Improving miRNA Delivery by Optimizing miRNA Expression Cassettes in Diverse Virus Vectors

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The RNA interference pathway is an evolutionary conserved post-transcriptional gene regulation mechanism that is exclusively triggered by double-stranded RNA inducers. RNAi-based methods and technologies have facilitated the discovery of many basic science findings and spurred the development of novel RNA therapeutics. Transient induction of RNAi via transfection of synthetic small interfering RNAs can trigger the selective knockdown of a target mRNA. For durable silencing of gene expression, either artificial short hairpin RNA or microRNA encoding transgene constructs were developed. These miRNAs are based on the molecules that induce the natural RNAi pathway in mammals and humans: the endogenously expressed miRNAs. Significant efforts focused on the construction and delivery of miRNA cassettes in order to solve basic biology questions or to design new therapy strategies. Several viral vectors have been developed, which are particularly useful for the delivery of miRNA expression cassettes to specific target cells. Each vector system has its own unique set of distinct properties. Thus, depending on the specific application, a particular vector may be most suitable. This field was previously reviewed for different viral vector systems, and now the recent progress in the field of miRNA-based gene-silencing approaches using lentiviral vectors is reported. The focus is on the unique properties and respective limitations of the available vector systems for miRNA delivery.

Keywords: miRNA cassettes, gene therapy, viral vectors, vector tropism

INTRODUCTION

NON-CODING RNA MOLECULES play an essential role in gene regulation of the cell via a mechanism called RNA interference (RNAi). The RNAi mechanism is evolutionary conserved in eukaryotes and triggers the sequence-specific inhibition or degradation of a single or a unique set of complementary mRNAs. Three small RNA classes have been described to participate in mammalian RNAi mechanisms: microRNAs (miRNAs),[1,2] endogenous small interfering RNAs (endo-siRNAs),[3] and PIWI-associated RNAs (piRNAs).[4] The endo-siRNAs and piRNAs are involved in suppression of transposable elements. The miRNAs are broadly involved in the regulated expression of multiple cellular genes and execute their effect at the post-transcriptional level.[5,6]

MiRNA-mediated regulation of gene expression plays a significant role in cell metabolism and cellular developmental and differentiation processes in mammals. More than a thousand human miRNAs have already been identified that are involved in the regulated expression of around 30% of all genes, which is a conservative estimate.[7] Because of the miRNA abundance and their important regulatory functions, these molecules have received much attention from the scientific community over the last decade. For instance, efforts have been made to explain the biological function of the natural miRNAs by identifying the target mRNA and the role in cellular physiology. On the other hand, the design of man-made miRNA mimics to impose control over gene expression became of interest for the control of newly introduced trans-

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genes in research and therapeutic applications. The development of vector-mediated RNAi allowed the establishment of durable gene silencing approaches, in particular for vector systems that are stably inherited by insertion into the host cell genome.[8–10] Especially attractive is the delivery of RNAi-inducing gene cassettes with viral vector systems. The use of different viral vector systems for miRNA delivery and their advantages and disadvantages will be discussed, followed by a more detailed discussion of the lentiviral vector system.

RNAi STRATEGIES

Since its discovery, RNAi was used frequently to knockdown specific genes for fundamental research, biotechnology applications, and therapeutic strategies.[11,12] The cellular RNAi pathway uses the nuclear Drosha and cytoplasmic Dicer enzymes to produce mature miRNA (Fig. 1, left pathway). The RNAi mechanism can also be induced to mediate transient gene silencing via transfected synthetic siRNA duplexes that are ~21 nucleotides complementary RNA strands with two-nucleotide 3′-overhangs. These artificial siRNA molecules are modeled after the natural miRNA duplexes formed upon Drosha/Dicer cleavage.[13–15] Man-made siRNAs are usually fully complementary to the mRNA target by their design, causing site-specific mRNA cleavage via interaction with the argonaute 2 (Ago2) protein. Nevertheless, the use of siRNAs has several disadvantages. First, degradation by RNase A-like nucleases causes a relatively short intracellular half-life. Second, it may also be difficult to deliver the siRNAs efficiently to specific target cells, especially primary cell types.[16–19] Perhaps most importantly, siRNAs can only cause a transient silencing effect because their intracellular concentration drops upon cell division.

This led to the development of gene cassettes for short hairpin RNA (shRNA) molecules (Fig. 1, right pathway) that are processed into siRNAs. Such constructs encode short transcripts that typically fold ~19–29 base pairs shRNAs, which mimic the pre-miRNA molecules with a base-paired stem, small loop and 3′-terminal UU overhang.[8–10] RNA polymerase III promoters (H1, U6, or tRNA) usually control the expression of such shRNA constructs.[8,10,20,21] RNA polymerase III is specialized in the production of high levels of small cellular transcripts and uses exact initiation and termination signals (four to six consecutive U residues) that result in synthesis of a defined shRNA molecule.

