Mammalian RNAi: a practical guide

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Silencing of gene expression by RNA interference (RNAi) has become a powerful tool for the functional annotation of the Caenorhabditis elegans *and* Drosophila melanogaster *genomes. Recent advances in the design and delivery of targeting molecules now permit efficient and highly specific gene silencing in mammalian systems as well. RNAi offers a simple, fast, and cost-effective alternative to existing gene targeting technologies both in cell-based and in vivo settings. Synthetic small interfering RNA (siRNA) and retroviral short hairpin RNA (shRNA) libraries targeting thousands of human and mouse genes are publicly available for high-throughput genetic screens, and knockdown animals can be rapidly generated by lentivirus-mediated transgenesis. RNAi also holds great promise as a novel therapeutic approach. This review provides insight into the current gene silencing techniques in mammalian systems.*

INTRODUCTION

RNA interference (RNAi) is a sequence-specific gene silencing process that occurs at the posttranscriptional level (1,2). The RNAi machinery is thought to represent an ancient, highly conserved mechanism that defends the genome against viruses and transposons. The effectors of RNAi are short (21–28 nucleotides) double-stranded RNA (dsRNA) molecules. These small interfering RNAs (siRNAs) are processed from longer precursors by a cytoplasmic ribonuclease called Dicer. The antisense strand of the siRNA serves as a template for the RNA-induced silencing complex (RISC) to recognize and cleave a complementary messenger RNA (mRNA), which is then rapidly degraded.

Long (typically 500 bp or more) dsRNAs can induce efficient and highly specific gene silencing when introduced into worms, flies, or plants (1,3). In mammalian cells, molecules of the same length are recognized by the RNA-dependent protein kinase (PKR) and elicit an interferon response, which results in the general inhibition of protein synthesis (4). However, it has been demonstrated that short (<30 bp) synthetic dsRNAs can trigger sequence-specific knockdown of gene expression without PKR activation in cultured mammalian cells (5,6). This breakthrough discovery provided the base for the development of RNAi as a powerful and widespread reverse genetics tool for the functional annotation of mammalian genes leading to genome-wide phenotypic screens in cell culture and the creation of knockdown mice (7,8).

This review will focus on the practical aspects of RNAi conducted in mammalian systems, emphasizing the design, delivery, and functional validation of targeting molecules.

PLANNING AHEAD

In mammalian RNAi experiments, two parameters primarily determine the choice of targeting molecule and delivery method: the cell type and the desired duration of the silencing effect (Figure 1). For example, if one is interested in the short-term consequences of knocking down a particular gene in an easy-to-transfect cell line, probably the most effective approach is to use synthetic siRNAs. When the goal is long-lasting gene silencing, it is more appropriate to use short hairpin RNAs (shRNAs) expressed from plasmids or, in the case of hard-to-transfect cells, from lenti- or retrovirus-based vectors.

Appropriate controls should be incorporated into each experiment (8,9), typically including si/shRNAs against genes that are not expressed in mammalian cells [e.g., luciferase, green fluorescent protein (GFP)]. Similarly, phenotypic changes associated with the knockdown of a gene should be corroborated with multiple targeting molecules against the same gene to exclude off-target effects. Finally, whenever possible, rescue experiments should be performed to demonstrate specificity, for example by expressing the targeted gene carrying a silent mutation in the siRNA binding region.

MATERIALS AND METHODS

siRNAs: The Fast Track

siRNAs are 21- to 22-nucleotide-long dsRNA molecules that contain a 19-bp core sequence and two unpaired nucleotides at each 3′ end (1). Because of their small size, the chemical synthesis of siRNAs is relatively easy, and several companies offer them delivered in ready-to-transfect format and, upon request, with chemical modifications that increase the stability

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and/or specificity of the molecules in cells. The final cost of a pool of functionally validated and chemically modified siRNA in a quantity that is suitable for a few in vitro experiments may be over $1000, but the fast processing time and rapid and efficient knockdown of the target gene are very appealing.

There are means of generating siRNAs other than chemical synthesis. One possibility is in vitro transcription of the target cDNA followed by cleavage by recombinant Dicer or bacterial RNase III (10,11). This method is very simple, does not require siRNA design, and the resulting pool of molecules can be easily purified and transfected into cells, which makes it an excellent choice for the generation of siRNA libraries (Figure 2A). Additionally, Dicer cleavage provides the siRNA in its natural form to the RISC complex. The drawbacks of this method are the lack of sequence information about the produced individual siRNAs and the potentially increased risk of off-target effects. However, it has been shown that distinct pools of siRNAs generated from two cDNAs sharing 82% identity induced knockdown in a highly target-specific manner (12).

Another option is in vitro transcription of well-defined short sequences from viral promoters with the corresponding viral polymerases, like T3, T7, or SP6, that do not require complex initiation and termination signals for transcription and do not add a long poly(A) tail to the end of the transcript. Cellular Pol III polymerases have similar features (see shRNAs: The Long-Term Solution section), and it has been shown that siRNAs can be readily produced by in vivo transcription from two opposing Pol III promoters flanking the template sequence (13). Thousands of promoter-template-promoter expression cassettes generated by PCR in a high-throughput fashion have been recently used in an RNAi screen (13).

