MicroRNAs and the Regulation of Vector Tropism

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Despite being small (~22 nt) microRNAs (miRNAs) profoundly influence tissue-specific gene expression by interacting with complementary target sequences in cellular messenger RNAs, impairing their translation or marking them for early destruction. Recent work has shown that tissue-specific miRNAs offer a versatile target that can be exploited to control the tropisms of gene expression vectors and of replication-competent viruses. The principle of incorporating miRNA targets into vector genomes to control their tropisms was first demonstrated for nonreplicating lentiviral and adenoviral vectors, with subsequent extension of these studies to replication-competent (oncolytic) picornaviruses, rhabdoviruses, and adenoviruses. In contrast to previous targeting approaches, miRNA targeting looks set to be applicable across the entire spectrum of viruses and gene expression vectors. Here we provide a critique of the literature relevant to this new and rapidly developing field of endeavor. We also examine the possibility of engineering viruses for expression of tropism-regulating miRNAs.

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INTRODUCTION

The engineering of designer viruses for use as viral vaccines, expression vectors, and as oncolytic viruses has been underway for many years. Engineering has focused predominantly upon targeting tissue tropism to specific cells/tissues, limiting/enhancing viral immunogenicity, increasing potency, and decreasing toxicity all to suit the specific virus and application.

Regulation of virus host range is of particular importance. For best therapeutic benefit, gene therapy vehicles should be targeted specifically such that they transduce or infect target cells while avoiding sequestration in other organs or toxicity from infection of unwanted cells. Many methods have been used to target the tissue tropism for gene therapy. However, many of these apply only to nonreplicating vectors and almost all tend to be viral class specific (**Figure 1**).

Current methods to target tropism include transcriptional targeting whereby host transcription factors are employed to select for specific tissues or cell types,[1] transductional targeting,[2] whereby viruses are modified to be selective for specific cells at the level of entry and translational targeting that exploits defective interferon (IFN) signaling in cancer cells.[3] Though all the aforementioned modalities prove very efficacious under certain circumstances, they are decidedly lacking in a number of ways.

Transcriptional targeting applies only to viral vectors that rely upon the host DNA polymerase for replication purposes and is not applicable to a large majority of emerging vectors, as they are often RNA viruses driven by viral RNA–dependent polymerases. Transductional targeting is theoretically possible for all viruses, but requires a large amount of space to accommodate coding sequences for retargeted attachment proteins within the viral genome and is often extremely inefficient. Translational targeting has only been applied to vectors employed for cancer gene therapy, and only when there is a defective IFN response within the particular cancer.

All targeting paradigms to date have been tailored very specifically to particular, singular vector systems. Very few, if any, targeting methods can be applied to all viral vectors, replicating or otherwise. Targeting viruses to be microRNA (miRNA) responsive, however, may be the first blanket method of altering tissue tropism. miRNA targeting involves engineering the viral genome to contain miRNA target (miRT) elements that can then be recognized and regulated by endogenous cellular miRNAs or, possibly, viral miRNAs. Viruses of each Baltimore class should be susceptible to miRNA-mediated attack, though at different places within the viral life cycle. miRNA-mediated targeting should avoid any size restrictions, as miRT elements are not traditionally in excess of 24 nt. While miRTs could possibly decrease antigen presentation that could dampen an antiviral immune response, miRNA targeting should appease many safety concerns such as those arising when modifying viruses by transductional means (which theoretically can increase pathogenicity by expanding host range to cells that are not normally susceptible to viral infection) because tropism is being restricted. Here we describe the strategies that have and can be used to engineer viruses to be recognized and regulated by miRNAs.

miRNAS: BIOGENESIS AND REGULATORY FUNCTIONS

miRNAs are ~22-nt regulatory RNAs that act post-transcriptionally to influence a diverse and expansive array of cellular functions. First identified in *Caenorhabditis elegans* for their role in specifying cell fate,[4] miRNAs are now known to act, among other functions, in disease pathogenesis,[5] cancer,[6] and the inflammatory response.[7] Through base pairing with complementary regions [most often in the 3′ untranslated region (3′UTR) of cellular messenger RNA (mRNA)], miRNAs can act to suppress mRNA translation or, upon high-sequence homology, cause the catalytic degradation of mRNA.

