



# RNAi and Gene Therapy: A Mutual Attraction

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**The phylogenetically conserved cellular phenomenon of RNA interference (RNAi)—the sequence-specific post-transcriptional silencing of gene expression mediated by small double-stranded RNAs—holds substantial promise for basic research and for drug development. Particularly attractive from a medical standpoint is the juxtaposition of new RNAi methodology with established gene transfer strategies, especially viral vectors for efficient and tissue-specific RNAi delivery to patients. Here, we summarize the latest experimental and clinical advances in RNAi-based gene therapy approaches. We briefly portray emerging nonviral strategies for siRNA transfer, before comparing the three viral vectors currently predominantly developed as shRNA delivery vehicles, adenovirus, lentivirus, and adeno-associated virus (AAV). Moreover, we describe the most clinically**

**relevant genetic, acquired or infectious targets being pursued for therapeutic purposes. Specifically, we assess the use of vector-mediated RNAi for treatment of viral processes, solid cancers, lymphoproliferative disorders, and neurodegenerative and ocular diseases. In addition, we highlight further emerging applications, including stem cell therapies and animal transgenesis, as well as discuss some of the potential pitfalls and limitations inherent to the individual approaches. While we predict that eventual schemes will be shaped by our increasing understanding of the complexities of human RNAi biology, as well as by progressive refinements of viral shuttle designs, the potential scientific and medical benefits from a successful marriage of RNAi and gene therapy seem enormous.**

## Introduction

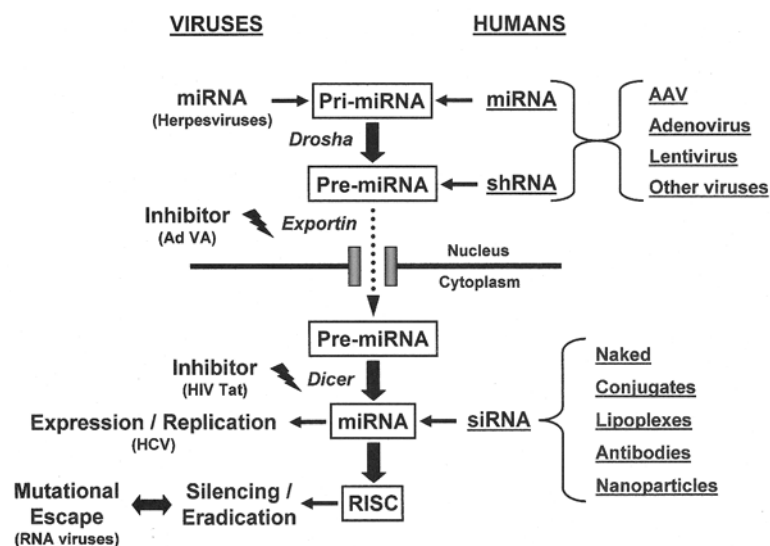
In the past 5 years, numerous groups have initiated a flurry of efforts to merge two of the most exciting and promising technologies in biomedicine, gene therapy and RNA interference (RNAi). Gene therapy, on the one hand, intends to provide therapeutic merit by introducing genetic material (DNA or RNA) encoding a protein that is missing or defective into a patient's cells or tissues. A hallmark of gene therapy is the efficient delivery of these nucleic acids via the use of shuttle vectors, which can be either nonviral, such as liposomes or nanoparticles, or derived from genetically modified viruses. Among the latter, adeno-associated virus (AAV), adenovirus (Ad) and lentivirus have emerged from numerous pre-clinical or clinical studies as the most robust and versatile option. For instance, AAV vectors were used to deliver the human factor IX gene to patients with hemophilia B,<sup>1</sup> and numerous children with genetic immunodeficiencies have been successfully treated with retroviral therapy.<sup>2</sup> Yet, isolated recent vector-related adverse events indicate that gene therapy is still facing obstacles and has not yet lived up to its promises.<sup>2,3</sup>

RNAi, on the other hand, describes the sequence-specific silencing of gene expression triggered by short double-stranded RNAs. First described in worms, insects and plants as an innate antiviral defense scheme, RNAi was later also found to be active in higher organisms (albeit likely not part of the immune system).<sup>4,5</sup> Mammalian and human RNAi pathways begin in the nucleus, with the expression of primary microRNAs (miRNAs). In a complex enzymatic cascade, they are cleaved into precursor miRNAs, exported to the cytoplasm, further processed by Dicer nuclease, and

then incorporated into a multiprotein complex (RNA-induced silencing complex [RISC]) together with a target mRNA (**Figure 1**).<sup>5</sup> MicroRNAs often have multiple sequence mismatches with their target and induce silencing through an as-yet unknown translational mechanism. However, there are increasing reports of miRNAs with perfect mRNA target complementarity and the ability to trigger its degradation.<sup>6</sup> It is believed that hundreds of miRNAs may be encoded in the human genome and that thousands of genes may be regulated at some level through either of the two mechanisms. A more detailed description of miRNA function is beyond the scope of this article but is found in excellent reviews.<sup>5,6</sup> What makes RNAi highly interesting from a therapeutic standpoint is the extreme robustness of gene silencing, which exceeds that previously observed with any other comparable inhibitors, e.g., antisense or ribozymes. In fact, a rapidly increasing number of reports are proving RNAi's potential to suppress virtually any gene, from cellular oncogenes or viral genes, to endogenous transcripts of more basic interest.