The use of siRNAs relies on efficient delivery into cells, but there are several cell types that are partially or completely refractory to transfection. In addition, because siRNAs are quickly diluted in dividing cells, the silencing effect usually lasts for a few days at most; and it can be maintained for a week or more in nondividing cells or when chemically stabilized molecules are used. Both of these limitations can be overcome by using shRNAs and viral-transduction methods.

shRNAs: The Long-Term Solution

shRNAs are produced as single-stranded molecules of 50–70 nucleotides in length and form a stem-loop structure in vivo. A 5- to 10-nucleotide loop connects the two complementary 19- to 29-nucleotide-long RNA fragments that create the double-stranded stem by base pairing. shRNAs are typically synthesized in vivo by transcription of complementary DNA sequences from Pol III promoters (1). Pol III-mediated transcription initiates at a well-defined start site and terminates at the second residue in a stretch of four or more thymidines, producing a non-poly(A) transcript. Additionally, Pol III promoters are active in all cell types, permitting the constitutive expression of shRNAs. After transcription, shRNAs are recognized and cleaved at the loop by Dicer and enter the RISC as siRNAs (2).

In most cases, the shRNA-encoding DNA fragment is made of two chemically synthesized 50- to 70-nucleotide-long oligonucleotides that are annealed and then ligated into a vector. This is a relatively straightforward method, although the synthesis of such long oligonucleotides is error prone. Alternatively, a Pol III promoter-shRNA template cassette can be assembled in a two-step PCR that uses shorter primers (14,15). The PCR product can be either cloned into a vector or directly transfected into cells.

Parallel synthesis of thousands of oligonucleotides in situ on microarray platforms may represent the future direction for the high-throughput generation of genome-wide shRNA libraries. In a very recent study, 96-mer single-stranded DNA molecules were synthesized by ink-jet technology, released from the glass slide by chemical treatment, PCR-amplified and digested with the appropriate restriction enzymes in pools, and finally cloned into a vector (16). The rate of recovery

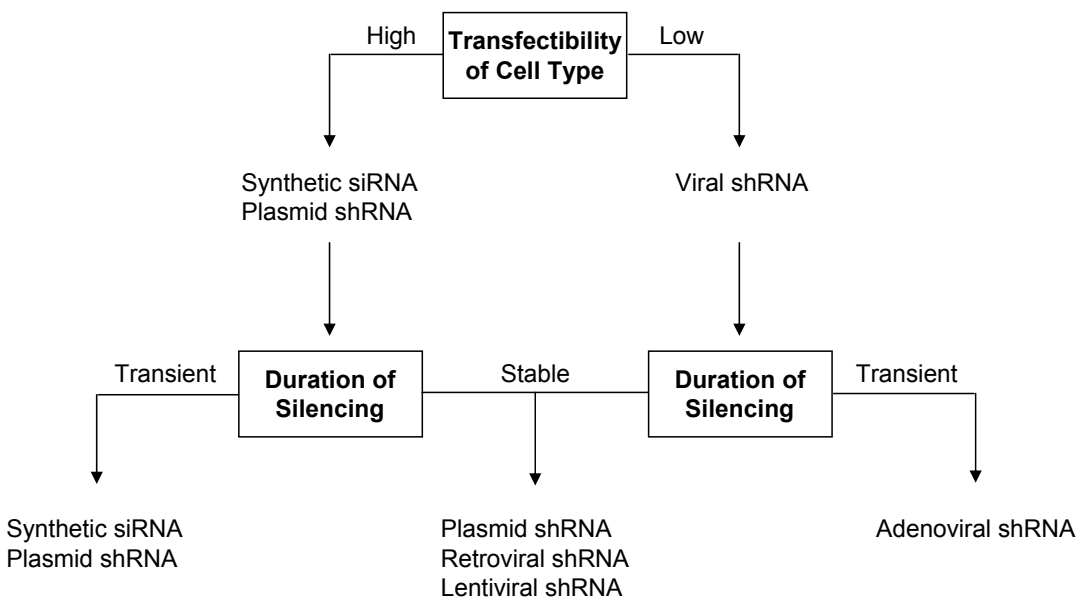

Figure 1. Selection of targeting molecules and delivery methods for mammalian RNA interference (RNAi). siRNA, small interfering RNA; shRNA, short hairpin RNA.

of correct shRNA sequences was similar or superior to those synthesized by conventional methods.

shRNAs can be generated from the target cDNA by enzymatic digest as well (17–19). In these methods, the cDNA is first cut into smaller pieces, then the fragments are ligated to a hairpin adaptor containing the *Mme*I recognition sequence (Figure 2B). This enzyme cleaves 20 nucleotides 3′ from its site, leaving a sticky end where another adaptor is attached. The single-stranded hairpin structure is then amplified to yield the double-stranded DNA, which is subsequently cloned into a vector (Figure 2B). Notably, however, it is likely that not all potentially effective target sequences will be incorporated into shRNA templates and that some targeting constructs generated with these approaches may have off-target effects.

The major advantage of vector-based RNAi is the achievement of long-lasting gene silencing with shRNAs expressed from stably transfected plasmids or from integrated retro- or lentiviral vectors (4,7,20). These tools are indispensable for conditional and inducible RNAi both in cell-based and in vivo settings and will be discussed in detail in the following paragraphs.