Optimization of the original shRNA design was achieved by incorporation of certain miRNA-derived characteristics.[22–24] Compared to the perfectly base-paired shRNAs (Fig. 1, right pathway), modified structures were designed that closely resemble the natural miRNAs by introducing typical pri-miRNA specifics such as imperfect hairpin structures with bulges and mismatches, larger loops, flanking sequences, and mismatches. The mature miRNA strands are usually designed with perfect complementarity to the target, thereby inducing mRNA cleavage. Artificial miRNAs, like their natural counterparts, are frequently transcribed from a promoter for RNA polymerase II such as the strong immediate early promoter of the cytomegalovirus (CMV).[25] However, RNA polymerase III promoters were also used.[26] RNA polymerase II promoters have certain benefits over RNA polymerase III systems, including regulated and tissue-specific expression,[27,28] and inducible RNA polymerase III systems have also been designed.[29,30] Regulated shRNA expression is crucial, as high-level constitutive shRNA synthesis can trigger serious adverse effects. Artificial siRNA/shRNA molecules can target unwanted mRNAs (off-targeting)[31] or induce immunological responses that cause toxicity.[32–34] High level shRNA expression can cause morbidity and mortality in mouse models,[35,36] but these adverse effects are usually dose-dependent. These results suggest that one or more key components of the RNAi pathway in mammalian cells may become saturated. Two key factors of the RNAi pathway, Ago2 and Exportin-5 (Fig. 1, left pathway), have indeed been shown to become rate-limiting upon shRNA overexpression, resulting in considerable disruption of the cellular miRNA pathway.[35,37]

VIRAL VECTOR SYSTEMS

Viral vectors have emerged as attractive vehicles for the delivery of transgenes to particular target cell types. In the simplest version, the pathogenic genes are removed from these viruses and replaced by the therapeutic gene(s). One vector may be more suitable than other systems, depending on the specifics of the transgene cassette, the therapeutic purpose (acute or chronic), and the cell type that is targeted. The properties of four popular viral vector systems that can facilitate high level transgene and miRNA expression are briefly presented: adenovirus and adeno-associated virus, retrovirus, and the subclass lentivirus (Table 1).

VECTORS BASED ON ADENOVIRUS

Members of the *Adenoviridae* family are nonenveloped viruses from with a linear dsDNA genome that is characterized by terminal inverted repeats of 37 kb.[38] Adenoviruses cause 5–10% of upper respiratory tract infections in children.