## Nonviral RNAi Delivery Strategies

At present, the major hurdle hampering the successful clinical translation of RNAi remains the need for effective and safe vehicles for systemic and targeted RNAi delivery in patients. One strategy is to use chemical or physical carriers for transfer of small interfering RNAs (siRNAs).<sup>6,7</sup> These mimic mature miRNAs in length (approximately 21 nucleotides), but are double-stranded and have perfect target complementarity, and thus mediate mRNA cleavage. RNAi induction through siRNAs holds particular promise for spe-



**Figure 1. Human/mammalian RNAi pathway and therapeutic options.** Shown in the center is the enzymatic cascade leading from nuclear expressed primary micro-RNAs (pri-miRNAs) to cytoplasmically active mature miRNAs. The three key cellular enzymes/proteins in this pathway (Drosha, Exportin and Dicer) are shown in italics. The right side summarizes options to reprogram this endogenous pathway for therapeutic intervention: viral vector-mediated nuclear expression of miRNAs or shRNAs, or alternatively, cytoplasmic delivery of siRNAs (mimicking mature miRNAs). The different vector options are described in the text. The left side shows examples of pathogenic viruses as some of the most clinically relevant RNAi targets, and ways how they have evolved to hijack and control human RNAi mechanisms. These include the expression of virally encoded miRNAs, or the exploitation of cellular counterparts, as well as the inhibition of key steps in the RNAi pathway. If successful, therapeutic RNAi can lead to the suppression of target (viral) gene expression (and virus replication). However, in particular RNA viruses can counteract RNAi by mutational escape. Abbreviations: RISC, RNA-induced silencing complex; Ad VA, adenovirus VA RNA; HIV Tat, HIV Tat protein; HCV, hepatitis C virus.

cific diseases in accessible tissues, e.g., the eye or the lung. Indeed, respective clinical phase 1/2 trials have already been initiated for age-related macular degeneration<sup>8</sup> or Respiratory syncytial virus (RSV) infection.<sup>9,10</sup> A common result of all trials was that the three different lead siRNAs were found to be safe and well tolerated in more than 100 healthy adults. Nonetheless, despite these encouraging findings with locally delivered siRNAs, there remain only a few demonstrations of systemic *in vivo* efficacy from siRNAs to date, with a single study in a nonrodent animal model. The reason is that effective delivery of siRNAs, especially to tissues deep within the body, remains a major challenging hurdle for the clinical transition of the approach. Consequently, a large number of different strategies to improve siRNA pharmacology, i.e., stability, potency, specificity, safety and delivery, are currently being evaluated in small animal models. A detailed discussion of the various siRNA formulations reported in the last few years is beyond our scope but can be found in recent specific reviews.<sup>6,7</sup> Briefly, one can distinguish various types of siRNA conjugates, including cholesterol and higher cho-

lesterol-lipoprotein complexes.<sup>11</sup> Because they mediate efficient siRNA uptake into the liver, cholesterol conjugates and derivatives are promising for the treatment of hepatic diseases, such as hypercholesterolemia. Similarly, siRNAs were encapsulated in stable nucleic acid lipid particles (SNALPs) and used to suppress apolipoprotein B (apoB) in mice and nonhuman primates. In a notable study, Zimmermann and colleagues achieved more than 90% apoB silencing in cynomolgous monkeys, following a single intravenous siRNA administration.<sup>12</sup> Yet, as with all other reported *in vivo* siRNA applications to date, the effect was detected for only less than 2 weeks, leaving the potential of siRNAs for treatment of chronic diseases unclear. Other siRNA formulations reviewed in detail elsewhere<sup>7</sup> include their complexation with diverse cationic peptides and polymers, to yield nanoparticles ideally with target cell specificity. A most notable example in this category is a recent study by Kumar et al, who created a chimeric peptide consisting of 29 residues from rabies virus glycoprotein (RVG) fused with 9 arginines.<sup>13</sup> The latter mediated siRNA binding by charge interaction, whereas the expected function of the RVG moiety was to enable transvascular siRNA-peptide delivery to the brain. Indeed, four intravenous injections of the siRNA complex induced robust protection against fatal viral encephalitis in approximately 80% of mice for a month. An intriguing further, related strategy is the noncovalent binding of siRNAs to antibody-protamine fusions.<sup>14</sup> While the protamine component attaches to the siRNA, the antibody part (e.g., a Fab fragment) potentially mediates specific binding to cells expressing proteins (e.g., encoded by a viral target or by an oncogene).