The shRNA-coding sequences can be also used as unique identifiers, dubbed molecular barcodes in genome-wide RNAi screens, where hundreds of constructs are analyzed in pools (20,21). One strand of each of the oligonucleotide pairs used for the generation of the shRNA library can be spotted on a glass slide to produce a DNA microarray (Figure 2C). After infection, the barcode can be PCR-amplified from genomic DNA, labeled with a fluorescent dye, and subsequently hybridized to the complementary oligonucleotide array. The intensity of the signal indicates the relative abundance of each barcode in a population. This approach permits the detection of both gain and loss of signal from a given shRNA, thus allowing the simultaneous identification of genes with positive and negative effect on the phenotype of interest (20). Other investigators found that specifically designed 60-mer oligonucleotides incorporated into the vector backbone show a better signal-to-noise ratio than shRNA oligonucleotides when hybridized to the corresponding microarray (22).

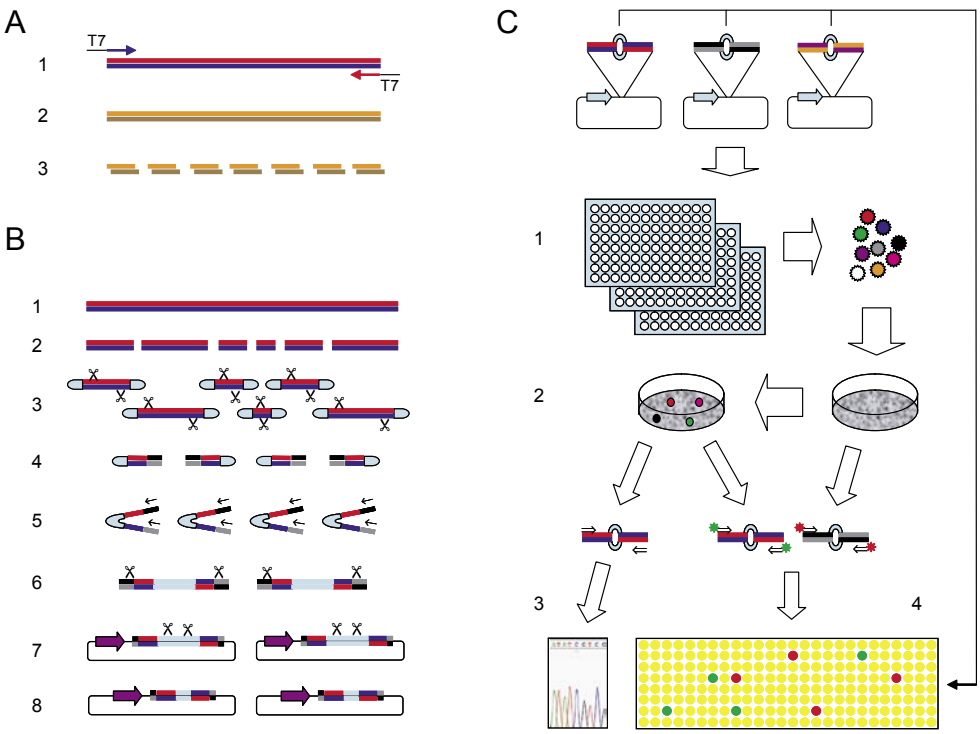

Figure 2. Methods for the generation of small interfering RNA (siRNA) and short hairpin RNA (shRNA) libraries and an in vitro RNA interference (RNAi) screen. (A) Generation of siRNAs by endoribonuclease cleavage (adapted from Reference 12). cDNAs are first PCR-amplified with primers containing the T7 promoter sequence (1) then transcribed in vitro with the T7 polymerase (2). Long double-stranded RNA (dsRNA) molecules are digested with recombinant *Escherichia coli* RNase III (3), and siRNAs are purified with an affinity column. (B) Generation of shRNA libraries from cDNAs (adapted from Reference 18). Full-length cDNAs (1) are randomly fragmented by DNase I treatment (2). Hairpin-adaptors, containing the *Mme*I recognition site, are ligated to each side of the fragments (3). *Mme*I cleaves 20 nucleotides 3′ from its recognition sequence, yielding equal-length pieces. After ligation of another adaptor (4), primer extension converts the stem-loop structure to double-stranded DNA with inverted repeats flanking the hairpin sequence (5). The products are first inserted into retroviral vectors downstream of a Pol III promoter (6), and then the length of the hairpin is adjusted by restriction digest (7). (C) An RNAi screen and the use of molecular barcodes (adapted from Reference 21). shRNA vectors are distributed in 96-well plates, and plasmid DNAs from each plate are pooled together for the production of high-titer polyclonal virus (1). Cells are infected and subjected to experimental conditions (2). The identity of shRNAs in the surviving cells is determined by sequence analysis of PCR-amplified genomic DNA fragments (3). Alternatively, PCR-amplification is performed with fluorescently labeled primers (green label for treated cells, red label for control cells) and the products are hybridized to the barcode oligonucleotide array (4). The array contains one strand of each of the hairpin oligonucleotides. Green signal indicates enrichment of a particular shRNA during the course of experiment, while red signal means the loss of cells expressing the corresponding shRNA under the assay conditions.

PHILOSOPHY BEHIND siRNA DESIGN: FROM EMPIRICISM TO RATIONALISM

The first successful mammalian RNAi experiments revealed great variations among the silencing potential of siRNAs targeting different regions of the same gene. To achieve efficient gene silencing, it has been suggested to select 19-nucleotide-long sequences in the coding region flanked by AA at the 5′ and TT at the 3′ end (23). Furthermore, a 30%–70% GC content was found to be advantageous for the internal stability of the siRNA. However, even by adhering to these empirical guidelines, this and other studies concluded that the overall fraction of efficient siRNAs was only

20%–25%. Later it was demonstrated that siRNAs targeting the untranslated regions (UTRs) can also silence gene expression effectively and the presence of 5′ AA and 3′ TT bases flanking the core 19-mer target sequence appeared to be dispensable (24).