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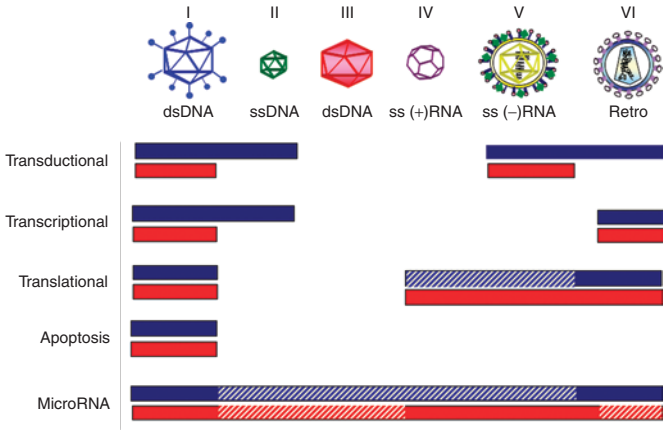

Figure 1 Targeting techniques applicable by viral class (where I–VI indicate Baltimore classification). Blue bars represent efficient targeting in replication-defective vectors. Red bars represent efficient targeting in replication-competent viruses. dsDNA, double-stranded DNA; ssDNA, single-stranded DNA.

Cellular miRNAs are derived from RNA polymerase II (pol II) transcribed RNA in the nucleus, most often from intronic or UTRs of mRNA.[8] miRNAs originate from larger precursor molecules characterized by a requisite secondary structure referred to as the primary miRNA that includes an imperfect ~80-nt stem loop (**Figure 2**).[9] This secondary structure is recognized and cleaved by the nuclear RNase III Drosha when coupled with its essential nuclear cofactor DGCR8, and this cleavage gives rise to a precursor miRNA, essentially a ~60-nt hairpin loop.[10] The precursor miRNA is then exported out of nucleus by the exportin-5 pathway[11] and is recognized and cleaved by a second cellular RNase III, Dicer.[12] Dicer cleavage liberates a duplex 20–24-nt RNA intermediate (double-stranded miRNA), and helicase activity of the Dicer complex then allows the incorporation of one of the RNA strands (typically the one with greater 5′ thermal instability) into the RNA-induced silencing complex (RISC).[13] This RNA (the mature miRNA), then acts to guide the recognition of target mRNAs,[14] while the "passenger strand" is degraded.

Sequence complementarity in the 7-nt "seed region" [base pair (bp) 2–8 of the miRNA] is essential for recognition of miRNA and its target.[12] In humans, it is predicted that there are >400 miRNAs,[15] many of which can be expressed in excess of 1,000 copies/cell.[16] A large number of these miRNAs are differentially expressed in different tissues and cell lineages such that they regulate tissue-specific gene expression.[17]

Lately, much focus has come upon the interplay between viruses and miRNAs. Well known for their ability to act in the antiviral response of plants and invertebrates, miRNAs were first assumed to play a similar role in higher metazoans. Debate is still ongoing, however, as to the role of mammalian miRNAs in the antiviral response: some claim definite antiviral activities for host miRNAs[18,19] while other carefully conducted studies dispute this claim.[20,21] It has been proposed that viruses are under selective pressure to avoid sequence homology to cellular miRNAs in tissues in which they replicate.[22] However, at least one virus has been shown to utilize a tissue-specific miRNA to enhance its replication in target tissue.[23]

