this can be achieved by having a U at the 5′-end of the guide, while ideally having a G at the guide position hybridizing with the 5′-end of the passenger strand (position 20 in 22-nucleotide guides)²⁴.

Off-target effects linked to endogenous miRNAs can arise through: (1) saturation of miRNA biogenesis leading to aberrant processing of endogenous miRNAs; (2) competition of experimental and endogenous RNAi triggers for incorporation and retention in RISC; and (3) undesired processing of shRNA precursors yielding unintended guide sequences. Specifically, the biogenesis of endogenous miRNAs can be perturbed by the accumulation of toxic precursors (primary (pri)-miRNAs, pre-miRNAs and stem-loop structures), saturation of miRNA processing (involving the Drosha and Dicer complexes), and overload of nuclear export^{19,45}. Embedding shRNAs into miRNA backbones alleviates the saturation of the pre-miRNA export machinery by reducing the concentration of precursor molecules or by promoting direct shuffling by the microprocessor complex¹⁹. Similarly, whereas bulk-transfected siRNAs can outcompete endogenous miRNAs for RISC loading⁴⁶, single-copy use of miRNA-embedded shRNAs leaves the endogenous miRNA population unperturbed⁴⁷.

Furthermore, unspecific cleavage by Drosha²⁴ or Dicer⁴⁸, and potential redirection of silencing targets through adenosine-to-inosine editing of precursor transcripts⁴⁹, can cause

off-target effects through the generation of unintended guide sequences. For Dicer, processing accuracy relies on the loop positioning and the presence of internal bulges⁴⁸, which can easily be addressed with an appropriate shRNA design. Lastly, general cell perturbations produced by high doses of exogenous vectors should be avoided using single-copy conditions. Hence, the optimal shRNA shows minimal cross-hybridization with non-desired transcripts, is embedded in an accurately and efficiently processed backbone, and induces potent knockdown at low concentration to minimize sequence-dependent and general off-target effects.

The power of pooled shRNA screens

High-throughput RNAi screens have enabled the rapid functional annotation of mammalian genomes (reviewed in ref. 50) and uncovered components of signalling pathways as new therapeutic targets⁵¹. One way to perform RNAi screens is by using genome-wide siRNA or shRNA collections in 'one-by-one' assays, where single RNAi triggers are introduced into cells in a multiplexed fashion. Although powerful, such screens require substantial infrastructure and are expensive to run. Another approach is to perform pool-based shRNA screens, taking advantage of the ability to deliver mixtures of shRNAs targeting different genes into cell populations by viral transduction. Here, shRNA libraries are delivered into cells such that, on average, each cell expresses only one shRNA of the population (single-copy). Stable integration into the genome prevents dilution of RNAi triggers through cell replication and enables shRNAs to be quantified by deep-sequencing. Following a perturbation (such as growth in culture or treatment with a drug), transduced cells are analysed to assess how the representation of each shRNA changed relative to the control condition. Hence, each cell serves as an individual test tube to determine how suppressing gene function influences phenotypes in a highly multiplexed fashion.

Pool-based shRNA screens require an enrichment step that selects for or against cells acquiring a particular phenotype. This can involve enrichment for cells expressing a reporter using flow cytometry, or simply be based on the competitive fitness of cells in culture. For example, the sought phenotype could produce a fitness advantage under a particular experimental condition. In this vein, shRNA libraries have been introduced into non-transformed cells to identify shRNAs that enhance oncogenic transformation *in vitro*, leading to the identification of new tumour suppressors⁵² and unravelling the organization of cancer genomes⁵³. In other instances, studies have looked for shRNAs that confer a fitness disadvantage, identifying tumour maintenance genes and pinpointing potential drug targets in cancer.

Examples of the latter involve efforts to identify specific vulnerabilities in cancer cells expressing mutant Ras oncoproteins, which so far have been refractory to the development of molecularly targeted therapies. In theory, shRNAs specifically depleted in cells expressing mutant Ras would identify cancer-specific drug targets. Several research groups performed screens with this goal, each finding potential drug targets for Ras-mutant cancer, although without overlap. This divergence remains to be fully understood, but it is possible that Ras synthetic lethal interactions are context-dependent — and, indeed, each group used different cell systems and assay conditions. Another explanation is that different RNAi

libraries were employed and that not all relevant targets were adequately inhibited to score in each assay, reflecting limitations of the reagents used.

