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Vector-based RNAi approaches for stable, inducible and genome-wide screens

Gwen D. Fewell and Karin Schmitt

Open Biosystems Inc. 6705 Odyssey Drive, Huntsville, Al, 35806, USA

RNA interference (RNAi) has revolutionized the study of biology and offers numerous applications in basic biology as well as in drug discovery research. Since the discovery of RNAi, several tools have been developed to enable loss-of-function studies in mammalian systems. The efficacy of RNAi is dependent on specific and versatile RNAi triggers that have evolved to enable transient, stable and *in-vivo* applications. Recently developed genome-wide short hairpin RNA (shRNA) and microRNA-adapted short hairpin RNA (shRNAmir) libraries incorporate advances in shRNA design and molecular 'barcodes' to enable more complex RNAi screens and the opportunity to progress to more complex genetics in whole animals.

RNA interference (RNAi) is an evolutionarily conserved, genetic surveillance mechanism that permits the sequence-specific post-transcriptional down-regulation of target genes. The discovery of RNAi as a biological response to double-stranded RNA (dsRNA) was first made in the nematode *Caenorhabditis elegans*. Injecting dsRNAs into the worm was found to silence genes that had complementary sequences to those of the introduced dsRNA [1]. An RNAi pathway has since been shown to be present in many, if not most, eukaryotes [2]. The mechanics of the RNAi pathway have been intensively studied and several reviews have been published [3,4]. In brief, dsRNA is processed into short interfering RNA (siRNA; ~22 nucleotides in length) by the RNase III enzyme Dicer. These siRNAs are incorporated into an effector complex called the RNA-induced silencing complex (RISC) that identifies and silences the complementary mRNA. Since its discovery, RNAi has been shown to have important roles in diverse biological processes including developmental regulation, antiviral defense and chromatin modeling. As a technology, RNAi offers enormous potential, not only as a tool in biological research but also as a therapeutic approach for silencing disease-causing genes. Recent advances in the design of vector-based synthetic RNAi triggers have contributed to advancing applications for whole-genome loss-of-function screens and complex genetics in animal models.

Corresponding author: Fewell, G.D. (gfewell@openbiosystems.com)

The evolution of synthetic silencing triggers for mammalian RNAi

Long dsRNA (>200 bp) can induce specific RNAi in worms, plants and flies; however, in mammalian cells long dsRNA triggers a potent interferon response and nonspecific inhibition of transcription and translation [5]. A biochemical understanding of the RNAi pathway was crucial to realizing that dsRNA shorter than 30 bp could be used successfully to trigger an RNAi response in mammalian cells. Three types of small RNA can be used to perform RNAi in mammalian cells: chemically synthesized siRNA, short hairpin RNA (shRNA) cloned into plasmid DNA vectors and microRNA-adapted short hairpin RNA (shRNAmir) also cloned into plasmid DNA vectors.

Short interfering RNA

siRNA was the first synthetic silencing trigger used successfully in mammalian cells to knockdown transiently the expression of target genes [6]. siRNAs are short dsRNAs that are typically 19–22 bp in length with an overhang of two nucleotides on either end, including a 5′ phosphate group and a 3′ hydroxyl group. siRNA typically is chemically synthesized or can be generated by RNase III digestion of dsRNA. It can be introduced into cells using standard transfection approaches and cause transient silencing of the targeted gene. The transient-only knockdown, lasting 3–5 days in culture, makes the siRNA approach unsuitable for the analysis of the long-term effects of gene silencing. Another limitation

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associated with siRNA is the variability of transfection efficiencies in different cell lines. Many cell lines, including primary and non-dividing cells, are difficult to transfect at the efficiencies required to elicit knockdown phenotypes.

Vector-based RNAi triggers: first-generation shRNA and second-generation shRNAmir

The discovery of microRNA (miRNA), an endogenous trigger of the RNAi pathway, resulted in the development of another generation of silencing triggers called short hairpin RNA (shRNA), modeled on miRNA hairpin precursors and expressed from DNA vectors [7,8]. Box 1 summarizes our current understanding of miRNA biogenesis. First-generation shRNA are modeled on precursor microRNA (pre-miRNA) and are cloned into viral vectors, where they are transcribed under the control of RNA Polymerase III (Pol III) promoters [9–11]. shRNAs are produced as single-stranded molecules of 50–70 nucleotides in length, they form stem–loop structures, exit the nucleus, are cleaved at the loop by the nuclease Dicer and enter the RISC complex as siRNAs. As the understanding of miRNA biogenesis advanced, new generation shRNAmir triggers were developed. shRNAmir are expressed as primary-miRNA (pri-miRNA) transcripts. These constructs were created by redesigning the well-studied human miRNA, miR-30, to express an artificial siRNA/miRNA. The stem of the primary miR-30 transcript was replaced with gene-specific duplexes for different target genes (Figure 1) [12–14]. This design does not affect normal miR-30 maturation and allows endogenous miRNA processing to produce mature siRNAs.

The shRNAmir design harnesses endogenous enzymatic processing by the RNase III Drosha, which increases subsequent Dicer recognition and specificity. shRNAmir triggers enter the RNAi pathway ahead of either shRNA or siRNA, and are processed by both Drosha and Dicer, leading to more siRNAs produced in the cell that are available for incorporation into the RISC complex for target mRNA degradation (Figure 2) [12–16].