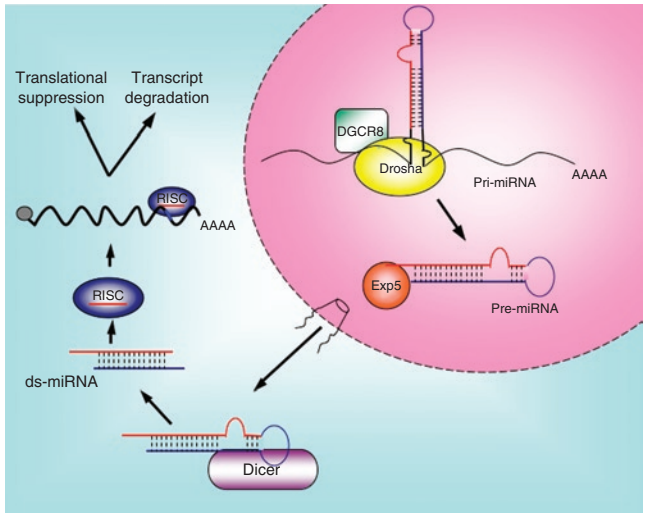

Figure 2 Biogenesis and processing of human microRNAs (miRNAs). DGCR8, DeGeorge syndrome critical region 8 (protein); ds-miRNA, double-stranded microRNA; Exp5, exportin-5; pre-miRNA, precursor microRNA; pri-miRNA, primary microRNA; RISC, RNA-induced silencing complex.

Because of the highly differential tissue expression of many miRNAs, it was proposed that cellular miRNAs could be exploited to mediate tissue-specific targeting of gene therapy vectors.[24] By engineering tandem copies of target elements perfectly complementary to tissue-specific miRNAs within viruses and vectors, multiple groups have shown that host miRNAs can regulate exogenously introduced transgene expression[24,25] and even viral gene products.[26–28] These demonstrations not only show the potential for miRNA-mediated direction of tissue tropism, but lend credence to the potential ability of miRNAs to act on mammalian viruses *in vivo*, though viruses may then be evolutionarily directed to avoid sequence complementarity.

Target sequences for cellular-encoded miRNAs have recently been used in the design of targeted gene therapy vectors and safer oncolytic viruses with strong initial results. Viral miRNAs, however, have not yet been utilized in the design of engineered viruses. With road maps emerging from basic science, it is likely that virally encoded miRNAs could soon be used in gene therapy applications.

Here, we examine ways in which miRNAs have and can be employed to target viral tissue tropism and examine the feasibility of utilizing virally encoded miRNAs to decrease viral immunogenicity, as well as increase persistence of gene expression for improved therapeutic benefit of replacement gene therapy.

VIRUSES AND RNA INTERFERENCE

In many ways, miRNA-mediated silencing might be called the endogenous cellular RNA interference (RNAi). Once outside the nucleus, miRNA biogenesis becomes virtually indistinguishable from RNAi.[29] The largest difference between endogenous miRNAs and introduced small interfering RNAs (siRNAs) is in the amount of homology that directs RISC to act on a target. miRNAs often interact with much less homology to suppress the translation of cellular mRNA, while siRNAs are designed to be perfectly complementary to the target such that transcript cleavage is more likely to occur.

In fact, RNAi has been optimized using information derived from the cellular processing of miRNAs. While first-generation gene silencing used transfected si/shRNAs,[30] suppression of gene expression was too transient for many systems. Gene transfer vectors encoding short hairpin RNAs (shRNAs) driven by pol III promoters formed second-generation gene-silencing systems.[31] However, shortly thereafter it was found that the use of pol II promoters as used in miRNA biogenesis were often equally, if not more, efficient in functional gene silencing.[32] Now in addition to transcribing shRNAs in the same method as miRNAs, expression cassettes containing the flanking regions of well-characterized miRNAs are often used to further enhance the efficacy of exogenously introduced shRNAs.[33]

With the great success of RNAi in functional analyses, it followed that RNAi could work equally well as an antiviral therapy. *In vitro*, it appears that all viruses may be susceptible to RNAi.[34] With almost every class of virus now having been tested (and antiviral effects seen in all cases), it has become clear that RNAi does inhibit viral replication. Because of this, it was thought that siRNAs might be delivered transiently for acute viral infections (particularly for respiratory viruses) and retro/lentiviral vectors might provide a method to counteract effects of the persistent infections of hepatitis B virus, hepatitis C virus, and human immunodeficiency virus for clinical benefit. And indeed, there are clinical trials underway that employ RNAi for these very means.[35]