Nonetheless, several hurdles and questions remain with this approach, as with all other siRNA formulations reported to date. They include the need for a better understanding of the mechanism of siRNA uptake and intracellular processing uncertainties about the therapeutic window, including the breadth of applicability to multiple clinically relevant targets and toxicities commonly associated with lipid-nucleic acid complexes, as well as issues from commercial and regulatory perspectives. Another major limitation to the clinical use of siRNAs is their typically very transient nature, with intracellular siRNA concentrations and silencing effects usually declining within a 2-week period. Thus, treatment of chronic ailments, such as cancer, infections or neurodegenerative disorders, will either require repetitive siRNA treatment or, preferably, long-term RNAi from a persisting and regulated template. This need has further

sparked the recent enthusiasm to combine the benefits of RNAi, efficacy and specificity, with the advanced delivery options provided by viral gene therapy vectors.

### **Viral Vectors for RNAi Expression**

The design of viral RNAi vectors became possible with reports that the RNAi machinery can be activated via the promoter-driven expression of short hairpin RNAs (shRNAs).<sup>15</sup> In these, two complementary target-specific strands are covalently linked via a brief hairpin stretch, yielding transcripts mimicking nuclear processed miRNAs and as such able to engage RNAi. Notably, the minimal length of a typical RNAi cassette permits shRNA expression even from the smallest of all viral vectors (AAV), and thus permits a vector choice based on other criteria. One vital parameter is shRNA integration versus episomal persistence of shRNAs embedded within an expression cassette, while further critical aspects are inherent virus immunogenicity or tropism. Obviously essential is also the efficacy of the recombinant virus in a given cell or tissue. Not only will this determine the strength of target gene suppression, but it might also increase the risk of unwanted side effects from shRNA expression. This became particularly apparent in our own recent study, where we over-expressed shRNAs in the murine liver from an optimized and utmost efficient AAV serotype 8 vector.<sup>16</sup> In fact, transduction by this vector (when used at high doses) was so efficient that the encoded shRNAs saturated the endogenous hepatic miRNA pathway, resulting in cytotoxicity and frequent animal morbidity. Fortunately, this adverse effect could be avoided by the use of judiciously selected shRNAs and lower vector doses. Notably, these conditions still permitted complete and persistent liver transduction from a single intravenous vector dose, resulting in stable *in vivo* suppression of hepatitis B virus in our mouse model.<sup>16</sup> Moreover, tremendous effort is currently being put into the development of new shRNA promoter systems for use from viral vectors, including constitutively and ubiquitously active variants, or, most recently, spatio-temporally controlled promoters.<sup>17</sup> The latter especially will further increase the safety of RNAi gene therapy approaches, as they will provide an optimal level of exogenous control over shRNA expression and thus target gene silencing.

At this point, the three predominating viral vectors for RNAi expression are Ad, lentivirus and AAVs.

#### **Adenovirus**

Adenoviruses (Ad) are double-stranded DNA viruses able to package approximately 8 up to approximately 30 kb of foreign DNA, depending on the design as first-generation or helper-dependent (devoid of most viral genes) vectors. The main advantages of Ad for shRNA expression are the very robust expression, plus the ease and efficacy of vector production (at least first-generation Ad). Ad is particularly attractive for RNAi gene therapies of cancer, due to the

feasibility to generate “tumor-specific” variants that conditionally replicate in, and lyse, transformed cells. Indeed, several reports have already validated the outstanding promise of oncolytic Ad vectors for tumor-restricted shRNA expression.<sup>18</sup> However, Ad vectors also have a series of limitations which might preclude them from many other applications, including strong immunogenicity and typically short duration of shRNA expression. Moreover, the large packaging capacity of gutless Ad vectors might be a problem for small shRNA cassettes, as they might jeopardize genetic vector stability.<sup>19</sup> Finally, there is evidence from our own work and others that first-generation Ad vectors may contain properties and/or stimulate cellular processes that block or counteract RNAi-mediated gene silencing.<sup>20,21</sup>

#### **Lentivirus**

Lentiviruses (LV) belong to the retroviruses and carry two copies of a single-stranded RNA genome in an enveloped capsid. Engineered in their most advanced and safest form, “self-inactivating” (SIN) vectors, LV can deliver approximately 9 kb of foreign DNA.<sup>22</sup> Remarkable features that make LV highly attractive for therapeutic RNAi of chronic diseases are the abilities to infect dividing and nondividing cells, and to stably persist through genomic integration. Of further use is the option to pseudotype LV with VSV glycoprotein, which broadens tropism and supports uptake into otherwise refractory cells, such as human hematopoietic or embryonic stem cells.<sup>23</sup> However, production and use of LV vectors are associated with safety concerns, including creation of replication-competent wild-type-like viruses or insertional mutagenesis. Moreover, LV vectors might become silenced long term or upon segregation into new generations of LV-transgenic animals.<sup>24</sup>