The shift from empirical to rational siRNA design began with a better understanding of the strand selection mechanism by the RISC (25,26). Analysis of hundreds of functional siRNAs revealed that these molecules have a reduced thermodynamic stability at the 5′ end of the antisense strand relative to the 3′ end within the RNA duplex (25,27). This asymmetry allows for the unwinding of the dsRNA from one end preferentially and ensures that only one strand enters the RISC (26). These findings predicted that design of si/shRNA molecules with a thermodynamically unstable 5′ antisense strand will increase silencing efficiency and reduce off-target effects (25,26). A comprehensive study statistically analyzed 180 siRNAs that were designed to systematically target every other position in a defined coding region of two genes (28). By grouping the most effective and the least efficient siRNA molecules, the authors identified those parameters that significantly contributed to the silencing potential of siRNAs (Table 1). By assigning a numeric score to each of these parameters (-1, 0, or +1) a maximum score of 10 can be obtained for each sequence. These investigators showed that selection of siRNAs with a score of 6 or more significantly increases the probability of efficient gene silencing (28). However, the authors also pointed out that not all the assayed siRNAs followed these rules, and some efficient molecules had low scores, while others with high scores performed poorly. Notably, previous reports suggested that the efficacy of silencing is affected by the accessibility of the target sequence, and therefore, secondary structure predictions of the mRNA may further improve si/shRNA design (29,30).

Table 1. Criteria for the Rational Design of siRNAs[a]

Criterion	Score if True	Score if False
30%–52% GC content	1	0
A or U at positions 15–19	5 (1 for each)	0
No internal repeats (predicted hairpin T_m < 20°C)	1	0
A at position 19	1	0
A at position 3	1	0
U at position 10	1	0
A or U at position 19	0	-1
No G at position 13	0	-1

T_m, melting temperature.
[a]Adapted from Reference 28.

Furthermore, two very recent reports demonstrated that 27-mer siRNAs and synthetic shRNAs with a 29-mer stem perform significantly better than the corresponding 19-mer-based molecules (31,32). These longer dsRNAs are more effective, especially at very low concentrations, permit greatly prolonged gene silencing, and in some cases, allow efficient knock-down through target sites that are refractory to RNAi with traditional siRNAs. The superior activity of longer siRNAs and shRNAs can probably be attributed to the importance of Dicer-mediated cleavage and loading of effector molecules into the RISC; while the longer molecules are quantitatively cleaved by Dicer to 21- to 22-nucleotide products, traditional siRNAs and shRNAs containing a 19-mer stem are not substrates of Dicer and probably enter the RISC by another mechanism (31,32). A puzzling issue is whether it is possible to apply the rational design rules—that have been worked out on siRNAs with 19-mer target sequences—to the longer effector molecules. Based on the analysis of the Dicer-processed synthetic shRNA sequences, Siolas and colleagues suggest increasing the length of the selected 19-mer sequence by adding one base upstream and nine bases downstream of the corresponding target region for the generation of the 29-mer shRNA stem (31).

There are several publicly accessible algorithms to help researchers design siRNAs and shRNAs (Table 2). Most of them incorporate these newly established rules, and some also perform a homology search of the predicted targeting sequence against the GenBank®, or more frequently, the UniGene database. This step is very important to reduce the chance of off-target effects. Although it was shown initially that siRNAs with only one mismatch to their cognate target

Table 2. Useful Web Sites for the Design of Effective si/shRNAs

Company or Lab	URL
Ambion	www.ambion.com/techlib/misc/siRNA_finder.html
Dharmacon	design.dharmacon.com/rnadesign/default.aspx
Hannon Lab	katahdin.cshl.org:9331/portal/scripts/main2.pl
Integrated DNA Technologies	scitools.idtdna.com/RNAi
Invitrogen	rnaidesigner.invitrogen.com/sirna
McManus Lab	web.mit.edu/mmcmanus/www/home1.2files/siRNAs.htm
Qiagen	www1.qiagen.com/Products/GeneSilencing/CustomSiRna/SiRnaDesigner.aspx
Sfold Algorithm	sfold.wadsworth.org/index.pl
Tuschl Lab	www.rockefeller.edu/labheads/tuschl/sirna.html
Whitehead Institute	jura.wi.mit.edu/siRNAext

siRNA, small interfering RNA; shRNA, short hairpin RNA.

Table 3. Comparison of Commonly Used Functional Validation Methods for Mammalian RNAi

Method	Detection Level	Advantage	Disadvantage	Throughput
Northern blot	Endogenous mRNA	Easy	RNA isolation	Low
qRT-PCR (TaqMan® or SYBR® Green)	Endogenous mRNA	Sensitive, quantitative	RNA isolation, primer design	High
QuantiGene® www.genospectra.com	Endogenous mRNA	Sensitive, quantitative, works on crude lysate	Cost	High
Western blot, IF, ELISA, FACS, etc.	Endogenous protein	Easy	Antibody availability	Low
Western blot, IF, etc., on epitope tag	Exogenous fusion protein	Same antibody for detection	In-frame cloning, restricted target region	High
Fluorescent/enzymatic reporter assay	Exogenous protein (translated from chimeric mRNA)	Entire cDNA can be targeted, only reporter is translated		High

mRNA, messenger RNA; qRT-PCR, quantitative reverse transcription PCR; IF, immunofluorescence; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting.

sequence failed to induce silencing, recent findings show this may not always be true (33). Therefore, it is best to select those candidate si/shRNA sequences that have at least three mismatches to any gene other than the one targeted. It is also advisable to avoid polymorphic regions, unless allele-specific silencing is the scientific goal. Some researchers prefer sequences that are identical in different species (e.g., human, mouse, and rat), so one si/shRNA can target the same gene in different organisms. Finally, when designing oligonucleotides for shRNAs, it is important to avoid stretches of four or more T nucleotides in order to prevent the premature termination of transcription by Pol III.