Despite *in vitro* success with RNAi as an antiviral therapy, many barriers exist to true therapeutic benefit. Foremost among these is viral escape from siRNAs. It is now clear that viruses can escape siRNA silencing, not just through mutation to avoid sequence complementarity to the introduced antiviral siRNAs,[36] but also by altering the secondary structure around the areas in which there is homology between virus and siRNA.[37] In addition to escape, toxicity and delivery are barriers to the success of RNAi as an antiviral therapeutic. These same challenges encumber the use of miRNAs for targeting means in gene therapy.

RANGE OF TARGET ELEMENTS

Target elements to multiple miRNAs engineered within a single vector can increase gene silencing in a single cell lineage[38] or act to silence in multiple tissues.[39] The exact target of choice becomes very specific to the cell type and tissue one wishes to focus upon. Most tissues such as brain, lung, spleen, liver, and others have multiple miRNAs that are much more abundant than in any other cell type (**Table 1**). Skeletal muscle, for example, has an increased abundance of miR-1, miR-133a, and miR-206 over all other cell types.[40] Heart muscle is differentiated by increased expression of the three skeletal muscle miRNAs, as well as an additional heart-specific miRNA, miR-208.[41] Every tissue has a unique miRNA expression profile that can be used as a database for the design of targeted gene therapy vectors.

In addition to having tissue-specific signatures, miRNAs are also known to, in certain instances, have cancer-specific signatures (**Table 2**). Oncogenic miRNAs are found to be highly enriched in tumors, while tumor suppressor miRNAs are ubiquitously expressed in normal tissue, but specifically downregulated in cancer.[42] miRNAs can even be associated with the exact stage of a cancer, some perhaps acting to suppress metastasis.[43]

Table 1 Tissue-specific miRNA expression

Tissue/lineage	miRNA	Reference
Brain	miR-124a,b, miR-125, miR-128, miR-132, miR-134, miR-135, miR-138, miR-153	17,63–66
Colon	miR-143, miR-194	67,68
Heart	miR-1, miR-133a, miR-206, miR-208	41,68
Hematopoietic	miR-142 5p, miR-142 3p, miR-181, miR-195, miR-221, miR-222	68–70
Kidney	miR-192, miR-194, miR-204, miR-215, miR-30b,c	64,71
Liver	miR-122a, miR-152, miR-199, miR-215	64,67,72
Lung	miR-130, miR-24, miR-32	64
Ovary	miR-189	68
Pancreas	miR-216, miR-375	73,74
Skeletal muscle	miR-1, miR-133a, miR-206	68
Spleen	miR-127, miR-150, miR-151, miR-212	17,64,67
Stomach	miR-148	67
Testis	miR-204	68

Table 2 Oncogenic and oncosuppresive miRNAs

Cancer	miRNA	Reference
Breast cancer	miR-125b,[a] miR-145,[a] miR-21,[a] miR-155[a]	75
Burkitt's lymphoma	miR-155[a]	76
Chronic lymphocytic leukemia	miR-15,[a] miR-16[a]	77
Colorectal cancer	miR-143,[a] miR-145[a]	78
Glioblastoma	miR-21,[b] miR-221,[b] miR-128,[a] miR-181[a]	79,80
Hepatocellular carcinoma	miR-125,[a] miR-199,[a] miR-224,[b] miR-18[b]	81
Neuroblastoma	miR-9[a]	82
Thyroid carcinoma	miR-146,[b] miR-221,[b] miR-222[b]	83
Lung cancer	let-7,[a] miR-17–92[b]	84,85

[a]Downregulated (oncosuppressive). [b]Upregulated (oncogenic).