Recent technological advances have improved the accuracy of pooled shRNA screens. Next-generation sequencing has largely replaced array-based approaches to quantify shRNA representation⁵⁴. Using this method, a screen looking for sensitizers to the BRAF inhibitor PLX4032 in *BRAF*^{V600E}-mutant colorectal cancer cells identified the epithelial growth factor (EGF) receptor as a combination target in this disease⁵⁵. In a large-scale effort, Project Achilles is using the same strategy to identify genetic vulnerabilities across many cancer cell lines⁵⁶. Immunohistochemistry-based high-content analyses can also be integrated into RNAi screens (reviewed in ref. 57). Although methods relying on microscopy remain restricted to one-by-one approaches⁵⁸, more basic features such as cell staining or total cellular fluorescence of immuno-detected antigens can be assayed by fluorescence activated cell sorting (FACS) and are compatible with pooled screening.

Bioinformatics tools can improve RNAi screening results by overcoming pitfalls of imperfect reagents. One simple approach maps all guide and passenger seed sequences of the used RNAi triggers to the transcriptome and questions whether scoring sequences enrich for a specific (non-target) transcript⁵⁹, thus revealing off-target genes. Although straightforward, this strategy accounts only for sequence-based effects and requires user-defined classes of scoring and non-scoring sequences. For large-scale shRNA data sets obtained through screening of dozens of cell lines, statistical analysis of the results can reduce experimental noise by computationally discarding data from reagents that are not efficient at inhibiting their target or likely to induce off-target effects. The ATARiS software achieves this by identifying patterns in RNAi data across multiple samples and computing phenotypic ‘gene solutions’ using only data from shRNAs it deems consistent⁶⁰. In contrast to seed sequence mapping aimed at identifying off-target genes, ATARiS looks for ‘real’ solutions by ignoring noise and improving screen analysis without requiring predetermined classes or assumptions about the cause of off-target effects.

Computational approaches can further facilitate the integration of genomic data across different platforms, to link loss-of-function phenotypes with mutational status, copy number and gene expression data. In the long run, this will lead to more coherent results across research groups and enable the generation of databases describing how shRNA-based perturbations of gene function affect cellular phenotypes (for a first attempt, see ref. 61). Similarly to sequencing databases existing today, such efforts will result in easily accessible resources for data mining to improve our understanding of normal homeostasis and disease. Yet, the realization of the full potential of RNAi as a tool for high-throughput screening will require the establishment of highly potent and specific genome-wide shRNA libraries.

Most current RNAi libraries contain reagents that have not been validated under the conditions at which they are used in screens (single-copy), making the interpretation of negative results inconclusive. One way to address this shortcoming is to generate large shRNA libraries in which a particular gene is targeted many times^{33,62}, although this has trade-offs in reducing the representation of any given shRNA and/or restricting the number of genes that can be assayed simultaneously. Alternatively, shRNA libraries can be

assembled from 3–5 shRNAs per gene that have been functionally pre-validated. The RNAi ‘Sensor Assay’ described above allows massively parallel identification of potent shRNAs²⁴, providing a strategy for the generation of functionally validated shRNA libraries. Such libraries will not only enable comprehensive pooled RNAi screening with reduced off-target effects, but would also be a boon for systems biology, as the ability to interpret positive and negative results will enable the identification of all key functional nodes in various cellular phenotypes.

Opportunities and caveats of *in vivo* RNAi

Animal models provide an immense resource to investigate the aetiology of human diseases, and have greatly contributed to the establishment of conventional and targeted therapeutics (reviewed in ref. 63). Mouse models enable gene-function studies to be conducted in a physiological environment that takes a decisive role in the initiation and progression of tumours as well as their response to therapy⁶⁴. Until recently, RNAi in the context of the whole organism was limited to non-vertebrate models. However, the optimization of shRNA technology now enables loss-of-function studies also in mice and, with some additional manipulation, shRNA libraries can be used in forward genetic screens to reveal unanticipated gene combinations influencing *in vivo* phenotypes.