BOX 1

miRNA biogenesis

The endogenous miRNA biogenesis pathway includes three distinct RNA intermediates: a primary miRNA (pri-miRNA) transcript, a precursor miRNA (pre-miRNA) transcript and the mature miRNA. Initially, miRNA genes are transcribed by an RNA polymerase II (Pol II) promoter into long pri-miRNA transcripts. The RNase III enzyme Drosha in the nucleus cleaves the stem to generate a ~65-nucleotide pre-miRNA intermediate. The pre-miRNA is transported to the cytoplasm by the nuclear export factor exportin-5 [47–50], where it interacts with Dicer and gets processed into ~22-nucleotide duplex mature miRNAs. The miRNA duplex then interacts with the RNA induced silencing complex (RISC) components, including Argonaute-2, which selectively incorporate the RNA strand that has a less tightly base-paired 5′ end [51–53]. Activated RISC can then downregulate the expression of homologous mRNAs via either translational repression or mRNA cleavage, depending on the level of complementarity between the miRNA and the target mRNA.

siRNA or shRNA/mir for RNAi studies: making the choice

One of the first choices to make in an RNAi-based experiment is whether to trigger suppression using siRNA or vector-based shRNA or shRNAmir. Although siRNAs are easy to use and often produce good knockdown, their effects are transient and are restricted by the rate of cell division, their delivery to hard-to-transfect cell lines and their limited functionality *in vivo*. siRNAs are also subject to

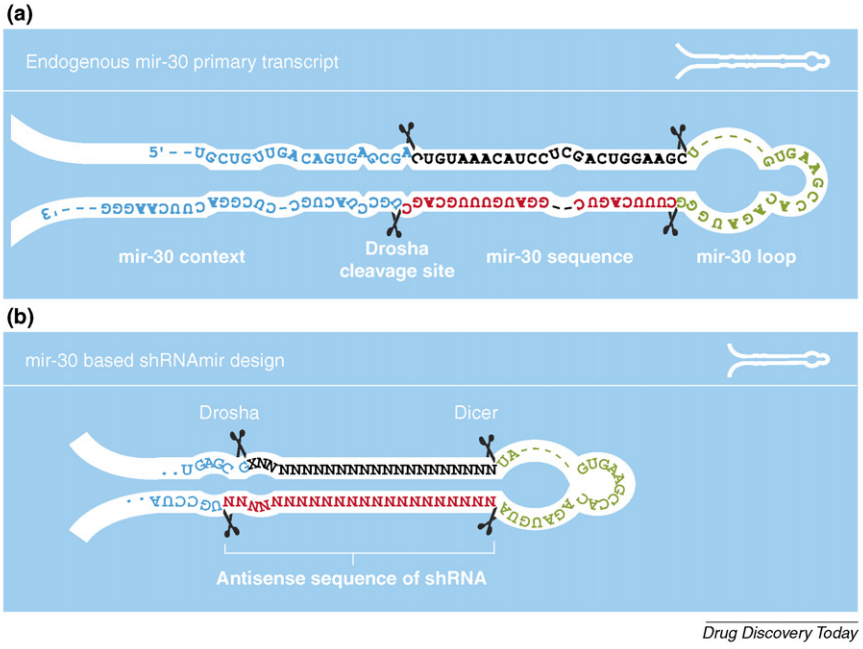

FIGURE 1

Second-generation shRNAmir design is based on the primary miRNA-30 (miR-30) transcript. (a) Endogenous miR-30 primary transcript. **(b)** shRNAmir expressed from a miR-30 context. The mature miR-30 sequence has been replaced with a gene-specific duplex.