Tumor suppressor miRNAs are known to be downregulated (or completely deleted) in breast,[44] lung,[45] and colorectal cancers[46] and expression of perhaps the most well defined of the tumor suppressor miRNAs, miR-15 and miR-16, are altogether lost in chronic lymphocytic leukemia.[47] Incorporation of tumor suppressor target elements within a vector could restrict expression in normal, untransformed tissue while allowing expression in tumors lacking these miRNAs. Targeting by this means could theoretically be transferred to a vector with toxicity to any cell type, providing a potential ubiquitous new way of conferring specificity.

While miRNAs almost always act to mediate post-transcriptional silencing, in at least one case, viral replication has actually been shown to be contingent upon a cellular miRNA.[23] Hepatitis C Virus replicates exclusively in the liver. While *in vivo* this was attributed to liver-specific receptor expression, virus propagation *in vitro* that circumvented virus–receptor interactions (by way of replicon RNA transfection) was still exceedingly difficult. It has

now been shown that the liver-specific miR-122 positively affects the accumulation of hepatitis C virus RNA.

While a mechanism for this positive regulation by miR-122 has not yet been defined, the concept has large implications for the design of miRNA targeted vectors. Oncogenic miRNAs could mediate the targeting of cancer-specific vectors and the wide database for tissue-specific miRNAs could now not only be used for vector restriction from those tissues, but actual targeted expression.

DESIGNING THE REGULATORY INSERT

Rough outlines are beginning to emerge on the best way to employ miRNAs for targeting (or restriction purposes). It has been shown that while four tandem copies of a single target work better than two copies or one copy, it does not hold that increasing numbers of targets will always translate to increased gene silencing.[39] In actuality, increasing the number of miRTs within a single transcript has been shown to cause a decrease in miRNA function.[48] In these cases, multiple RISCs, all guided by the same miRNA guide strands may bind to a single transcript creating what some have termed to be a "sponge" effect, whereby miRTs sequester all-sequence complementary miRNAs, thus severely impairing function. miRNA-mediated suppression of vector-delivered gene expression substantially decreases in this situation and may be more pronounced in the case of imperfect complementarity between target and miRNA. In addition, the normal function of these cellular miRNAs can be completely inhibited. In what may be an extreme example, impaired endogenous miRNA function by oversaturation of the exportin-5 pathway was shown to cause a fatal liver toxicity in mice.[49]

Much work remains to be done on the exact number of copies and the spacing elements between tandem copies of miRT elements that will prove most efficacious for vector targeting. In addition, to avoid problems for rescue and manufacturing, production cell lines should either lack the miRNA to which the corresponding target has been engineered or be treated with miRNA inhibitors.

Tissue-specific miRNAs are expressed at different copy numbers in different cell lineages, and it has been proposed that a threshold copy of miRNAs must be reached to achieve appreciable gene silencing.[39] miRNA abundance alone does not necessarily make a good target, however. Even when delivered in equimolar amounts, different miRNAs will regulate the expression of a perfectly homologous target engineered into the same position in a transcript to different extents.[38] While it is not clear why, the actual miRNA sequence itself does appear to affect the extent to which a target RNA is regulated. In some cases, it appears that perfect sequence matches may not be ideal.

The reigning dogma in miRNA regulation had always been the higher degree of homology between miRNA and target, the better. A perfect match in the seed sequence is obligatory, while higher degree of complementarity tends to increase the likelihood of catalytic mRNA cleavage between bp 10 and 11 by Argonaute 2 in the Dicer complex.[12] However, in the absence of a formal proof, it remains a possibility that some miRTs might prove better as imperfect matches, engineered to include miRNA/miRT mismatch or even bulge sequences. In addition to questions as to whether the ideal sequence to best regulate a vector is the position in which a target(s) should be introduced.