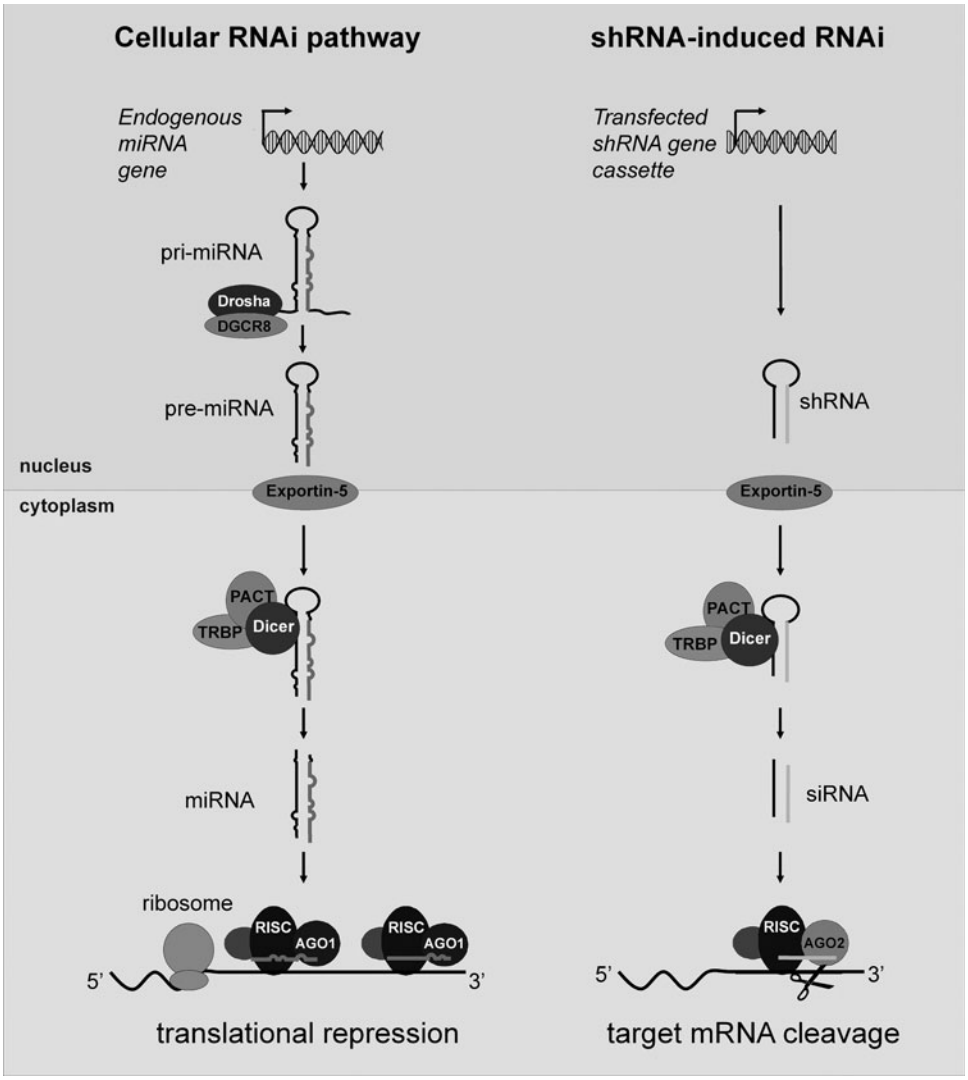

Figure 1. The RNA interference (RNAi) pathway. Illustrated are the endogenous miRNA pathway (left side) and the man-made exogenous short hairpin RNA (shRNA) pathway (right side). The cellular gene and the transgene, respectively, are transcribed into the pri-miRNA transcript and the shRNA precursor. Pri-miRNAs are processed by DGCR8/Drosha into pre-miRNAs and subsequently by Dicer/TRBP/PACT into miRNA duplexes with imperfect ~22 base pairs. Exogenous shRNAs are directly processed by Dicer/TRBP/PACT. The miRNA and siRNA duplexes are subsequently incorporated into RISC, and one strand (thick-lined RNA strand) of the duplex directs this complex toward complementary mRNA targets. A typical miRNA causes translational repression by binding to multiple partially complementary target sites in the mRNA. In exceptional cases, a near perfect base pairing complementarity of the miRNA and mRNA will result in cleavage of the latter. Similarly, siRNAs are designed to be fully complementary to the target mRNA, which is inactivated by cleavage.

Initial binding to the cell occurs by interaction with the ubiquitously expressed coxsackievirus B receptor (CAR), and cell entry is triggered by binding to integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$.[39,40] Adenoviral vectors are attractive delivery tools due to the relatively ease of manipulation and the ability to transduce a broad variety of quiescent and proliferating cells without integrating its cargo into the host genome.[41] Serotypes 2 and 5 are frequently used for vaccination and gene delivery purposes. The initial adenoviral vectors were deficient for the E1 gene and provided a large packaging capacity of 8 kb. To reduce the chance of evoking immunostimulatory responses further, second-generation adenoviral vectors were designed by omitting the E2, E3, and E4 genes. Next-generation "gutless" adenoviral vectors are completely stripped of all viral proteins and encode only the packaging signal and regulatory elements, which results in an increased packaging capacity of ~37 kb.[42,43] The major complication in vector production is that a helper virus is needed for vector propagation. Reduced stimulation of the immune response by gutless adenoviral vectors can lead to reduced vector toxicity and prolonged transgene expression.[44,45] The adenoviral gene therapy vector is ideal for

Table 1. *Viral vector systems*

Viral vector	*Advantages*	*Shortcomings*
Adenovirus	High vector titers, high efficiency Efficient uptake in dividing and nondividing cells Insert capacity (max 8 kb, extended to 37 kb in "gutless" vectors)	No integration, short-term expression Requires repeated administration[a] High immunogenicity
Adeno-associated virus	High vector titers, high efficiency Uptake in dividing and nondividing cells Relatively low toxicity, non-pathogenic Remains predominantly episomal Low risk of insertional mutagenesis	Requires helper virus for replication Time-consuming production protocol Limited insert capacity (max 3–5 kb)
Retrovirus	Low immune response in host Modest insert capacity (max 8 kb) Integrates into genome	Low vector titers Incorporates into dividing cells only Restricted tropism (expanded by pseudotyping) High risk of insertional mutagenesis
Lentivirus	Uptake in dividing and nondividing cells Modest insert capacity (max 8 kb) Integrates into genome New generation is self-inactivating for safety	Low vector titers Restricted tropism (expanded by pseudotyping) Risk of insertional mutagenesis

[a]Episomal DNA is rapidly lost from dividing cells but may be retained by nonmitotic cells.

short-term use because recombinant adenoviral vectors are replication deficient and do not integrate into the host genome.[46,47] The major shortcoming these vectors is the potent induction of immune responses (adaptive and innate), which may impose cell toxicity and thereby limit the gene transfer efficiency, thus hampering repeated use.[48–50]

Adenoviral vectors can be used for delivery of shRNA/miRNA expression cassettes to specific cells *in vitro*[51–54] and *in vivo*.[55–58] The first *in vivo* study using adenoviral vectors demonstrated reduced expression of the target gene in the liver and brain,[57] arguing that adenoviral-mediated RNAi approaches may be of special interest for neurodegenerative disorders.[59] Adenoviral vectors can deliver shRNA cassettes to liver cells and induce gene silencing for five weeks without perturbing or saturating the endogenous miRNA silencing machinery.[60,61] Due to its large cargo capacity, adenoviral vectors are especially suited for the simultaneous delivery of protein and sh/miRNA cassettes. Several publications used of adenoviral vectors for transgene expression of shRNA or miRNA molecules,[62–66] and these studies are covered in excellent recent review articles.[67,68]