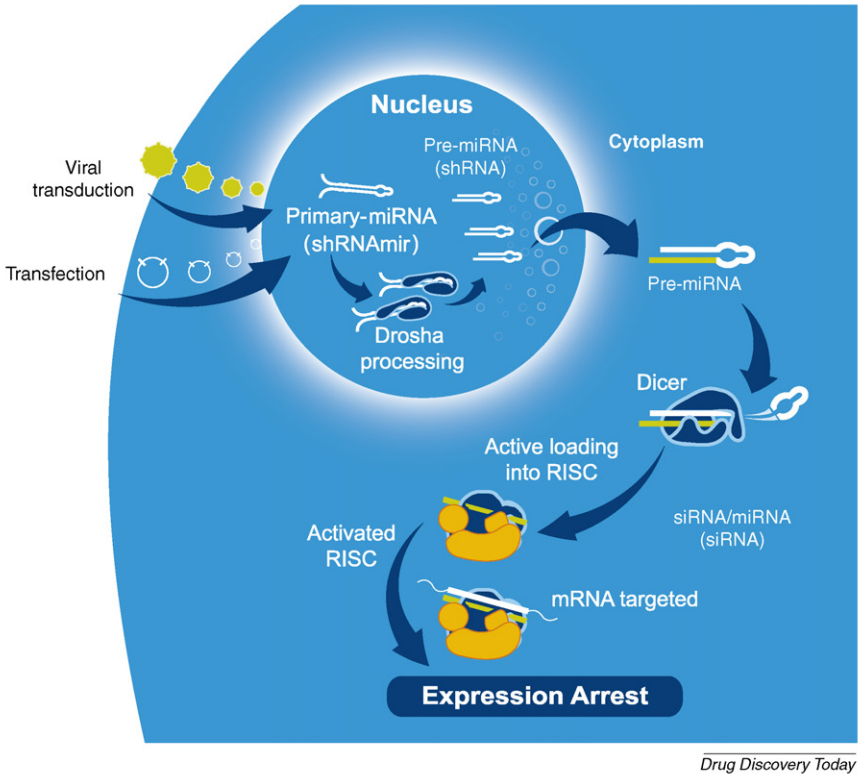

FIGURE 2

The endogenous miRNA processing pathway is used for shRNAmir processing. shRNAmir triggers are modeled on primary miRNA and processed by Drosha and Dicer to produce mature siRNA that targets a complementary mRNA. shRNA are approximately modeled on precursor miRNA and are processed by Dicer to produce mature siRNA. Chemically synthesized siRNA enter the RNAi pathway post-Dicer cleavage and incorporate into RISC to target complementary mRNA. All three RNAi triggers use the endogenous RNAi pathway but have distinct entry points.

concentration-dependent off-target effects when used at the concentrations necessary to produce optimal knockdown [17]. shRNA and shRNAmir constructs expressed from viral vectors are more versatile, allowing transient transfection, stable integration, germline transmission and the creation of *in vivo* animal models (Figure 3). shRNA and shRNAmir cloned into lentiviral or retroviral vectors also have the advantage of infection-based delivery into most cell lines, including hard-to-transfect cells such as primary and non-dividing cells. shRNA and shRNAmir processing is rate limited by enzymatic processing within the cell and their knockdown effects are potentially less susceptible to the concentration-dependent off-target effects seen using synthetic siRNA. Lentiviral vectors in combination with second-generation shRNAmir design offer a powerful tool for RNAi studies. Whole-genome shRNAmir lentiviral libraries have recently been created using vectors [18] (Table 1) that incorporate green fluorescent protein (GFP) to track shRNAmir expression, molecular barcodes (See Box 2 for a definition and further explanation) for deconvoluting data from complex genetic screens, and can produce decreased gene expression even with a single integration event in the genome (single-copy knockdown), a feature important for complex (pooled) RNAi screens.

shRNAmir design: increased processed siRNA and knockdown efficiency

The performance of shRNAmir relative to first-generation shRNA was tested in a study comparing the overall siRNA generated from an identical target sequence for firefly luciferase (luc) [12]. The sequence was inserted such that an identical mature small RNA would be generated from each shRNA or shRNAmir construct after endogenous processing. HEK293 cells were transfected with shRNA–luc and shRNAmir–luc, and assayed for resulting cellular siRNA using northern blotting. Cells transfected with shRNAmir–luc contained ~12 times more siRNA than did cells transfected with shRNA–luc, suggesting that the shRNAmir was more efficient than the first-generation shRNA at being processed into siRNA. In a separate experiment using a fluorescent reporter assay for measuring knockdown in a functional assay, multiple shRNA and shRNAmir constructs directed against proteasomal genes were compared for their gene-silencing ability. The average percentage knockdown using multiple shRNA or shRNAmir constructs per gene was measured (Figure 4). Overall, shRNAmir produced an increased and more consistent knockdown compared with that produced by first-generation shRNA [12].

Applications of vector-based RNAi for the analysis of gene function

Genome-wide vector-based RNAi resources

Soon after siRNAs were successfully used for mammalian RNAi studies, vector-based shRNA and shRNAmir approaches were also shown to be very effective in gene silencing [19,20]. Since its first demonstration in a small-scale RNAi screen [10], several genome-wide vector-based RNAi resources have been developed (Table 1).

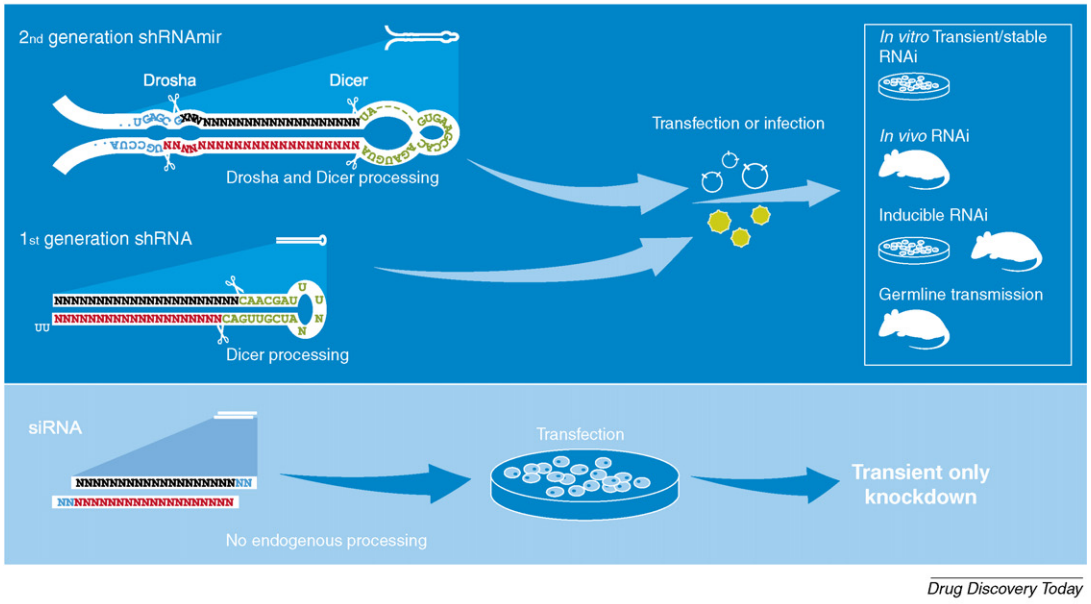

FIGURE 3

A comparison of siRNA, shRNA and shRNAmir silencing triggers. siRNA produces rapid, transient-only knockdown. shRNA and shRNAmir overcome several limitations inherent in siRNA, including the ability to generate stable knockdowns *in vitro*, transfection or infection options and the creation of *in vivo* animal models.