In nature, most miRTs have been found within the 3′UTR of cellular mRNAs. It is well known that siRNAs can be quite effective when targeted against any region of an mRNA, whether coding or not. As RNAi and miRNA regulation are quite similar, it seems likely that target elements introduced within any part of a vector genome have the potential to work, as long as the region in which it was present was actually transcribed and found outside the nucleus. Studies whereby targets were introduced within the 5′UTR of reporter mRNAs showed that there was no great difference in silencing efficiency.[50] It may be that the ideal spot for miRTs insertion relies not so much on the defined location (whether 5′ or 3′ untranslated or coding sequence), but upon the secondary structure of that location. Many studies have shown that target sequences present within regions with a great deal of secondary structure are ill recognized by miRNAs.[51,52]

miRNA-MEDIATED TARGETING OF NONREPLICATING VECTORS

Efforts to engineer viral RNA to be stably expressed or translated tissue-specifically have been underway for many years. In two examples, UTRs of two genes (*fibroblast growth factor-2* and *cyclooxegenase-2*)[53,54] were engineered into replication-competent adenoviruses to confer cancer-selective viral replication. While often the mechanism that made these RNA stability elements confer tissue specificity has been unclear, with developments in the understanding of miRNA function, it became apparent that cellular miRNAs present in some tissues presented a means to confer selective RNA stability.

With the demonstration that viruses are susceptible to RNAi-mediated attack, and with RNAi assuming more of the characteristics of true endogenously encoded miRNAs, it followed that viruses might be engineered to become miRNA responsive by engineering them to contain target elements for miRNAs.

In the first example of such engineering, it was hypothesized that poor specificity of a systemically delivered lentiviral vector caused the transduction of (hematopoietic) antigen-presenting cells such that an immune response was then generated against the delivered transgene. By engineering four perfect copies of a hematopoietic-specific miRT element within the 3′UTR of a transgene delivered by a lentiviral vector, the Naldini group was able to show that a hematopoietic-specific miRNA could recognize target elements within the transgene such that expression was squelched in hematopoietic cells, and thus no immune response to the transgene was generated.[24] This study represented the first conclusive evidence that viral vectors could be engineered such that they were restricted from expressing in cells bearing cognate miRNAs both *in vitro* and *in vivo*. They then significantly extended these findings to show that tissue-specific miRNAs from a broad array of cell types could suppress the expression of transgenes bearing sequence complementary miRTs and that there was a relationship between miRNA abundance within a cell and the extent to which that miRNA could function to suppress gene expression.[39]

Similarly, it was thought that miRNAs highly enriched in the liver could perturb severe hepatotoxicity associated with a replication-deficient adenoviral vector. Adenovirus vectors encoding the

thymidine kinase gene from herpes simplex virus can act as potent anticancer agents when used in combination with the prodrug ganciclovir.[25] By incorporating tandem copies of the miRT for the liver-specific miR-122a, it has been shown that liver expression can be reduced up to 1,500-fold without affecting tumor transduction, resulting in roughly equivalent suicide gene–mediated cancer therapy with markedly reduced liver toxicity.

miRNAs represent a new paradigm for restricting tissue tropism of viral vectors. By increasing specificity, potency can potentially be increased without the worry of toxicity from transduction of off-target cells.

miRNA-RESPONSIVENESS IN REPLICATING VIRUSES

Nonreplicating viral vectors transduce cells to express a transgene of interest aiming to mimic physiologically normal expression. In these cases, robust expression is wanted as long as it is not at the expense of normal cellular function. Replicating viruses used for oncolytic cancer therapy or for attenuated vaccines, however, act much differently. In these cases, robust viral expression is wanted to act lytically on cancer cells (*i.e.*, oncolytic virotherapy) or generate a healthy immune response (vaccines).

Replicating viruses in many cases may represent a larger hurdle in terms of gene expression, able to completely overtake cellular machinery for DNA or RNA synthesis. In addition, the design for miRNA targeting may now have to be much more complex, as many viral genes are being expressed as opposed to a single transgene in the case of nonreplicating expression vectors. While miRTs themselves may be readily transferred without sequence change among viruses of different families and thus be one of the first pantropic targeting modalities, the actual placement may need to be very specific to the virus/family.