VECTORS BASED ON THE ADENO-ASSOCIATED VIRUS

Adeno-associated virus (AAV) represents a nonenveloped member from the *Parvoviridae* family with icosahedral capsids. AAV contain a single-stranded DNA (ssDNA) genome of 4.7 kb flanked by inverted terminal repeats that are required for replication of the genome, its packaging, and integration. AAV vectors can transduce both dividing and nondividing cells. Wild-type AAV is dependent for its replication on a helper virus such as adenovirus or herpes simplex virus. In the absence of a helper virus, the AAV particle releases its genome that persists in episomal forms, and sometimes the virus remains latent by integrating its DNA into the AAVS1 region of chromosome 19. Infection of the cell by a helper virus can rescue the latent state.[69] The AAV Rep proteins are essential for targeted integration. In recombinant AAV (rAAV), the Rep protein is deleted from the viral genome but can be supplied in trans. Consequently, integration of rAAV is less efficient than wild-type AAV and not targeted to chromosome 19.

High-level and durable gene expression was reported in the mouse for diverse organs, including the lung, muscle, liver, brain, and eyes.[70–74] As with other integrating vectors, insertional mutagenesis remains a concern, especially for rAAV that lacks the specificity for chromosome 19. Wild-type AAV and rAAV integration has been linked to hepatocellular carcinoma in mice due to insertional oncogene activation,[75–77] but other long-term studies did not describe such a correlation.[78–82] Genome integration seems to occur preferentially at breaks in the host cell genome, including regions sensitive to endonuclease attack,[83] but other motifs have also been implicated, such as CpG islands,[84,85] sites of active transcription[84–86] and palindromic chromosome sequences.[87]

The 11 known AAV serotypes exhibit distinct host cell tropisms as well as varying immunological properties, such that one can pick the best match for a certain therapeutic application in a particular target cell type.[88] Pseudotyping of AAV allows vector retargeting to non-natural target cells or tissues.[89] An extensive set of capsid variants has been engineered to avoid neutralization by anti-

bodies that are directed against a particular capsid, as this can significantly affect the *in vivo* transduction efficiency.[90,91]

The absence of AAV pathogenicity in humans makes them an attractive therapeutic vehicle. In contrast to other viral vectors, AAV is the only vector system for which the wild-type virus is not associated with human malignancies. AAV has a relatively small packaging capacity, which commonly suffices for sh/miRNA cassettes that are limited in size. Accordingly, many *in vivo* studies were performed with AAV-RNAi vectors for human cancers, muscular dystrophies, neurodegenerative disorders, and cardiac, retinal, metabolic, and infectious diseases (see Grim,[92] McCown,[93] and Borel *et al*.[94] for reviews).

RNAi-related toxicity and mortality were first observed in AAV-injected mice when the shRNA was overexpressed in the liver.[35] Toxicity was found to be dose-dependent and due to the constitutive high shRNA expression, leading to saturation of Exportin-5. Toxicity was also observed in the mouse brain for shRNAs against the Huntington gene that were delivered with an AAV-vector.[95] Toxicity was avoided by inserting the siRNA sequences in a backbone of a miRNA instead of a shRNA. The miRNA backbone caused reduced steady-state levels of the mature miRNA and prevented saturation of the RNAi machinery.[96] Injection of this AAV-miRNA vector caused effective gene silencing of the target in Purkinje cells, suggesting that miRNA approaches are suitable for therapy applications in the brain. A second study confirmed that artificial miRNAs outperform shRNAs in suppressing a photoreceptor gene in the retina both *in vitro* and *in vivo*.[97] These and other studies indicate the superiority of miRNA-based AAV approaches for some therapeutic applications with respect to the efficiency and safety.[98–101]

VECTORS BASED ON RETROVIRUSES

Retroviruses belong to the *Retroviridae* family and the enveloped virion particles contain a ssRNA genome of $\sim$10 kb. Retroviral vector (RV) systems are usually derived from Moloney murine leukemia virus, with a simple genome encoding the Gag, Pol, and Env proteins flanked by long terminal repeats (LTR).[102] The RNA genome is copied upon cell entry by the virus-encoded reverse transcriptase enzyme into dsDNA that subsequently integrates randomly into one of the host chromosomes. This retrovirus-specific property is obviously favorable for the durable expression of inserted transgenes, but also raises a specific risk. RNA packaging sequences and other regulatory elements are relatively well defined to allow the design of RV systems. All protein-encoding sequences can be replaced by foreign sequences, as the required viral proteins can be supplied *in trans* during vector production.