#### **Adeno-associated virus**

AAVs are single-stranded DNA viruses and some of the smallest vectors, with a packaging limit of approximately 5 kb. Notably, this is still sufficient to accommodate at least eight individual shRNA expression cassettes.<sup>25,26</sup> AAV vectors stably and efficiently infect a wide variety of dividing or quiescent cells, and have already been clinically studied in multiple tissues.<sup>1</sup> A unique AAV feature is the option to pseudotype the viral shRNA genome with any of the more than 100 naturally occurring, or with chimeric, synthetically generated capsids.<sup>27</sup> This permits retargeting of the shRNA cassette to specific cells or tissues, or repeated delivery of the same genome, by the switching of viral capsids. Moreover, AAV vectors mediate persistent gene/shRNA expression, but unlike LV or retroviruses, AAVs integrate at extremely low frequency. Beneficial from a safety aspect, this raises a need for multiple vector administrations in rapidly dividing tissues. Finally, AAVs can deliver substantially higher shRNA copy numbers per cell than LV (hundreds versus a single or few), and thus provide greater *in vivo* efficacy and a wider therapeutic window.<sup>25</sup>

## RNAi Gene Therapy Applications

Major targets for RNAi gene therapy that have emerged in the last 5 years are viral infections, cancer (solid or bloodborne), and neurodegenerative and ocular diseases. As the literature is already extensive and too complex for a complete discussion, we will limit ourselves to main principles, and refer the reader to reviews where appropriate.

### Viral infections

Human infection with viral pathogens presents an excellent target for therapeutic RNAi for several reasons. First and foremost, as exogenous sequences, viral nucleic acids (RNA or DNA) provide unique targets for highly specific RNAi, with potentially minimal risks of cellular off-targeting. This is particularly beneficial for the design of RNAi therapies against dissimilar viruses that infect the same tissue and cause similar diseases. An important example comprises viral hepatitis infections for which the pathogenic agents (e.g., hepatitis A, B, C, or delta virus) are genetically diverse.<sup>25</sup> Once a viral gene transfer vector has been optimized to deliver shRNAs to the commonly infected host cells (in this example, hepatocytes), it can readily be modified to specifically target the genome of any type of hepatitis virus. Second, all viral life cycles include RNA at some point, either in the form of genomic RNA, messenger RNA, or replicative RNA intermediates. The resulting susceptibility to RNAi offers the means to inhibit viral gene expression or to disrupt viral replication, thus causing a slowing or a complete cessation of the viral life cycle. An exciting idea is that for viruses with an RNA genome, such as SARS, hepatitis C virus (HCV), or HIV, therapeutic RNAi could destroy the viral genome itself and thus potentially rid the body of virus altogether. However, this concept is highly controversial, as the viral genome might actually remain shielded from RNAi in the capsid or in cellular compartments.<sup>20,21</sup> Moreover, evidence is currently emerging that at least some human viruses might have evolved mechanisms to counteract antiviral RNAi (Figure 1).<sup>20,21</sup> Third, some target viruses have already been developed as RNAi transfer vectors, most notably, HIV.<sup>28</sup> Ironically, this allows their use as shuttles for antiviral RNAi to the same cells as those infected by the parental viruses, and thus provides an optimal, pretailored RNAi delivery system. It is not surprising that every class of human virus, whether it is based on DNA or RNA, or on single- or double stranded genomes, has been RNAi-targeted at least in laboratory studies. A number of recent comprehensive reviews are available.<sup>20,21,25,26</sup>

One clinically pertinent viral target for which new treatment options are urgently needed are HBV and HCV. More than 500 million people worldwide are chronic carriers of at least one type of hepatitis virus, and many will ultimately die from infection complications.<sup>20</sup> This somber prognosis is due to the facts that both viruses respond very poorly to conventional therapies, and that there is no vac-

cine for HCV (unlike HBV). To begin to evaluate HBV as an RNAi gene therapy target, we have recently engineered double-stranded AAV serotype 8 (AAV-8) vectors to express our most potent anti-HBV shRNAs.<sup>16</sup> In systemically infected HBV-transgenic mice, we achieved a near complete reduction of HBV protein and mRNA expression for 5 months.<sup>16</sup> A later study with a similar vector confirmed the exceptional potential of AAV-8 for antiviral RNAi in the liver,<sup>29</sup> and further supports the development of AAV pseudotypes for *in vivo* RNAi. Of interest, a phase 1 clinical trial using a DNA plasmid-based vector for delivery of an anti-HBV shRNA expression cassette to the liver has been approved by the U.S. Food and Drug Administration (FDA) (C. Satish, personal communication). It will be important to compare the efficacy and persistence of this nonviral approach to the AAV-8 studies.