The most frequently used homology search is Basic Local Alignment Search Tool (BLAST), although there is some dispute about whether this is the most appropriate algorithm. It is advisable to use the BLAST option "search for short, nearly exact matches" that is set with a low "Word Size" (=7) and a high "Expect" value (=1000) as default to make the search more suitable for short sequence matches.

FUNCTIONAL VALIDATION: THE PROOF IN THE PUDDING

Not all rationally designed si/shRNAs will knock down gene expression at the same level, and therefore, it is very useful to evaluate the silencing capability of several candidates before committing to the actual experiment. This is particularly relevant in the case of in vivo studies, where strict ethical issues and financial constraints limit the number of animals that can be used and where results may only be obtained months after starting the experiment. Moreover, having a set of targeting molecules with different silencing potential can be a valuable tool for generating a series of hypomorphic alleles (34).

Table 3 lists the most commonly used assays to evaluate gene silencing. Whenever possible, detection of knockdown at the endogenous protein level is the preferred method, although it requires good antibodies against the target and is not well-suited for high-throughput applications. Another option is cloning the target gene's coding sequence (CDS) in frame with a small antigen epitope (Myc, HA, FLAG, His, etc.) and determine the extent of silencing on the overexpressed protein with the antibody against the tag. Instead of a small epitope, fluorescent (e.g., GFP) or enzymatic (e.g., luciferase, LacZ) reporters can be used as fusion partners. In a recently reported high-throughput system, targeting molecules and vectors encoding for the GFP-target fusion protein were spotted onto a glass slide on which cells are then seeded in a medium containing the transfection reagent. After this reverse transfection procedure, fluorescence intensity in each spot of the glass slide can be detected by automated microscopy (35).

Alternatively, a target cDNA can be cloned downstream of the translational stop codon of a reporter. Upon transcription, this construct produces a chimeric mRNA, from which only the reporter is translated. Efficient targeting of the cDNA of interest leads to degradation of the entire mRNA, and consequently, no reporter product is produced (Figure 3). Co-expression of a second reporter allows normalization for transfection efficiency. This method does not require in-frame cloning, permits targeting regions other than the CDS, and is particularly useful if the overexpressed protein product of the target gene would otherwise influence the survival or proliferation of the transfected cells. Subcloning, however, is a rate-limiting step, and usually only a few restriction sites are available for this purpose.

To facilitate high-throughput cloning and assay conditions, our group has adapted a commercially available luciferase reporter vector (psiCHECK™-2; Promega, Madison, WI, USA) to Gateway® technology (Invitrogen, Carlsbad, CA, USA), by placing a destination cassette 3′ to the translational stop of *Renilla* luciferase (P. Sandy, unpublished data). Gateway-cloning allows in vitro recombination-based transfer of DNA from one vector to another without the use of restriction enzymes. Notably, more than 15,000 sequence-verified, mouse and human full-length cDNA clones are publicly available from the I.M.A.G.E. Consortium,

and a significant number of them are cloned in Gateway-compatible vectors (36). Alternatively, cDNAs cloned in different vectors can be PCR-amplified with primers containing the short recombination sites and transferred into the Gateway-compatible reporter construct. The silencing potential of targeting molecules can be assessed by measuring luciferase activity 24–48 h after cotransfection with the luciferase-target reporter construct. Sample analysis can be efficiently performed in 96-well plates and typically requires <200 ng transfected DNA/well (P. Sandy, unpublished data).

CONSTITUTIVE, INDUCIBLE, AND CONDITIONAL SYSTEMS FOR IN VITRO AND IN VIVO RNAi

RNAi has become a valuable tool for studying the biological consequences of gene inactivation in animal models as well. RNAi can be achieved locally by delivering siRNAs, shRNA-expressing plasmid DNAs, or viral particles directly into the target tissue or organ, as has been demonstrated for the retina (37), the brain (38,39), and muscle (40). Ex vivo infection of bone marrow cells followed by injection into lethally irradiated mice has also proven effective to induce stable gene knockdown in hematopoietic cells (34). Systemic RNAi is usually attempted by hydrodynamic tail vein injection, in which a saline solution containing siRNA or the shRNA vector is rapidly introduced into the blood stream of the animal (41). In most cases, highly perfused organs (e.g., the liver) take up the injected material; therefore, it is not surprising that most systemic RNAi experiments targeted genes in liver-associated diseases, including fulminant hepatitis (42) and hepatitis B virus infection (43). The main drawbacks of this method are the physiological shock associated with the rapidly (10–15 s) administered large volume (mL) of solution that may result in cardiac arrest and the large quantity (μg) of targeting molecules that are expensive and may have off-target effects at such a high concentration. There are several significant improvements for the more effective delivery of siRNAs into target tissues, including the use of cationic polymers and liposomes for packaging and chemical modifications that increase the half-life of the molecules (44–51). In a recent study, it has been shown that intravenously administered, cholesterol-conjugated siRNAs targeting apolipoprotein B, a protein implicated in the metabolism of cholesterol, significantly reduced serum lipoprotein levels (52). These findings and other on-going phase I clinical trials demonstrate that RNAi may soon become a relevant therapeutic tool in humans.