Despite RV integration, persistent transgene expression is not warranted because the transgene may be transcriptionally silenced over time.[103,104] A significant safety concern is RV integration in or near unwanted genome sites. In fact, several patients treated with a RV developed T-cell leukemia in a gene therapy trial for X-linked inherited immunodeficiency in which hematopoietic stem cells (HSCs) are modified.[105–107] The leukemic event was linked to proto-oncogene LMO2 activation by adjacent RV integration. Dysregulation of host cell gene expression can occur because retroviruses were found to integrate preferentially near the start sites of transcription.[108–111] The presence of transcriptional enhancers in the RV can result in enhanced expression of cellular (proto-) oncogenes.

Self-inactivating (SIN) vector systems were designed to improve the safety profile.[112] SIN vectors carry an inactivating 3′ LTR deletion that is transferred into the 5′ LTR during vector amplification, which results in inactivation of this promoter. The vector requires an internal promoter to drive transgene expression. In addition, transcriptional enhancers that can affect cellular gene expression were removed. A significant limitation of RVs is the inability to infect quiescent cells due to an inability of the infecting particle to enter the nucleus, as the membrane is disassembled exclusively during mitosis.

Despite early disappointments, two recent Phase I clinical trials with RVs showed that *ex vivo* transduction of CD4+ T cells as well as CD34+ HSCs, followed by re-infusion of the transduced cells, is feasible and safe.[113,114] A Phase II trial confirmed safety of such a gene therapy, but also demonstrated a lack of efficacy of RVs that encode antiviral ribozymes for HIV-1 infected individuals.[115] Despite other RV-miRNA applications,[116] most recent studies have shifted toward the use of lentiviral vectors.

VECTORS BASED ON LENTIVIRUSES

Lentiviruses represent a subgroup of the *Retroviridae* family that includes the human immunodeficiency virus type 1 (HIV-1). HIV-1 encodes the standard RV proteins Gag, Pol, and Env, but also the regulatory Tat and Rev proteins and the accessory Vif, Vpr, Vpu, and Nef proteins. Tat is the viral trans-activator that induces HIV-1 transcription from the LTR promoter. The Rev protein

is responsible for export of singly spliced and unspliced HIV-1 transcripts from the nucleus.[117–120] All protein-coding information is deleted in the HIV-based lentiviral vector (LV), leaving only the necessary regulatory sequences. LVs have been developed based on a variety of immunodeficiency viruses of human (HIV-2), simian (SIV), horse (EIAV), bovine (BIV), and feline (FIV) origin.[121–125]

A main benefit of LV over RV vectors is that the former are able to transduce dividing and nondividing cells. LVs allow persistent transgene expression by stably integrating into the host DNA, although transcriptional silencing may occur over time.[126] LVs have a better safety profile than RV vectors because they favor integration within active transcriptional units, thereby reducing the risk of insertional oncogenesis.[127–129] In addition, safer SIN-based LV variants were designed. Both RV and LV are amenable to pseudotyping, meaning that heterologous envelope proteins can be accommodated. Pseudotyping with pantropic envelopes such as the vesicular stomatitis virus G protein can mediate viral entry into a wide variety of cells that otherwise would be refractory to infection, including hematopoietic and embryonic stem cells.[130]

The CD4+ T cells of HIV-infected patients were treated *ex vivo* in a clinical trial with a LV that encodes an anti-HIV antisense transcript and reinfused into the patients after *ex vivo* expansion. The integration sites of the vectors were mapped in CD4+ T cells, and no adverse events were reported.[131,132] These results confirmed the previously described HIV-1 integration profile and thus further backed up the safety profile of LVs.[131,133] Several Phase I gene therapy trials were performed with LV to modify CD34+ HSC. Patients received a LV expressing a TAR RNA decoy, a ribozyme against CCR5 mRNA and an anti-tat/rev shRNA. This treatment was both feasible and safe, although no therapeutic effect could be documented.[134,135] The other trial concerned two children with adrenoleukodystrophy (ALD), a lipid-storage disease of the brain. Disease progression was stabilized, and transduced stem cells showed polyclonal reconstitution of diverse cell subsets, including monocytes that migrate to the brain to become astrocytes that will express the therapeutic protein.[136] The ALD trial also raised safety concerns due to detection of common insertion sites (CIS) of the LV. CIS are typically associated with insertional mutagenesis in mouse models and humans.[137–141] A similar LV integration profile was observed in xenotransplanted immunodeficient mice.[142] However, these CIS differed from the previously detected genotoxic CIS by clustering in mega-base-wide chromosomal regions, whereas genotoxic CIS were confined in contracted genome regions, always targeting a single gene. Recent LV successes in HSC gene transfer include trials in patients with metachromatic leukodystrophy and Wiskott–Aldrich syndrome.[143,144] The combined data indicate that LV-mediated gene therapy is safe and effective, but this should be confirmed in larger patient groups and with extended follow-up.