A second virus for which impressive preclinical efficacy of RNAi gene therapy was recently documented is HIV. John Rossi's group reported a potent anti-HIV viral vector, based on lentiviruses and expressing three effective entities: an shRNA to suppress HIV *tat/rev*, a nucleolar localizing decoy to sequester HIV Tat, plus a ribozyme to cleave the cellular CCR5 coreceptor.<sup>28</sup> The vector is thus an intriguing example for combinatorial antiviral RNAi. This triple vector was highly efficient in primary hematopoietic cells, where it mediated complete protection against HIV, but its real potential will be assessed in its looming clinical evaluation. In this trial, the vector will be *ex vivo* delivered to HIV patient-derived hematopoietic stem cells, which will then be re-infused to assess therapeutic benefit. The approach is particularly promising due to the combined targeting of multiple aspects of the HIV life cycle, which should prevent mutational HIV escape and thus overcome a problem with present monotherapies.<sup>20</sup>

This impending trial will in fact represent the first clinical testing of vector-mediated RNAi gene therapy and is thus highly anticipated in the field. Yet, it is not the first trial of antiviral RNAi, in view of the ongoing phase 1 evaluation of siRNAs against RSV. Unlike HIV, RSV has a limited duration of infection, and might not necessarily require gene therapy because of its tropism for the easily accessible lung, which makes it amenable to inhibition with locally delivered siRNAs.<sup>9,10</sup> Another example is herpes simplex virus 2, whose vaginal transmission can be blocked topically with siRNAs.<sup>7</sup> Both viruses might still require RNAi gene therapy, in case siRNA persistence proves to be limiting.

### Cancer

A second promising target for RNAi gene therapies is human cancer. As the literature is growing rapidly, we refer the reader to a set of excellent reviews.<sup>30-32</sup>

In general, one can distinguish three classes of RNAi targets for cancer therapies: genes that are part of cancer-associated cellular pathways, those that play a role in tu-

mor-host interactions, or those that mediate resistance to chemo- or radiotherapy. In particular, the latter are emerging targets for combinatorial RNAi (coRNAi), where vectors co-express shRNAs against the cancer-causing gene itself, as well as against further genes whose inhibition might yield additive or synergistic therapeutic effects. However, the most obvious targets are oncogenes themselves that are causally linked to malignant transformation, and that are either amplified, mutated, result from chromosome or gene rearrangement, or are exogenously introduced by transforming viruses in human tumors. Many of these targets have been studied *in vitro* with siRNAs, and an increasing number are now being validated *in vivo* using RNAi gene therapy.

For instance, Kock et al constructed a lentiviral vector to target *bcl-2*, whose overexpression especially in human gliomas mediates protection from apoptosis.<sup>33</sup> The group also made a second vector to express a secreted form of TRAIL, an extrinsic apoptosis-inducing agent. Co-infection of both vectors into glioma cells and transplantation into nude mice led to a near-complete remission of tumors. This is an impressive example not only of the potential of RNAi gene therapy for tumor treatment, but also of the power of combinatorial approaches. Similar data were reported, e.g., by Sumimoto et al, who used lentiviruses to co-suppress two genes (*BRAF* and *Skp-2*) that are frequently up-regulated and mutated in melanoma cells; they also observed superior anti-tumor efficacy from this combinatorial approach.<sup>34</sup> Another recently emerging candidate RNAi target is the *Hec1* gene, which is highly expressed in many cancers and crucial for chromosome segregation. Two studies using Ad, lentiviral or AAV vectors showed that *Hec1* depletion caused significant reductions in tumor sizes in various xenograft models.<sup>35,36</sup> *Hec1* has thus been suggested as a highly promising new target for treatment of gliomas, lung or pancreatic cancers.

As mentioned, alternative targets are those genes mediating host interactions for tumor formation or progression, e.g., those encoding angiogenesis factors. A pivotal and multiply tested example is vascular endothelial growth factor (VEGF). For instance, Shen et al showed that treatment of VEGF over-expressing human K562 cells with anti-VEGF shRNAs, followed by xenotransplantation, resulted in smaller tumors with decreased vessel densities.<sup>37</sup> In another, very elegant study, Yoo et al engineered an oncolytic Ad to express anti-VEGF shRNA.<sup>18</sup> As compared with a conventional Ad vector, the oncolytic shRNA construct mediated substantially greater antitumor effects in glioma xenografts, including extended animal survival.