One approach for *in vivo* RNAi involves *ex vivo* introduction of shRNAs into cultured cells before their transplantation. This method has been particularly powerful in the haematopoietic system and mammary gland, where stem or progenitor populations can be isolated, cultured and transduced with viral vectors before being transplanted into recipient mice to reconstitute the tissue of origin^{65,66}. Germline transgenic approaches have also shown promise, especially using strategies that combine engineered embryonic stem cells⁶⁷, tetracycline (tet)-regulated systems^{68,69} and RNAi technology to produce genetically engineered mice that reversibly express miRNA-embedded shRNAs from a defined locus following doxycycline (a tet analogue) treatment^{47,70–72}. In principle, these models allow genes to be conditionally suppressed throughout an entire organism or in a particular tissue using a ubiquitous or tissue-specific tet-transactivator, respectively. Such RNAi transgenics yielded insight into the roles of the *p53* and *APC* tumour suppressor genes during tumorigenesis^{47,70}, elucidated the function of an essential DNA replication protein⁷³, and are expected to further enhance our understanding of normal development and pathogenesis (reviewed in ref. 74).

Inducible shRNA transgenics can expedite the evaluation of putative drug targets. For example, RNAi transgenic mice conditionally suppressing the eukaryotic initiation factor *eIF4F* have been used to validate the translation initiation complex as therapeutic target in *Myc*-driven lymphoma⁷⁵. Although RNAi-mediated target knockdown can produce phenotypes different from those achieved by small-molecule or antibody-based target inhibition, such distinctions can usually be uncovered by further experimentation. One advantage of the RNAi approach is that it allows the suppression of a particular member of a multi-gene family to dissect the phenotypes observed with compounds binding several structurally similar targets, as has been shown by comparing the BET (bromodomains and extraterminal) domain inhibitor JQ1 (targeting the proteins Brd2, Brd3, Brd4 and Brdt) with

phenotypes produced by suppressing *Brd4* only⁷⁶. Analogous to small molecules, RNAi-based target inhibition is not a genetic null — it leaves the endogenous alleles intact. This facilitates the study of hypomorphic phenotypes⁷⁷ that may resemble drug-mediated target inhibition and enables reversibility.

Various *in vivo* RNAi screens have been performed using transplantation-based approaches to uncover novel tumour suppressors or oncogenes. In multiple positive selection assays, libraries targeting candidate tumour suppressor genes were introduced into premalignant cell types from the liver or haematopoietic system, and analysed for those shRNAs that were enriched following transplantation and tumour formation in recipient mice. Such studies validated many genes as novel tumour suppressors *in vivo*^{78–80}, and revealed that they can be physically linked⁸¹. Negative selection screens, that is, those that search for genes whose inhibition produces a selective disadvantage, have also been performed *in vivo*, although with additional challenges. In one example, a library of shRNAs targeting 1,000 cancer-related genes was employed to identify factors required for lymphoma cell proliferation *in vitro* and, in parallel, was used *in vivo* in a syngeneic murine lymphoma system⁵⁴. Interestingly, a large number of ‘hits’ were unique to the *in vivo* setting, highlighting the importance of an intact microenvironment. In other studies, pools of up to 500 shRNAs were introduced into human xenotransplants to uncover dependencies of certain breast cancers on increased metabolic pathway flux⁸², and pools of 1,500 shRNAs were used in screens to identify leukaemia-specific dependencies⁸³.

To induce gene alterations in the adult, transposon-based expression systems have recently been adapted to deliver shRNA libraries directly into the liver of whole animals⁸⁴. Although limited to certain tissues, this powerful method circumvents the need for *ex vivo* manipulation of cells and avoids cell-culture-based artefacts. Together, studies done so far suggest that *in vivo* RNAi screens are limited by the complexity of shRNA pools that can be surveyed in an animal owing to incomplete transplantation and engraftment of manipulated cells, or limited transduction efficiency of vectors delivered *in vivo*. Replicates help ensure that differences in representation are indicative of specific events, but careful attention to library complexity is crucial for the successful interpretation of such screens⁸⁵. If implemented properly, however, *in vivo* RNAi screens are an invaluable tool to identify and characterize drug targets or biochemical pathways in physiologically relevant models of human disease.