LV-induced shRNA expression can cause cytotoxicity in cell lines, as was described for AAV vectors. This effect depends on the shRNA sequence and dose.[145] Several basic and applied studies tested different LV–miRNA combinations.[146–149] For instance, Bcr-Abl oncogene expression was effectively controlled by LVs encoding three artificial miRNAs, thus preventing outgrowth of leukemic cells *in vitro* and *in vivo*.[150] Furthermore, LV-delivered anti-osteopontin miRNAs could inhibit cell proliferation *in vitro* and *in vivo* tumor growth of hepatocellular carcinoma.[151]

VECTOR PRODUCTION ISSUES

When using viral vectors for miRNA or shRNA delivery, it is crucial that production of the vector is not influenced by insertion of the RNAi-inducing elements. Each vector system will exhibit some unique advantages and disadvantages, but a universal issue that may hamper vector production is genomic instability, although the details will differ among vector systems. DNA packaging in optimized adenoviral vectors demonstrates a strict size limit (27–37.8 kb).[152,153] RNAi cassettes are normally small, such that DNA stuffers could be added to prevent destabilization of the capsid. LV titers were detectable, even with a viral RNA genome size of 18 kb that greatly exceeds that of the wild-type virus.[154] However, the titer was much reduced, suggesting that increasing LV size hampers RNA genome encapsidation. This may not be a serious problem because RNAi cassettes are relatively small, and even multiple RNAi cassettes can be accommodated based on size arguments in the LV system. The recently described fourth generation of LV may facilitate longer inserts.[155,156]

Repeated-sequence motifs can also have a negative impact on the genetic stability of RVs and LVs. Retroviruses are known to be recombination prone, and repeats in RVs can thus trigger sequence duplication or deletion during transduction of target cells.[157–160] For instance, it was demonstrated that combinatorial RNAi cassettes with repeat H1 promoter sequences are not stable, resulting in deletion of one or multiple shRNA cas-

settes by recombination.[161,162] Removal of such repeat sequences, for example by use of multiple different promoter elements, did indeed increase the vector genome stability,[163] although repeat-associated problems were not reported for DNA virus vectors, but homologous recombination on repeat sequences can in theory occur during production of DNA vectors.

Mammalian cells sense a viral infection by the innate immune system and respond by induction of the interferon (IFN) response.[164–168] Accumulating evidence indicates that other antiviral defense mechanisms are also triggered, including the RNAi pathway.[169–176] To counteract such cellular responses, mammalian viruses frequently encode IFN antagonists.[177] Some of these IFN antagonists also act as potent RNA-silencing suppressors.[169,171,172,178] Vector production can be regulated by innate antiviral mechanisms in mammalian cells, especially when the viral counter defense mechanism is absent from these vector systems compared to the fully equipped wild-type virus. Provision of RNAi-silencing suppressors *in trans* can indeed improve the production of LV, adenoviral vectors, and especially Sindbis virus vectors.[179]

Several RNAi-specific problems may be encountered when viral vectors encode shRNAs or miRNAs. First, high transgene expression levels may impact on viability of the vector-producing cell and the eventual amount of vector that is produced. Overexpression of the vector-encoded shRNA or miRNA can cause activation of the IFN response and related toxicity, saturation of the endogenous miRNA pathway, or off-target effects. Such effects can easily be missed, as producer cells are usually thrown away after vector production. Second, specific RV and LV problems may arise when a shRNA/miRNA is introduced in the viral RNA genome. LV production requires transient co-transfection of the shRNA/miRNA-LV and packaging constructs that yield the structural proteins.[180] The full-length viral RNA genome encoding the RNAi cassette will be expressed by RNA polymerase II during vector production. Besides the RNA genome, the shRNA/miRNA will be simultaneously transcribed from its own promoter and processed into a mature siRNA/miRNA that can target the complementary sequences in the LV RNA genome (Fig. 2, left and right panels: self-targeting). Self-targeting of the vector RNA genome can theoretically cause a drop in the yield of produced vector.

Contradictory reports are present in literature on the impact of this "self-targeting" by LV-encoded shRNAs. It was suggested that self-targeting does not take place, as the target sequence is masked in a stable RNA structure and thus is shielded from the RISC complex.[181,182] There is indeed good evidence in the literature for a major suppressive effect of stable target RNA structure on RNAi efficiency.[183,184] However, another study showed dramatic titer reductions when shRNAs were produced by LV.[185] The cause of these differences has not yet been resolved, but likely relates to the different LV systems and/or cell types used in these studies. Dramatically low titers of dsRNA-encoding LV were reported due to RNAi-mediated cleavage of the RNA genome during vector production.[182] For miRNA-encoding LVs, it remains to be investigated whether self-targeting is excluded, since miRNAs are typically less tightly structured and thus likely more vulnerable to such attack.