These resources target every gene in the human and mouse genomes with multiple shRNA or shRNAmir already cloned into viral-vector-based formats. These easily renewable resources can be used in transfection-based as well as infection-based assays and most importantly make *in vivo* and inducible RNAi applications routine.

Two studies published in 2004 [9,11] reported on the construction of genome-wide libraries of retroviral-based shRNA reagents and their application in large-scale vector-based RNAi screens. The shRNA library developed by Bernard's laboratory consists of ~23,000 constructs targeting ~7900 human genes. This library was used in a large-scale screen [11] to identify modulators of p53-dependent proliferation. A similar screen was also performed using a first-generation shRNA library constructed by Hannon's laboratory [9]. With the rapid advances in our knowledge of miRNA biogenesis, this first-generation shRNA library was recently replaced by a second-generation shRNAmir version targeting the human and mouse genomes. These libraries were created in a retroviral [19], as well as a lentiviral, vector format [18] (see http://codex.cshl.org/scripts/newmain.pl and/or http://www.openbiosystems.com/RNAi for more details). These shRNAmir libraries target the complete human and mouse genomes (28,500 genes) with a goal of providing at least 150,000 shRNAmir (6–10 shRNAmir/target) per genome. The performance of the Hannon–Elledge retroviral human shRNAmir library was successfully validated in a functional screen designed to report defects in human proteasome function [12]. In another effort, the RNAi

TABLE 1

Publicly available genome-wide arrayed vector-based RNAi resources

Collection	Current coverage/goal	Source	Refs
Human			
Hannon–Elledge whole-genome retroviral shRNAmir library	~100,000 constructs targeting 28,500 genes Goal: 150,000 shRNAmir targeting 28,500 genes	http://www.openbiosystems.com http://codex.cshl.edu/scripts/newmain.pl	[12,14]
Hanon-Elledge whole-genome lentiviral shRNAmir library	~20,000 constructs targeting 10,000 genes Goal: 150,000 shRNAmir targeting 28,500 genes	http://www.openbiosystems.com http://codex.cshl.edu/scripts/newmain.pl	[18]
The RNAi Consortium (TRC) lentiviral shRNA library	~60,000 constructs targeting ~13,000 genes Goal: 75,000 shRNA targeting 15,000 genes	http://www.openbiosystems.com http://www.broad.mit.edu/rnai_platform	[21]
Netherlands Cancer Institute retroviral shRNA library	22,000 constructs targeting ~7900 genes	http://www.biomedicalgenetics.nl/Members/Bernards/bernards.html	[11]
Mouse			
Hannon-Elledge whole-genome retroviral shRNAmir library	~100,000 constructs targeting 28,500 genes Goal: 150,000 shRNAmir targeting 28,500 genes	http://www.openbiosystems.com http://codex.cshl.edu/scripts/newmain.pl	[12,14]
The RNAi Consortium (TRC) lentiviral shRNA library	47,000 constructs targeting ~11,000 genes Goal: 75,000 shRNA targeting 15,000 genes	http://www.openbiosystems.com http://www.broad.mit.edu/rnai_platform	[21]

BOX 2

Molecular barcodes

Vector-based RNAi triggers (shRNA and shRNAmir) can be screened in pools because they can be uniquely tagged with a sequence that functions as a molecular 'barcode'. This can be the shRNA/mir targeting sequence itself or an additional unique nucleotide sequence. These unique molecular barcode sequences can be used to identify shRNA/mir that has integrated into a cell by PCR using primers that flank the unique barcode sequence.

For a typical pooled screen using the barcode study design, cells are infected with a pool of shRNA/mir; following culture over several days, genomic DNA is extracted, amplified, labeled and hybridized to a microarray that has probes directly complementary to the shRNA hairpin or a unique barcode that is part of the vector construct. This microarray hybridization allows the identification and relative representation of each individual shRNA/mir to be measured within a population of transduced cells.

Consortium (TRC), which aims to provide a lentivirus-based shRNA library targeting the human and mouse genomes, is part of a public–private consortium based at the Broad Institute (Cambridge MA, USA). Current plans include producing shRNA to target 15,000 human and mouse genes with a goal of five shRNA per target gene (see details at http://www.broad.mit.edu/genome_bio/trc/ and/or http://www.openbiosystems.com/RNAi). The use of the human TRC library was also recently demonstrated in a viral screen based on high-content imaging to identify genes required for mitotic progression in human cancer cells [21].