The future of shRNA technology

Further understanding of RNAi biology will lead to an enhanced ability of producing potent shRNAs with fewer off-target effects. For example, beyond redirecting miRNA duplexes into different Ago proteins^{86,87}, endogenous miRNAs show mismatches and bulges at various positions for largely unknown reasons. Future shRNAs might feature such modifications to increase potency while concomitantly reducing off-target effects (discussed for siRNAs in ref. 88). Furthermore, functionally validated shRNA libraries will enable the interpretation of negative results and reduce the amount of false positives in screens through more stringent cutoff thresholds. At the same time, library generation will profit from advanced ‘on-chip’ oligonucleotide synthesis, enabling customized pools to be rapidly

built⁸⁹ and adapted to new experimental designs. With falling costs and increasing sequence quality, such libraries will eventually eliminate the advantage of sequence-verified clone archives.

While past advances in shRNA technology have improved proliferation-based screens, current developments will continue the trend towards more elegant readouts such as reporter-based assays and high-content screens, which promote a more physiologically relevant analysis of biological phenotypes. In parallel, *in vivo* applications will benefit from enhanced delivery and expression systems that avoid unintended tissue perturbation, for example through the use of potent single-copy shRNAs.

Increasing technical sophistication will expand RNAi beyond protein-coding genes. Indeed, shRNAs can be used to repress any sufficiently long transcript present in the cytoplasm and have been employed to suppress cytoplasmic large intergenic non-coding RNAs (lincRNAs)^{90,91}. Although several nuclear RNAi pathways have been discovered, including piRNAs involved in transposon defence and co-transcriptional gene silencing mechanisms enabling heterochromatin formation in plants (reviewed in ref. 92), it is unknown whether exploitation of these pathways may enable suppression of nuclear non-coding RNAs in vertebrates.

Advanced genome editing technologies such as insertional mutagenesis screens relying on haploid cells, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeat (CRISPR)-based systems will complement gene silencing methods and broaden our toolkit to understand and tackle human disease. Although these alternative approaches are powerful, they do irreversibly modify the genome and come with their own set of strengths and weaknesses⁹³. Hence, the future will involve combinations of strategies, and improvements of each technology will be necessary to assemble a complete set of tools for the functional annotation of the human genome.

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Figure 1.

Discovery and development of RNA interference (RNAi). Ever since the discovery of RNAi as an endogenous mechanism that fine-tunes gene expression, efforts have been made to exploit it experimentally to silence genes of choice for both research and therapeutic purposes. Pigmentation defects observed in petunias engineered to overexpress key enzymes in the flavonoid biosynthesis pathway were among the first experimental hints of a post-transcriptional gene silencing mechanism mediated by mRNA degradation^{94,95}. Several years later, *lin-4*, a gene known to time *Caenorhabditis elegans* larval development, was found to code for a pair of small RNAs that regulate LIN-14 protein levels^{96,97}. Although regulatory roles for RNAs had been proposed earlier^{98,99}, *lin-4* and its prototype counterpart *Xist*, a long non-coding RNA involved in mammalian X chromosome inactivation, were traditionally regarded as exceptions to the prevailing model of gene expression control by protein transcription factors. *lin-4* is now recognized as the founding member of a class of small non-coding RNAs called microRNAs (miRNAs) that, in turn, were found to regulate the expression of at least one third of all human genes^{100–102}. Decisive for the boom of experimental RNAi was the discovery of a sequence-specific gene silencing mechanism mediated by double-stranded RNA (dsRNA) in the nematode *C. elegans*¹⁰³. Genetic and biochemical studies subsequently revealed insight into the molecular underpinnings of the RNAi pathway and uncovered analogous mechanisms in many eukaryotic organisms, enabling their exploitation for loss-of-function genetics in vertebrate systems. Shown here are selected theoretical, experimental and technical achievements.

Figure 2.