RV and LV with a miRNA cassette face yet another problem. The vector RNA can be cleaved by Drosha (Fig. 2, left panel only: Drosha cleavage of vector genome). It was demonstrated that knockdown of Drosha can indeed increase the titer of such vectors.[186,187] When comparing a large series of LV constructs, a dramatically low titer was measured for LVs that express one or multiple artificial miRNA(s), but this reduction was largely due to the internal Pol II promoter element.[186] The titer could be restored almost completely by deletion or replacement of this promoter. It is possible that transgene expression from the CMV promoter is favored over RSV promoter-driven expression of the vector RNA genome (Fig. 2, left panel only: promoter interference).[188,189] Such effects have indeed been described for RV vectors.[190]

In special cases, the RNAi reagents could attack the vector backbone to cause a reduction in titer. Because the LV system is based on the HIV-1 genome, anti-HIV shRNA and miRNA molecules can theoretically also target HIV-derived sequences in the vector (Fig. 2, left and right panels: vector targeting). It was shown that anti-HIV shRNAs indeed cause a severely titer reduction when the LV or packaging construct is targeted.[181,186] Restoration of the vector production titer can be obtained via inhibition of the RNAi pathway, either by production of an excess of shRNAs to saturate the RNAi pathway or by expression of a siRNA/shRNA against Dicer or Drosha. To prevent vector targeting, one could also select shRNAs that target HIV-1 sequences that are absent from the LV genome and the Gag-Pol vector that is required for vector production. To prevent targeting of the packaging construct, a Gag-Pol construct optimized for human codons can be utilised.[191] At last, the usage of an alternative LV system can be considered, for example based on HIV-2, SIV, FIV, or BIV.

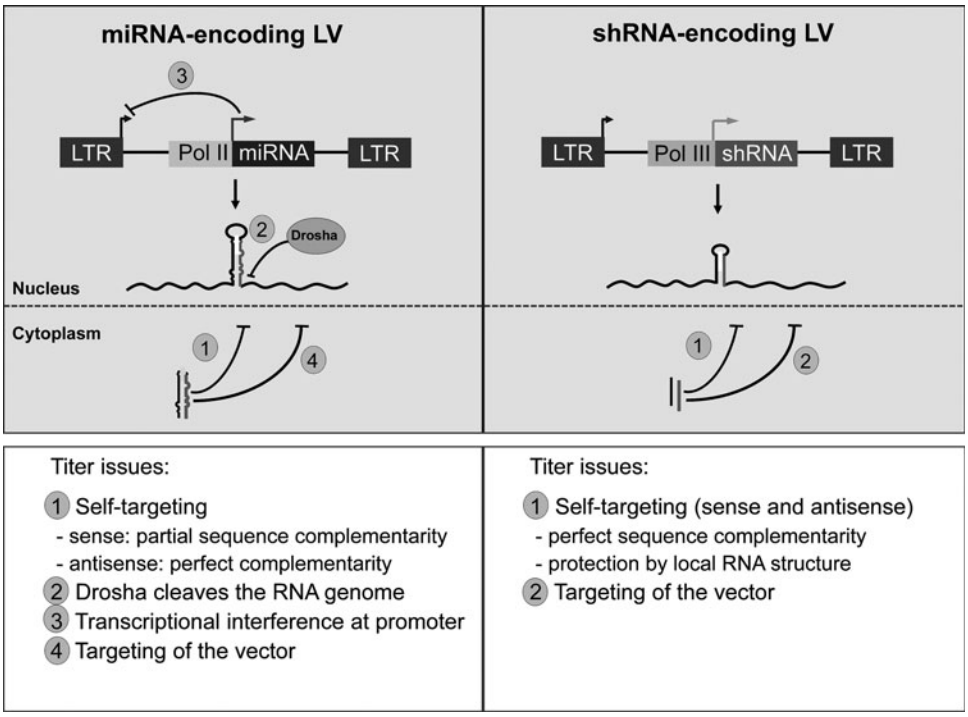

Figure 2. Titer issues in lentiviral vectors (LV) expressing miRNA or shRNA. LV encoding miRNAs can potentially cause self-targeting due to expression of the mature miRNA that can target the homologous sequences in the miRNA encoding LV genome during vector production (mechanism 1, self-targeting) If the miRNA cassette is cloned in the sense orientation in respect to the vector RNA genome, then the target will be partially complementary to the miRNA, whereas the antisense miRNA cassette in the LV will have a perfect complementarity with the expressed miRNA. The LV genome encoding the miRNA can be recognized and cleaved by the Drosha during vector production (mechanism 2, Drosha cleavage). As the LV genome and the miRNA are both transcribed from an RNA polymerase II promoter, promoter interference could possibly reduce the titer (mechanism 3, promoter interference). In case of lentiviral RNAi vectors that target HIV-1, the mature anti-HIV-1 miRNA can target sequences from the HIV-1 based LV genome (mechanism 4, vector targeting). LV encoding shRNA (right side) could also potentially target their own complementary sequences as part of the LV genome (mechanism 1, self-targeting). However, this is not likely to occur in shRNA expressing LV due to occlusion of the target in a tight hairpin structure. Anti-HIV-1 shRNAs can possibly target similar sequences present in the LV genome.

CONTROLLED TRANSGENE EXPRESSION VIA miRNAs

The focus of this review will now be expanded by discussing novel technologies to regulate the expression of transgenes or viral vectors by means of introduced miRNA target sequences. Because most miRNAs have a distinct cell type or tissue expression profile, this approach allows one to control the cellular tropism of genes, vectors, oncolytic viruses, and viral vaccines. By incorporating miRNA targets, mRNA expression becomes vulnerable to RNAi control in the cells programmed to express this particular miRNA.