We now have access to several genome-wide RNAi libraries in different vector systems (Table 1). The design algorithms have been independently derived and differ widely, including in the use of the microRNA context; there are numerous differences in the details of the vector backbones and other vector elements that have been incorporated. The studies described above demonstrate the use of genome-wide shRNA/shRNAmir libraries for large-scale screens in mammalian cells and have since become a valuable resource for gene discovery and analysis of gene function [10,22,23]

Positive and negative genetic screens using molecular barcodes

RNAi studies using vector-based shRNA or shRNAmir are typically carried out in an arrayed format (i.e. one construct per well). However, given the availability of the various libraries in viral vectors, a collection of shRNA/mir containing viruses (pool) can be used to infect a cell culture simultaneously. Following infection,

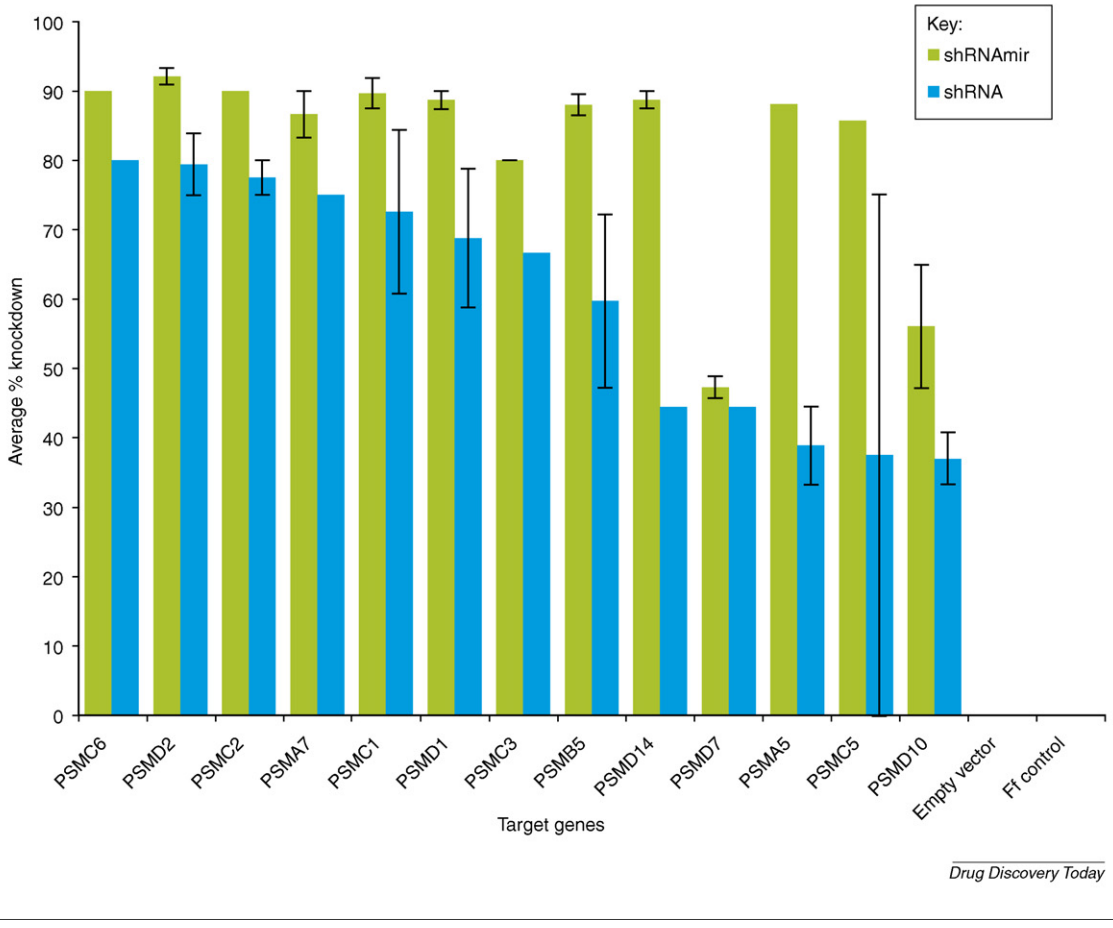

FIGURE 4

A comparison of knockdown efficiency of shRNA and second-generation shRNAmir. The data plotted is an average of the percentage knockdown produced by multiple shRNA and shRNAmir per target gene tested. Firefly luciferase (Ff control) and empty vector were used as negative controls and did not produce any knockdown. Adapted, with permission, from Silva *et al.* [12].

the shRNA or shRNAmir sequences will get integrated, along with an antibiotic selectable marker, into the genome infected cells. One of the requirements for carrying out a screen in a pool is that each shRNA or shRNAmir (i.e. hit in a screen) can be unambiguously identified. For that purpose, the first large-scale mammalian shRNA libraries described above incorporate a molecular 'barcode' that makes a pooled screen feasible for several experimental set-ups. The use of this molecular barcode technology for genetic screens has long been established in the yeast community [24]. Using a pooled format makes it possible to carry out large screens, even for the average researcher, by reducing the cost as well as employing assay systems that cannot be adapted to a larger throughput as required for genome-wide arrayed screens.

The first demonstration that pooled screening using molecular barcodes was feasible for large-scale mammalian RNAi screens came from a positive-selection screen that identified new tumor suppressor genes [22]. Specifically, a previously established human primary epithelial cell model was infected with a pool of retroviral shRNA from the Hannon library that contained 28,000 shRNA clones targeting ~9000 human genes. Each shRNA was linked to a unique nucleotide sequence (barcode) that could be used to monitor the abundance of a specific shRNA construct in a complex population of cells via micorarray hybridization. The read-out for this study was a transformed anchorage-independent growth phenotype. Approximately 100 anchorage-independent colonies were pooled and analyzed using a barcode microarray chip to identify all shRNA constructs that were part of this pool. As a proof-of-principle experiment, the same set of clones was also analyzed by sequencing to identify the exact shRNA sequences. Both methods are useful approaches for analyzing the hits from a positive-selection screen and yielded similar results, identifying ~25 unique candidate tumor suppressor genes. Among the list of candidate genes, the authors were able to identify two known tumor suppressor genes, as well as uncovering a tumor suppressor role for REST/NRSF, a transcriptional repressor of neuronal gene expression. Having a complete set of genome-wide shRNA constructs available made it possible to carry out this unbiased genetic screen and rapidly identify genes with new and unanticipated tumor suppressor functions that had gone undetected using other approaches [22,23].