Endogenous RNAi pathways and their use as tools for gene silencing. The RNAi machinery can be engaged at different points of the pathway by various forms of endogenous or exogenous double-stranded RNA (dsRNA) molecules to trigger the suppression of homologous genes. Like most endogenous miRNAs¹⁰⁴, synthetic miRNA-based shRNAs are expressed from RNA polymerase II (Pol II) promoters as primary miRNA transcripts (pri-miRNAs) that are recognized as natural substrates of the RNAi pathway. In contrast, stem-loop shRNAs are expressed as shorter precursor miRNAs (pre-miRNAs) mostly from promoters dependent on the activity of RNA polymerase III (Pol III) and thus skip the initial Drosha-mediated step of miRNA biogenesis. Different types of chemically synthesized short interfering RNAs (siRNAs) are transduced into cells as small RNA duplexes and enter the pathway at or after the Dicer stage (see Table 1 for details) to be directly incorporated into the RNA-induced silencing complex (RISC). Largely based on the degree of homology between the RNAi trigger and its target transcript, target knockdown occurs either through Ago-mediated mRNA degradation or translational repression and deadenylation.

Figure 3.

Best practices for efficient and specific stable RNAi. For loss-of-function studies, RNAi has become an indispensable tool that enables functional gene annotation through stable and reversible gene silencing. RNAi can mirror gene loss during disease progression or mimic pharmacological target inhibition even where no such drug currently exists. Despite this potential, the design of potent and specific shRNAs is not trivial and careful experimental design is required to obtain robust results. For single-gene studies, the use of multiple shRNAs targeting the same gene can help experimentally reject phenotypes associated with off-target silencing. Likewise, multiple RNAi transgenic animals expressing shRNAs targeting the same gene can confirm on-target specificity of phenotypes. With pooled RNAi screens, special attention must be given to the complexity of shRNA libraries to ensure sufficient representation of each shRNA throughout the experimental procedure. Furthermore, for pooled screens and transgenic animals, shRNAs must be potent enough to induce a phenotype when expressed from a single genomic integration (that is, ‘single-copy’). Ultimately, the successful design of an experimental RNAi system includes selection of the right target transcript (region), choice of an appropriate expression system, informed selection of candidate shRNAs, and functional validation of knockdown efficiency. Using both biological and computational tricks to minimize system noise, RNAi results can be enhanced even following the experimental assay (for example, by analysing only those cells that productively express an shRNA). However, as most safeguard mechanisms are system-dependent, they require their implementation to be planned already during the experimental setup.

Figure 4.

Ramifications of off-target effects. When interpreting RNAi results, off-target effects remain a key concern. However, these can be minimized with an appropriate shRNA design and implementation of certain experimental precautions. Most off-target effects fall into one of three large categories: (1) sequence-based homology with non-target transcripts; (2) aberrant miRNA biogenesis of endogenous or exogenous RNAi triggers; and (3) general perturbations affecting cell homeostasis. The diagram depicts the experimental precautions (red circles) that can minimize the various ramifications of each class of off-target effects.

Table 1

Endogenous and synthetic RNAi triggers.

	siRNA	Stem-loop shRNA	miRNA-based shRNA	Endogenous miRNA	miR-451
Origin of RNAi trigger	Chemically synthesized	Transduced expression vector	Transduced expression vector	Endogenous transcript	Endogenous transcript
Expression *	Bolus transfection	Pol III promoter	Pol II promoter	Pol II promoter	Pol II promoter
Pri-miRNA processing	-	-	Drosha	Drosha	Drosha
Nuclear export	-	Exportin-5	Exportin-5	Exportin-5	Exportin-5
Pre-miRNA processing	Dicer†	Dicer	Dicer	Dicer	Ago2
Strand selection	Ago	Ago	Ago	Ago	Ago2
Target suppression	RISC	RISC	RISC	RISC	RISC
Mode of gene silencing *	Target cleavage	Target cleavage	Target cleavage	Target cleavage, translational repression and deadenylation	Target cleavage, translational repression and deadenylation

The table shows similarities and differences between siRNAs, stem-loop shRNAs, miRNA-embedded shRNAs and endogenous RNAi triggers.

* Only the most common forms of expression methods and modes of gene silencing are shown here.

† Only certain longer siRNAs require processing by Dicer. Ago, Argonaute. RISC. RNA-induced silencing complex.