Several groups have reported the successful generation of knockdown animals by transgenic RNAi approaches and the efficient germ line transmission of the shRNA-expressing transgenes (see below). Importantly, RNAi allows gene targeting in other important model organisms, such as rats, where conventional knockout technologies are not available.

Mouse embryonic stem (ES) cells can be electroporated with the appropriate transgene or transduced with a lentivirus-based vector (Figure 4). In both cases, the resulting clones can be assayed for transgene copy number, shRNA expression, and degree of knockdown (if the gene is expressed in ES cells) before proceeding further. Selected clones are then injected into diploid blastocysts that are subsequently transferred to pseudopregnant females. The resulting chimeric mice are then bred further to obtain germ line transmission of the shRNA vector. To bypass the chimeric stage, it is possible to generate entirely ES-derived mice by injecting the ES cells into tetraploid blastocysts (53) or by the tetraploid aggregation method (54). Standard transgenesis can also be used for introducing shRNA vectors into the germ line (Figure 4). In this case, a linearized DNA fragment containing the shRNA expression cassette is injected into the pronucleus of fertilized eggs that are then transferred to pseudopregnant females (55–57). Finally, lentiviruses carrying shRNA expression cassettes can be directly injected into the perivitelline space of single-cell mouse embryos that are then transferred into female recipient mice (58,59).

Although the speed and ease of generation of knockdown animals will likely accelerate the functional annotation of the mouse genome, this technology is still at an early stage and cannot be considered equivalent to the well-developed gene targeting approach. In vivo RNAi is, in fact, subject to the same limitations as cell-based RNAi, including the inability to achieve complete inhibition of gene expression and the risk of off-target and nonspecific effects. Furthermore, although transgenic shRNA expression has been observed in all tissues tested (Reference 58 and A. Ventura, unpublished data), it remains to be determined whether all mouse tissues are equally sensitive to RNAi. It is also likely that the degree of shRNA expression in a given cell type will be affected by the number of integrated RNAi vectors and the sites of integration. One way to avoid position effects is to target the shRNA expression cassette by homologous recombination in appropriate ES cells to a ubiquitously expressed locus such as the ROSA26. This approach can be particularly useful for creating epi-allelic series of hypomorphs, as the generated transgenic animals would differ exclusively for the shRNA sequences. Diminished shRNA expression due to epigenetic silencing of the promoter during development might also be circumvented by incorporating insulator elements into the targeting vectors.

A major limitation of unregulated RNAi technologies is that it cannot be applied to study the function of essential genes. Due to the dominant nature of RNAi, this problem is even more relevant for in vivo studies, since it prevents the generation of transgenic RNAi strains to analyze genes whose inhibition is incompatible with normal development or compromises fertility. To overcome this obstacle, a number of inducible and conditional RNAi systems have been recently developed. For example, several groups have generated modified Pol III promoters containing a tetracycline-doxycycline response element (tetO), which serves as a binding site for the Tet repressor (60–63). Introduction of these constructs into cells expressing the Tet repressor allows the isolation of

clones in which shRNA expression can be induced by the addition of doxycycline to the culture medium. Removal of doxycycline leads to reexpression of the targeted gene, albeit with a few days delay that is probably due to the relatively long half-life of siRNAs (63). Tet-inducible lentiviral RNAi vectors have been also reported (64). An interesting variation is to place the Tet-regulated Pol III promoter-shDNA cassette into the U3 region of the 3′ long terminal repeat (LTR) of the lentiviral vector (64). Because this region serves as template for the synthesis of its 5′ copy of the LTR during reverse transcription, the cassette is duplicated in the provirus, allowing higher rates of shRNA expression after induction.

As an alternative, a three-component ecdysone-inducible retroviral system is available for inducible RNAi (65). Two vectors encode the nuclear receptors/transcription factors chimeric ecdysone receptor (VgEcR) and retinoid X receptor (RXR). The third vector contains a GAL4-Oct-2 chimeric transactivator under the control of the ecdysone-inducible promoter and a modified U6 promoter with four GAL-4 binding sites in place of the enhancer. In the presence of the ecdysone-analog muristerone A, VgEcR and RXR dimerize and activate the GAL4-Oct2 chimeric gene. This in turn activates the U6 promoter and transcription of the shRNA. As with Tet-inducible systems, shRNA expression is reversible upon withdrawal of the inducer. The main advantages of this system over the Tet-inducible ones are that shRNA expression is more tightly regulated and the various components do not seem to have any adverse effects on their own. However, the requirement for three different retroviruses (and therefore, three different selection markers) makes it more cumbersome and significantly complicates its application to in vivo settings.