In a different type of genetic screen (a negative-selection screen), the goal is to discover synthetic-lethal interactions in the cell. Two genes are called synthetic lethal if the cells die when both genes are mutated but survive if either gene alone is mutated. Traditionally, these types of screen have been carried out in model systems such as yeast but it had not been possible to transfer the study design from yeast to mammalian cells. With the availability of vector-based genome-wide RNAi libraries and the incorporation of the barcode technology for a microchip read-out, negative-selection screens are now within reach for various applications in mammalian cells. Negative-selection screens can be set up to study potentially oncogenic pathways, for mechanism-of-action (MoA) studies or to look at the effect of drugs on cells 'sensitized' by integrating specific shRNA [22,23]. Although such studies are very powerful, they are technically challenging experiments, require extensive experimental controls and should be carried out in a repeat format.

The idea of perturbing a cell by a drug and following the effects mediated by the drug was successfully applied in a recent study. An RNAi screen was combined with molecular barcode technology to gain insight into the mechanism of action of nutlin-3, a small-molecule inhibitor of MDM2 that regulates the p53 pathway [25]. One potential approach to activate p53 in tumor cells is to disrupt the interaction between MDM2 and p53. This can be achieved using nutlin-3, which binds to MDM2 and thereby prevents its interaction with p53. The barcode screen was designed to identify important components of the p53 network mediating the cytotoxic effects of nutlin-3 in human tumor cells. Apart from p53, tumor protein p53 binding protein 1 (p53BP1) was identified as a crucial mediator of nutlin-3-mediated cytotoxicity. This study illustrates the use of a shRNA barcode screen to identify cellular components mediating drug cytotoxicity, resulting in a more complete understanding of drug action [25].

All experiments described above rely on constitutively expressed shRNA. However, inducible shRNAs will have to be used when investigating the function of essential genes using knockdown technology. A recently published study [26] reported an RNAi barcode screen to identify gene–gene interactions required for the proliferation and survival of cancer cells. Here, a doxycycline-inducible retroviral shRNA library targeting 2500 human genes was employed. This vector does not express the shRNA unless doxycycline is added. Two different types of diffuse large B-cell lymphoma were infected with the set of shRNA constructs. The abundance of a particular shRNA construct is compared using barcode microarray hybridizations in the induced and un-induced state separately for each cell line. Experimental outcomes for the two cell types (assumed to have accumulated different mutations) are compared. Any shRNA that knocks down genes critical for proliferation or survival of a particular cancer cell will be eliminated from the cell population. This screen identified 15 genes that inhibit proliferation and survival in the two large B-cell lymphoma cell lines. These types of studies are particularly powerful for uncovering oncogenic pathways that are essential for the survival of cancer cells. When carried out systematically, such experiments could provide us with a functional profile of cells in health and disease.

In vivo applications for RNAi

One of the most exciting applications of the vector-based RNAi technology is its use for the analysis of gene function *in vivo*. Several reports have already described shRNA-mediated knockdown in mice, using both conditional and conventional gene inactivation. A variety of technical approaches to the generation of transgenic mice have been reported, including pronuclear injection [27–29], transfection of plasmid-based shRNA constructs into embryonic stem (ES) cell lines followed by injection into blastocysts [30,31] and lentiviral infection of ES cells and embryos [32–36]. All of these approaches are based on the random integration of the shRNA construct into the mouse genome. Recently, another approach was published based on the integration of the shRNA transgene at a defined, non-random locus in the mouse genome [37], followed by tetraploid complementation to generate transgenic mice [38]. These reports show that transgenic RNAi can be used to inactivate endogenous genes in

mice. The further development of RNAi technology will make it possible to limit gene knockdown to certain tissues or developmental stages.

Inducible RNAi systems

Given the tremendous need for tools allowing the fast and efficient evaluation of gene function, drug-inducible control of gene expression in mammalian systems and especially *in-vivo*-based RNAi will rapidly become invaluable to the research community. A recent study by Dickins *et al.* [39] showed for the first time that the transcription of shRNAmir clones from Pol II promoters is sufficient for effective target knockdown. The demonstration of a Pol II promoter driving shRNAmir expression also makes it possible to achieve conditional knockdown because Pol III promoters do not lend themselves to conditional regulation. Further experiments showed that shRNAmir constructs under the control of a Pol II promoter can produce stable gene knockdown that can be regulated in cultured cells but they can also function effectively *in vivo* [39]. This might be attributed to the fact that endogenous miRNAs are initially transcribed by Pol II promoters to generate pri-miRNA transcripts. In this first proof-of-principle study, it was shown that a tightly regulated shRNAmir construct based on a tetracycline-responsive promoter system (regulated by changing doxycyline levels) and directed against the transformation related protein Trp53 can switch cultured mouse fibroblasts between a proliferative and senescent state; tumors induced by Trp53 suppression (and other cooperating oncogenes) regressed upon re-expression of Trp53. These experiments indicate that miRNA-based shRNA constructs under the control of Pol II promoters will be suitable for a variety of *in vivo* applications, including tissue-specific knockdown and *in vivo* forward genetic screens.