Another emerging strategy is to (co-) target genes that contribute to tumor resistance to chemotherapy or irradiation, e.g., those for multidrug resistance (MDR) proteins. For example, Pichler et al used retroviral vectors to transduce colon cancer cells with shRNA against P-glycoprotein (MDR1 product) and saw evidence for enhanced sensitivity to cytotoxic drugs.<sup>38</sup> Similarly, Zhang et al used Ad

vectors to deplete various cancer cells of HIF-1 $\alpha$ , a factor up-regulated in rapidly progressing tumors under conditions of hypoxia.<sup>39</sup> Interestingly, the Ad vector delayed growth of implanted tumor cells in mice, and co-irradiation gave a synergistic effect, thus further supporting the concept of coRNAi gene therapy. Comparable effects were seen by Lee et al with Ad vectors against insulin-like growth factor 1 in lung cancer cells.<sup>40</sup>

Finally, another critical target are fusion oncogenes, which are especially prevalent in lymphoproliferative cancers. These are highly attractive for RNAi therapies, as the chimeric mRNA is unique to tumor cells. A prototype example is the *bcr-abl* fusion oncogene in chronic myelogenous leukemias (CML) resulting from t(9;22) translocations. Sengupta et al used an Epstein-Barr virus vector to express shRNA against the *bcr-abl* junction in primary CD34<sup>+</sup> CML cells and saw an induction of apoptosis.<sup>41</sup> Interestingly, co-expression of a dominant-negative form of p27 (a cdk inhibitor) potentiated the effect, providing another example for coRNAi therapies. Similar data came from Chen et al, who used retroviral vectors to block the TEL-PDGFB $\beta$ R oncogene, another well-validated target in human leukemias.<sup>42</sup> Their vectors not only extended disease latency and survival of mice that received transplants, but also mediated synergism with imatinib, a small molecule inhibitor of tyrosine kinases. Notably, imatinib is in clinical use for leukemia treatment, but is hampered by low efficacy and resistance in many patients. Fusion oncogenes are also present and thus good targets in solid tumors, e.g., mucoepidermic salivary gland tumors or rhabdomyosarcomas (highly malignant soft-tissue tumors of childhood), as reported, for example, by Komiya et al<sup>43</sup> and Taulli et al.<sup>44</sup> Further examples for fusion gene targets are found in recent reviews.<sup>45,46</sup>

### Neurodegenerative disorders

Further impressive preclinical proof for the vast potential of RNAi gene therapy stems from small animal models of gain-of-function neurodegenerative disorders. Important examples are polyQ repeat disorders that are currently untreatable with conventional drugs, including spinocerebellar ataxias (SCA) and Huntington disease (HD). Further relevant targets are Parkinson disease and Alzheimer disease, as well as ALS (amyotrophic lateral sclerosis). All major viral vectors have been evaluated in the context of these diverse diseases and using appropriate small-animal models. The results were usually encouraging and ranged from improvements in motor coordination and muscle function to delayed disease onsets and progression rates and substantial extensions of lifespans. A detailed description of the various studies and concepts is found in recent, more specific reviews.<sup>47,48</sup> Generally, a question that remains open is whether the selected shRNAs would be tolerated in humans, considering they did not distinguish between the wild-type and mutant genes. A more feasible strategy will

thus be to design sequences that specifically target and down-regulate only the disease allele. What also frequently remained unclear was the ideal vector injection route (intraspinal or intramuscular). Of note, repeated intraspinal injections might not be feasible in humans.

### Ocular disorders

Various pathologic processes affect the human eye, and the organ itself is an isolated and accessible compartment, explaining why ocular diseases are actively studied RNAi targets. Thus far, multiple preclinical studies showed that local (intravitreal) injection of high siRNA doses result in diffusion throughout the eye and persistence in mice for more than 5 days.<sup>8</sup> We recently expressed shRNAs against basic fibroblast growth factor in the rat retina from AAV or lentiviral vectors (Paskowitz et al, in press). In particular, AAV-5 gave safe, efficient and long-term knockdown, validating the promise of RNAi gene therapy also in the eye. As a result, this scheme might eventually become relevant for persistent suppression of two of the most important ocular targets, VEGF and its receptor (VEGFR). Both are involved in neovascularization and responsible for many cases of blindness and visual morbidity. The therapeutic potential of ocularly delivered anti-VEGF/R siRNAs is currently being tested in two phase 1/2 clinical trials of age-related macular degeneration.<sup>8,49,50</sup> Thus far, these trials have primarily assessed siRNA safety, and efficacy and duration of siRNA effects remain to be tested. A comparison to RNAi gene therapy will be important, especially as the difficulties and morbidities associated with repeated intraocular infusions might be overcome with the ability to give a single administration of a persisting vector. Various other genes involved in ocular diseases and representing potential therapeutic targets include TGF $\beta$  and its receptors (fibrotic eye diseases), c-Jun (apoptosis in glaucoma), and p66Shc (oxidative damage).<sup>8</sup>

### Further RNAi Vector Applications

Below, we will briefly discuss three further, currently emerging applications of vector-mediated RNAi beyond gene therapy.

#### Segregated transgene expression

One of the biggest hurdles facing gene therapy is priming of the immune system against the newly introduced transgenes, resulting in host-mediated immune rejection and vector clearance, thus preventing stable gene transfer. Unfortunately, previous strategies to abrogate such responses, e.g., co-administration of immunosuppressive drugs or use of tissue-specific promoters (minimizing transgene expression in professional antigen-presenting cells, APCs), has had only limited success.