More recently, an inducible RNAi system utilizing the *lac* repressor has been described (66). In this case an *Escherichia coli* lacO element was placed between the TATA-box and the transcription start site of the H1 promoter to which the *lac* repressor binds, preventing transcription. Addition of isopropyl-1-thio-β-D-

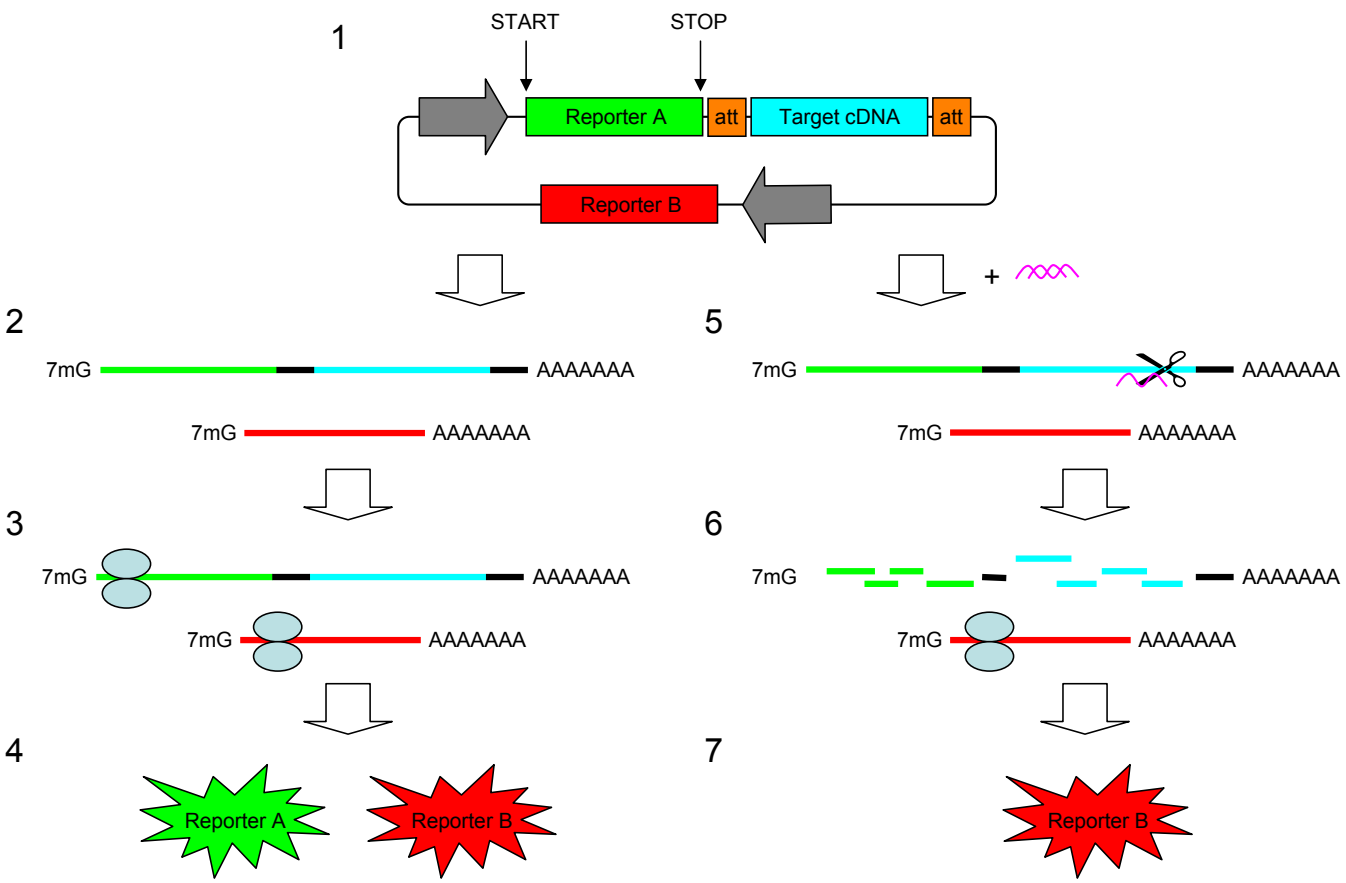

Figure 3. Dual reporter-based functional validation assay. The cDNA of a target gene flanked by recombination (att) sites is cloned downstream of the coding sequence of Reporter A in a Gateway-compatible validation vector (1). Reporter B serves as an internal control to monitor efficient delivery of the plasmid. After transfection, a chimeric mRNA consisting of Reporter A and target gene-specific sequences are transcribed along with the messenger RNA (mRNA) for Reporter B (2). Because of the presence of a translational stop codon in Reporter A and the lack of an internal ribosome entry site (IRES) between Reporter A and the target gene, only the two reporters are translated into proteins (3 and 4). In the presence of an effective small interfering RNA (siRNA) against the target gene (5), the chimeric mRNA is recognized and cleaved by the RNA-induced silencing complex (RISC; scissor), which leads to subsequent degradation of the entire message (6), and only Reporter B is translated (7). The difference between the ratios of the enzymatic or fluorescent activities of Reporter A and B in the absence and in the presence of target-specific siRNA correlates with the silencing potential of the siRNA.

galactopyranoside (IPTG) to the culture medium leads to dissociation of the repressor from the promoter and, consequently, to reversible shRNA expression and gene knockdown.

Conditional gene targeting using the Cre-lox system has become a widely used and powerful method for analyzing gene function in vivo. This technology is based on the ability of the Cre recombinase to catalyze the excision of any genomic region that is flanked by short (34 nucleotide) sequences termed loxP sites. Cre activity can be introduced by transduction of cells with recombinant adeno- or lentiviruses expressing the recombinase. More importantly, a growing number of mouse strains expressing Cre under the control of tissue-specific inducible or developmentally regulated promoters are available. Recently, our group and others have generated lentiviral RNAi vectors in which shRNA expression is controlled by a lox-stop-lox element. The element is placed between the TATA-box and the transcription start site of the U6 promoter and prevents expression of the shRNA until a Cre-mediated recombination event leads to its deletion (53,67). Conditional Cre-regulated gene silencing was demonstrated in cell culture (53,67) and in vivo by generating transgenic animals and crossing them to Cre-expressing mice (53). Other Cre-lox-regulated RNAi systems, based on slightly different strategies, have also been reported (68–70).