The outlook for vector-based RNAi

Genome-wide tools for mammalian RNAi are making possible complex and high-throughput experiments that were previously inconceivable. Although remarkable progress has been made in the past few years, there are still technological challenges to be overcome both in the design of effective RNAi triggers and their applications *in vitro* and *in vivo*. Second-generation design algorithms employ several rules on the basis of knowledge of endogenous RNAi pathways, yet not every shRNA or shRNAmir sequence is successful at knocking down gene expression. Researchers must continue to screen multiple siRNA or shRNA/mir per gene to identify at least two constructs that produce the same knockdown phenotype. Both genome-wide shRNA (TRC) and shRNAmir (Hannon–Elledge) resources have a goal of at least five constructs per gene. Additional design improvements will no doubt continue as the understanding of endogenous pathways advances. Off-target effects have been documented in several siRNA studies [40,41] but have not been reported in systematic studies using shRNA or shRNAmir. It is probable that shRNA and shRNAmir will elicit similar types of off-target effects but because they are dependent on endogenous processing by Drosha and Dicer (and are as such rate limited) they might not be subject to the concentration-dependent off-target effects seen using siRNAs [14]. Controls for all RNAi experiments are therefore very important and should include negative and positive controls as well as rescue experiments [42]. Genome-wide viral shRNA libraries incorporating advances in both shRNA design and molecular barcode technology enable large-scale RNAi screens that rely on subtle changes in fitness levels of cells under various environmental conditions. Such screens could even be carried out *in vivo* using lentiviral shRNA libraries that perform effectively at a single-copy level. Pooled screens, although powerful for rapidly conducting genome-wide studies, are technologically challenging; obtaining uniform pools of virus for pooled screening is important for success, as are robust design algorithms and shRNA/mir expression that enable gene knockdown at a single-copy level. Pooled screens might have a large number of false negatives but the ability to screen thousands of genes simultaneously is still very attractive. False positives are easier to analyze in further validation studies because all positive hits will be tested extensively in a set of secondary assays. The recent advances in vector-based RNAi presented here will enable us to perform large-scale loss-of-function screens in mammalian systems similar to those already proven to be very successful in model organisms [43–46]. Further advances in vector-based RNAi triggers will include inducible RNAi libraries that allow the progression to more complex genetics in animal models. These advancements will probably lead to the identification of novel targets and therapeutic strategies on the basis of new insights into complex genetic pathways.

References

1 Fire, A. *et al.* (1998) Potent and specific genetic interference by double stranded RNA in *Caenorhabditis elegans*. *Nature* 391, 806–811
2 Hannon, G.J. (2002) RNA interference. *Nature* 418, 244–251
3 Hannon, G.J. and Rossi, J.J. (2004) Unlocking the potential of the human genome with RNA interference. *Nature* 431, 371–378
4 Hammond, S.M. (2005) Dicing and slicing: the core machinery of the RNA interference pathway. *FEBS Lett.* 579, 5822–5829
5 Williams, B.R. (1997) Role of the double stranded RNA-activated protein kinase (PKR) in cell regulation. *Biochem. Soc. Trans.* 25, 509–513
6 Elbashir, S.M. *et al.* (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411, 494–498
7 Bartel, D.P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116, 281–297
8 He, L. and Hannon, G.J. (2004) MicroRNAs: small RNAs with a big role in gene regulation. *Nat. Rev. Genet.* 5, 522–531
9 Paddison, P.J. *et al.* (2004) A resource for large-scale RNA-interference-based screens in mammals. *Nature* 428, 427–431
10 Brummelkamp, T.R. *et al.* (2003) Loss of the cylindromatosis tumour suppressor inhibits apoptosis by activating NF-κB. *Nature* 424, 797–801
11 Berns, K. *et al.* (2004) A large-scale RNAi screen in human cells identifies new components of the p53 pathway. *Nature* 428, 431–437
12 Silva, J.M. *et al.* (2005) Second-generation shRNA libraries covering the mouse and human genomes. *Nat. Genet.* 37, 1281–1288
13 Boden, D. *et al.* (2004) Enhanced gene silencing of HIV-1 specific siRNA using microRNA designed hairpins. *Nucleic Acids Res.* 32, 1154–1158
14 Cleary, M.A. *et al.* (2004) Production of complex nucleic acid libraries using highly parallel *in situ* oligonucleotide synthesis. *Nat Methods* 1, 241–248
15 Siolas, D. *et al.* (2005) Synthetic shRNAs as potent RNAi triggers. *Nat. Biotechnol.* 23, 227–231
16 Gregory, R.I. *et al.* (2005) Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. *Cell* 123, 631–640
17 Persengiev, S.P. *et al.* (2004) Nonspecific, concentration-dependent stimulation and repression of mammalian gene expression by small interfering RNAs (siRNAs). *RNA* 10, 12–18