In a clever attempt to overcome neoantigen-specific immunity and to induce vector tolerance, Brown et al recently reported a new paradigm in gene therapy vector design which hinges upon the central roles for miRNAs in

establishing cell identity.<sup>51</sup> The group created a lentiviral vector in which a strong neoantigen (*gfp* reporter gene) was tagged with a miRNA target (mir-142-3p) specific to cells of the blood lineage. Indeed, in immunocompetent mice, *gfp* expression from systemically delivered vector was suppressed in hematopoietic lineages, but maintained in nonblood cells, especially hepatocytes and endothelial cells. In contrast, a nontagged *gfp* variant was totally cleared from the mice. Segregation of transgene expression from the tagged construct and abrogation of immune clearance were confirmed in transgenic mice, where Gfp was absent from APCs, but detected throughout the parenchyma of multiple organs.

It may be possible to adapt this novel strategy for the segregation of RNAi expression from systemically delivered gene therapy vectors. It should be feasible to tag shRNA expression cassettes with binding sites for miRNAs, resulting in detargeting of cells expressing these miRNAs and thus enhancing the stringency of RNAi gene therapy. Obviously, the success and versatility of this novel conditional strategy will depend on the specificity of the miRNA regulator, as well as on the discovery of further tissue-, development- or tumor-specific miRNAs.

#### Stem cell biology and therapies

Particular applications of RNAi technology that are still in their infancy but holds great potential are its use to dissect human stem cell biology, or to therapeutically modify these cells. Currently the most promising vectors for these goals are lentiviruses pseudotyped with the VSV-G glycoprotein, which provides a high tropism and transduction efficacy for mouse or human stem cells.<sup>23</sup> As already demonstrated, these vectors permit gene transfer to both types of stem cells, pluripotent embryonic stem (ES) or adult stem cells (which are more restricted in their differentiation potential), such as neural or hematopoietic cells.<sup>23</sup>

It is likely that a first possible application of vector-based RNAi will be gene discovery in ES cells, to unravel and eventually control the molecular mechanisms underlying, for example, self-renewal or pluripotency. Most helpful should be the combination of RNAi vector technology with high-throughput library screening approaches, as recently established by a variety of academic and commercial entities.<sup>52</sup> A critical, therapeutically most relevant goal should then be the identification of genes that mediate lineage commitment. There are in fact examples of genes that, when introduced into ES cells by conventional techniques, promote their differentiation, e.g., into hematopoietic progenitors.<sup>53</sup> Proof-of-concept for the usefulness of RNAi to achieve this goal was recently provided. One notable example with relevance to hematopoiesis came from Zou et al, who used transfected siRNAs to suppress the Pu.1 transcription factor in various murine ES cell-derived hematopoietic progenitor cells.<sup>54</sup> This resulted in their differentiation into progenitor B cells displaying characteristic cell surface markers and transcription factors.<sup>54</sup> This ex-

emphasizes the potential of RNAi as a surrogate genetic tool to force enrichment of cell types of interest from murine or human ES cells.

A second, therapeutically highly relevant use of vector-mediated RNAi is the genetic manipulation of ES cells for the prevention and treatment of human diseases. One possible strategy will be to isolate and clonally propagate stem cell pools from patients with a particular condition, followed by the identification, characterization and correction of the underlying genetic defects (e.g., via RNAi-mediated suppression of an abundant oncogene). The use of RNAi vector libraries should prove extremely useful, as they will facilitate large-scale gene discovery in stem cells. Moreover, RNAi vectors may become useful for ES cell-based vaccination against infectious diseases. One notable example already mentioned above is the use of a lentiviral RNAi vector to express anti-HIV shRNAs in human hematopoietic stem cells.<sup>28</sup> In the future, an improvement might be to instead target human ES cells, considering they can be grown indefinitely and to large numbers. Further looming vector RNAi applications in ES cells are reduction of their tumorigenic potential (by knocking down proliferation genes), or manipulation of their immune repertoire to minimize rejection in patients who undergo transplantation.<sup>55</sup>

### *Animal transgenesis*

An intriguing vector RNAi application with great future potential is its use for genetic engineering of animals (and plants) as an adjunct to conventional methods, such as creation of knockout mice via homologous recombination. Two critical limitations of the latter approach are lack of stable ES cells from most mammalian species apart from mice, and the high costs and low efficacies of alternative strategies, including somatic nuclear transfer from fetal fibroblasts. A further drawback is that gene function is entirely abrogated in knockout mice, which likely over-represents the natural variation in gene expression typical for human diseases. RNAi-mediated gene knockdown might provide a more pertinent genetic tool to capture and model pathogenic phenotypes, as it yields hypomorphic allelic animals with different degrees of gene deficiency, similar to the genetic profiles seen in human diseases.<sup>56,57</sup>