Brown *et al*. tried to confine transgene expression to hepatocytes by the use of tissue-specific promoter elements because transgene activity in antigen-presenting cells (APCs) could induce an immune response.[192,193] This approach could not block an immune response, and transgene-expressing hepatocytes were eventually cleared. Next, one tried the insertion of four fully complementary targets downstream of the transgene for miR-142-3p that is specific for the hematopoietic lineage. Inclusion of the miRNA targets caused profound suppression of transgene expression, but exclusively in hematopoietic cells, including APCs.[194] By successfully avoiding immune-mediated clearance of the vector, stable gene transfer was achieved in this mouse model.[195,196] Soon thereafter, target sequences of different cell-type-specific miRNAs were tested and promising results were reported, indicating that this is a broadly applicable approach.[197] The strategy was used to separate transgene expression in neurons versus astrocytes (miR-124), undifferentiated versus differentiated embryonic stem cells (miR-302), immature versus mature dendritic cells (miR-155), and lymphoid versus myeloid lineage cells (miR-223). Two different miRNA targets can be combined to restrict transgene expression to a specific cell type. Notably, these vectors did not perturb the endogenous miRNA pathway.

This research team also used miR-126 targets in a gene therapy for treatment of globoid cell leukodystrophy.[198] HSCs were treated *ex vivo* with LV that encodes the therapeutic galactosylceramidase (*GALC*) gene. This approach is toxic without miRNA targets because of *GALC* overexpression in HSCs and early progenitor cells. *GALC* expression was blocked by the inserted miR-126 targets in HSCs and early progenitors, but vigorous expression was preserved in the mature hematopoietic cell lineages. Elevated *GALC* levels were obtained in multiple tissues upon transduced HSC transplantation in mice, causing reduced malignancies and prolonged survival.

The same strategy has been applied to control a stem cell–based gene therapy. Several miR-181 targets were inserted in the LV that encodes tumor antigen receptors.[199] This miRNA is expressed in developing thymocytes, but its expression ceases in mature thymocytes. This property was used to restrict the LV to developing thymocytes in mice upon re-engraftment with modified bone-marrow cells. One could prevent the clonal deletion of autoreactive cells by avoiding early transgene exposure in developing thymocytes. The pioneering Naldini team published a review on this topic.[200]

CONCLUSIONS

The mechanism of RNAi allows the researcher to silence genes in a sequence-specific manner. This major breakthrough in molecular biology prompted the development of several technologies for basic research and therapeutic applications. It was realized that these technologies need further refinement when artificially induced RNAi were shown to cause serious side effects, for example off-target effects on unrelated genes or saturation of the RNAi pathway, thus affecting normal cellular physiology. Early RNAi experiments focused on siRNA inducers in transient systems and shRNA inducers in stably transduced cells. Improvements were reported for enhancement of the safety and efficacy by the use of artificial miRNAs. An important safety precaution concerns the implementation of inducible or tissue-specific promoters for miRNA or shRNA expression.

Future research may lead to the development of novel classes of more specific shRNA/miRNA reagents. One recent example started with the discovery of Dicer-independent miRNAs such as miR-451.[201,202] This lack of Dicer recognition is due to the too-short base-paired duplex.[203,204] Surprisingly, this class of miRNA is not destroyed but processed by other nucleases and is shown to be active in the RNAi pathway. In fact, the Ago2 enzyme as part of the RISC complex processes these short duplexes to activate the mature miRNA strand.[205,206] Independent studies led to the description of a strikingly similar phenomenon for shRNAs that are active, despite being too short for Dicer processing.[207] It was demonstrated that Ago2 is key in this alternative shRNA processing route.[208,209] Compared to conventional shRNA designs, this alternative Ago2 route activates the "opposite" RNA strand. This design was called "AgoshRNA", as Ago2 is required for both its processing and subsequent silencing action.[209] Most importantly, whereas siRNA and shRNA reagents have the potential to generate two active strands, the AgoshRNA produces only a single active strand, thus minimizing off-target effects. The AgoshRNA design may have additional advantages, including full activity in Dicer-minus cells.[210]

Viral vectors represent smart vehicles to transport RNAi-inducing gene cassettes into cells. The characteristics of different vector systems were discussed, in particular with regard to the expression of miRNA payloads. After several setbacks in clinical gene therapy trials using viral vectors, significant modifications were introduced to augment the safety profile of these vectors. Recent clinical studies indicate that these optimized approaches can be used in a safe manner to achieve a clinical benefit. Thus, the future also seems bright for miRNA-based therapeutic interventions using a variety of viral vector systems.

Progressive knowledge of RNAi pathways inside the cell has resulted in optimized RNAi inducers that combine enhanced safety and efficiency. Concurrently, the viral vectors were improved to exhibit augmented efficacy and better safety profiles. Further studies should reveal the potential for RNAi-based therapeutics to be applied in the clinic.

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AUTHOR DISCLOSURE

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