CONCLUSION AND PROSPECTS

The demonstration of efficient and sequence-specific gene silencing by synthetic siRNAs and expressed shRNAs has led to the beginning of a revolution in mammalian functional genetics. Advances in the design of targeting molecules and in their delivery methods have allowed the interrogation of several biochemical and signaling pathways relevant for basic physiological and complex

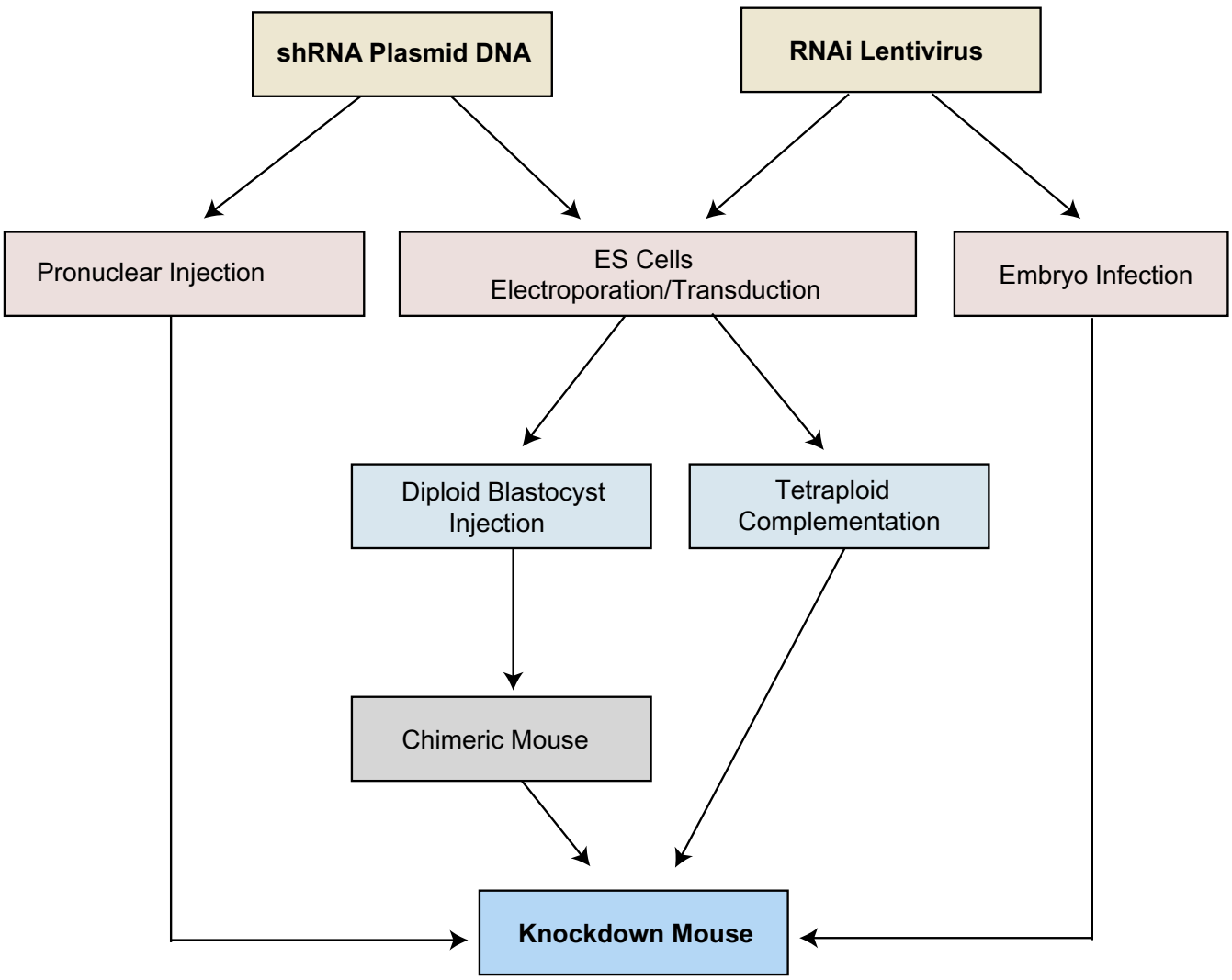

Figure 4. Generation of knockdown mice by various transgenic approaches. The fastest method is infection of single-cell embryos with high-titer lentiviruses. The advantage of using embryonic stem (ES) cells is that copy number and expression of the integrated short hairpin RNA (shRNA) can be monitored before blastocyst injection. RNAi, RNA interference.

disease processes. We expect that, in a few years, functionally validated targeting molecules will be available for all mouse and human genes, thus allowing genome-wide RNAi screens in various in vitro settings. Moreover, the advent of inducible and conditional RNAi systems, combined with improved transgenic methodologies, will undoubtedly facilitate the characterization of gene function in vivo. Further technical improvements in the delivery, stability, and specificity of siRNAs and shRNAs will likely establish RNAi as a standard tool in the treatment of human diseases.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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