A pivotal discovery in RNAi transgenesis was that lentiviruses permit *in vitro* delivery of shRNA cassettes directly into early-stage, pre-implantation mouse embryos. This now provides a simple and reproducible means to create knockdown mice that bypasses the need for pronuclear DNA injection.<sup>58</sup> The basic method of lentiviral RNAi transgenesis was established in mice,<sup>59,60</sup> and a plethora of later reports validated its usefulness in other mammals, including goats, pigs and bovines. An important recent pilot study by Dann et al validated the use of lentiviral RNAi transgenesis in rats; these animals are invaluable models for human diseases, but unfortunately not amenable to knockout technologies.<sup>61</sup> By targeting *Dazl*, a gene re-

quired for fertility, the group created hypomorphic rats with heritable defects in germ-cell development and viability, analogous to phenotypes in knockout mice.

From a gene therapy standpoint, most exciting are recent papers showing lentiviral RNAi transgenesis in livestock. The goal of those studies was protection against infection with viruses or prions, for which no pharmacologic compounds exist at the moment. Resistant livestock would thus be highly interesting for the production of biomedical products (animal pharming), as the risk of disease transmission to humans would be minimal. Several groups validated the idea of RNAi-based prion vaccination in mice,<sup>62</sup> goats or cattle.<sup>63</sup> Others created transgenic pigs and studied the epigenetic regulation of integrated vectors. Stable RNAi in pigs will likely become very valuable, as these animals could be used for cell-based therapies and organ transplantation. Finally, in this area, Dickins et al recently reported the first transgenic mice harboring an inducible and tissue-specific RNAi expression cassette.<sup>64</sup> Spatio-temporal RNAi transgenesis is a groundbreaking approach which will find numerous broad uses in basic research or in preclinical disease models.

### **Outlook: The Future of RNAi Gene Therapy**

The pace at which RNAi is currently progressing from basic research to potential clinical applications is astounding and unprecedented. Major players in this development are viral gene transfer vectors and established gene therapy protocols, whose juxtaposition with RNAi technology even further potentiates the power of the approach. In fact, the two concepts—mRNA inhibition/depletion (RNAi) and shRNA addition (gene therapy)—are mutually attracted and complement each other very well. Clearly, RNAi offers an exceptionally powerful means to suppress the expression and thus the function of disease-causing genes, and is superior to all similar methodologies previously tested in a gene therapy context. Over time, it is likely that accepted therapeutic practices will include both siRNA- and shRNA-mediated approaches dependent on the disease entity. We believe that RNAi gene therapies may become the predominant clinical scheme for many types of chronic human diseases, based on the inherent benefits of using viral vectors for shRNA transfer. Most notable are the options for systemic delivery and for tissue-targeting, as well as the potential to achieve lifelong RNAi persistence. As discussed above, a wealth of preclinical studies already prove the potential of RNAi gene therapy for the treatment of a variety of human genetic disorders, especially in conjunction with conventional options, such as cancer chemotherapy or antiviral immunotherapy. It is thus with the greatest excitement that we anticipate the results of the first clinical RNAi gene therapy trials, as well as the others which will follow.

Despite the overwhelming medical promise, it must not be overlooked that the combinatorial use of RNAi and gene therapy might also compound the potential adverse effects which both techniques might elicit. The possible

impediments to a broader use of the scheme include, first and foremost, issues related to vector and RNAi safety. For instance, more data from animal models are needed to fully assess the potential risk of insertional mutagenesis from integrating vectors, even those that remain mostly episomal, such as AAV. Also, the capacity of the various vectors and viral pseudotypes to escape pre-existing host immunity, or to avoid the induction of humoral or cellular responses, requires further thorough characterization (ideally in humans) and subsequent experimental fine-tuning. Likewise, there are pivotal concerns about the safety and true specificity of the current generation of RNAi triggers, especially as long-term data in animals are still very limited and preliminary. On a positive note, none of the reported *in vivo* studies could verify initial worries about an induction of interferon responses to shRNAs, and adverse off-targeting has likewise not yet been confirmed in animals. Nonetheless, because the field is still young, any evidence for adverse effects will indicate a need for sustained investigation into innate RNAi mechanisms as a prerequisite for the development of feasible and safe therapeutic strategies. In fact, the first *in vivo* evaluations of long-term RNAi gene therapy have raised new concerns about a possibly deleterious saturation of cellular RNAi pathways, especially those for miRNA processing.<sup>16</sup> Importantly, our own recent study and work from others indicated that this particular concern is readily overcome by the use of judiciously selected shRNA sequences and minimal, but effective viral vector doses.<sup>16</sup> Thereby, if designed and used correctly, RNAi gene therapy vectors can have an extremely wide therapeutic index. In fact, we are convinced that RNAi gene therapy remains the single most promising biomedical strategy for knocking down undesired gene expression to date, and we believe that the successful clinical implementation of RNAi gene therapy, and its eventual translation into tangible benefits, are merely a question of time.

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