18 Stegmeier, F. *et al.* (2005) A lentiviral microRNA-based system for single-copy polymerase II-regulated RNA interference in mammalian cells. *Proc. Natl. Acad. Sci. U. S. A.* 102, 13212–13217
19 Paddison, P.J. *et al.* (2002) Short hairpin shRNAs induce sequence-specific silencing in mammalian cells. *Genes Dev.* 16, 948–958
20 Brummelkamp, T.R. *et al.* (2002) A system for stable expression of short interfering RNAs in mammalian cells. *Science* 296, 550–553
21 Moffat, J. *et al.* (2006) A Lentiviral RNAi library for human and mouse genes applied to an arrayed viral high-content screen. *Cell* 124, 1283–1298
22 Westbrook, T.F. *et al.* (2005) A genetic screen for candidate tumor suppressors identifies REST. *Cell* 121, 837–848
23 Kolfschoten, I.G. *et al.* (2005) A genetic screen identifies PITX1 as a suppressor of RAS activity and tumorigenicity. *Cell* 121, 849–858
24 Winzeler, E.A. *et al.* (1999) Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. *Science* 285, 901–906
25 Brummelkamp, T.R. *et al.* (2006) An shRNA barcode screen provides insight into cancer cell vulnerability to MDM2 inhibitors. *Nat. Chem. Biol.* 2, 202–206
26 Ngo, V.N. *et al.* (2006) A loss of function RNA interference screen for molecular targets in cancer. *Nature* 441, 106–110
27 Hasuwa, H. *et al.* (2002) Small interfering RNA and gene silencing in transgenic mice and rats. *FEBS Lett.* 532, 227–230
28 Shinagawa, T. and Ishii, S. (2003) Generation of Ski-knockdown mice by expressing a long double-strand RNA from an RNA polymerase II promoter. *Genes Dev.* 17, 1340–1345
29 Coumoul, X. *et al.* (2005) Conditional knockdown of Fgfr2 in mice using Cre-LoxP induced RNA interference. *Nucleic Acids Res.* 33, e102
30 Kunath, T. *et al.* (2003) Transgenic RNA interference in ES cell-derived embryos recapitulates a genetic null phenotype. *Nat. Biotechnol.* 21, 559–561
31 Carmell, M.A. *et al.* (2003) Germline transmission of RNAi in mice. *Nat. Struct. Biol.* 10, 91–92
32 Rubinson, D.A. *et al.* (2003) A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nat. Genet.* 33, 401–406
33 Tiscornia, G. *et al.* (2003) A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering RNA. *Proc. Natl. Acad. Sci. U. S. A.* 100, 1844–1848
34 Ventura, A. *et al.* (2004) Cre-lox-regulated conditional RNA interference from transgenes. *Proc. Natl. Acad. Sci. U. S. A.* 101, 10380–10385
35 Szulc, J. *et al.* (2006) A versatile tool for conditional gene expression and knockdown. *Nat Methods* 3, 109–116
36 Kissler, S. *et al.* (2006) *In vivo* RNA interference demonstrates a role for Nrampl in modifying susceptibility to type 1 diabetes. *Nat. Genet.* 38, 479–483
37 Oberdoerffer, P. *et al.* (2005) Efficiency of RNA interference in the mouse hematopoietic system varies between cell types and developmental stages. *Mol. Cell. Biol.* 25, 3896–3905
38 Seibler, J. *et al.* (2005) Single copy shRNA configuration for ubiquitous gene knockdown in mice. *Nucleic Acids Res.* 33, e67
39 Dickins, R.A. *et al.* (2005) Probing tumor phenotypes using stable and regulated synthetic microRNA precursors. *Nat. Genet.* 37, 1289–1295
40 Birmingham, A. *et al.* (2006) 3′ UTR seed matches, but not overall identity, are associated with RNAi off-targets. *Nat Methods* 3, 199–204
41 Jackson, A.L. *et al.* (2006) Widespread siRNA off-target transcript silencing mediated by seed region sequence complementarity. *RNA* 12, 1179–1187
42 Echeverri, C.J. and Perrimon, N. (2006) High-throughput RNAi screening in cultured cells: a user's guide. *Nat. Rev. Genet.* 7, 373–384
43 Eason, R.G. (2004) Characterization of synthetic DNA bar codes in *Saccharomyces cerevisiae* gene-deletion strains. *Proc. Natl. Acad. Sci. U. S. A.* 101, 11046–11051
44 Baetz, K. *et al.* (2004) Yeast genome-wide drug-induced haploinsufficiency screen to determine drug mode of action. *Proc. Natl. Acad. Sci. U. S. A.* 101, 4525–4530
45 DasGupta, R. *et al.* (2005) Functional genomic analysis of the Wnt-wingless signaling pathway. *Science* 308, 826–833
46 Sonnichsen, B. *et al.* (2005) Full-genome RNAi profiling of early embryogenesis in *Caenorhabditis elegans*. *Nature* 434, 462–469
47 Lee, Y. *et al.* (2004) MicroRNA genes are transcribed by RNA polymerase II. *EMBO J.* 23, 4051–4060
48 Lee, Y. *et al.* (2003) The nuclear RNase III Drosha initiates microRNA processing. *Nature* 425, 415–419
49 Lund, E. *et al.* (2004) Nuclear export of microRNA precursors. *Science* 303, 95–98
50 Yi, R. *et al.* (2003) Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev.* 17, 3011–3016
51 Chendrimada, T.P. *et al.* (2005) TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature* 436, 740–744
52 Liu, J. *et al.* (2004) Argonaute2 is the catalytic engine of mammalian RNAi. *Science* 305, 1437–1441
53 Meister, G. *et al.* (2004) Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. *Mol. Cell* 15, 185–197