Picornaviruses represent possibly one of the least complex viral families. With a single-stranded positive-sense (+) RNA genome, targets placed anywhere within the viral genome also become part of the single-viral mRNA. Engineering the oncolytic picornavirus, coxsackievirus A21, to contain the muscle-specific miRTs corresponding to miR-133a and miR-206 within the 3′UTR effectively abolished replication *in vitro* in the presence of cognate miRNAs.[38] In addition, it protected mice *in vivo* from developing fatal myositis associated with the wild-type virus. Because muscle-specific miRNAs were not present in melanoma and myeloma xenografts of these mice, the miRNA-targeted coxsackievirus A21 fully retained its oncolytic potency and was shown to be a fully curative therapy. In this study, target retention was shown in a majority of treated mice to be fully intact out to 45 days post-treatment (at which time no tumor remained). However, analysis of creatine kinase, a quantitative serum marker of muscle damage showed a small, but statistically significant increase in muscle damage that accumulated over time as a small number of mice mutated the miRT insert.

miRT mutation and deletion is of particular concern, and indeed has been shown in the regulation of the closely related picornavirus, poliovirus (PV). In an effort to control poliomyelitis caused by the virus, a miRNA-targeted PV that has been previously shown to be highly sensitive to the miRT insertion[26] (though mutations through extended passage could overcome miRNA-mediated regulation) was investigated.[55] It was hypothesized that incorporation of the neuronal miR-124T or the tumor suppressor let-7aT would curb viral replication in the brain, while still allowing for the generation of a protective immune response within mice transgenic for the PV receptor. Even with the incorporation of only two miRTs within the virus, PV receptor transgenic mice were shown to be completely protected from poliomyelitis when given up to 10^8 plaque-forming unit intramuscularly (intracerebral and intranasal administration were not reported), demonstrating proof of principle for the generation of safe *de novo* vaccines by way of miRT incorporation.

In a similar study, vesicular stomatitis virus was engineered to contain three copies of let-7a within the 3′UTR of the viral M gene, which is responsible for the inhibition of the cellular IFN response.[27] let-7a is a member of the let-7 family of tumor suppressor miRNAs and should act to inhibit viral replication in a broad variety of normal cells. Through incorporation of its miRT, it was thought that tumor selectivity could be generated and, indeed, the viral output was decreased by three logs *in vitro* in cells expressing the let-7a miRNA. It is possible that targeting by this method could restrict acquired neuropathology, which is found commonly in animal models of vesicular stomatitis virus oncolysis. To examine this relationship, a total of six mice were inoculated intranasally with viruses containing miRTs with perfect matches to let-7a or with a mutated miRT insert and followed for 15 days, with only one mouse in either group succumbing to what is presumed to be neurotoxicity. In this regard, it is difficult to conclude definitively that neurovirulence was affected to any appreciable extent. The ability of this miRNA to provide a more pronounced response may have been limited by the placement of the miRT within the virus, among other factors. In this example, only one of five viral genes was targeted, by a target that did not fully extinguish the expression of a reporter gene *in vitro*.

Oncolytic adenoviruses have now been engineered to be miRNA responsive as well as their replication-defective counterparts. In a recent study, incorporation of the liver-specific miR-122T within the viral gene E1A decreased E1A mRNA expression *in vitro*, which may markedly reduce liver toxicity *in vivo*, though this has not yet been examined.[28] The development of an oncolytic adenovirus with reduced hepatotoxicity may be of particular importance, as it is perhaps the furthest clinically advanced virus.

UNANSWERED QUESTIONS

Negative-strand RNA viruses (*i.e.*, vesicular stomatitis virus) might be intrinsically harder to control via miRNA targeting than other viruses such as coxsackievirus A21 or PV. Because picornaviruses contain a single (+) RNA genome, both viral mRNA and genome are potentially targeted by an miRT in the same orientation. Vesicular stomatitis virus, however, has genome and mRNA in different orientations and transcribes viral genes as distinct mRNAs.[56] In addition, the genome is protected by a ribonucleoprotein complex that surrounds the viral RNA such that RISC-mediated attack may not occur.

Viral escape may present another obstacle for the success of miRNA-mediated targeting. In this respect, many of the problems with the use of siRNA as an antiviral therapeutic may also apply to the use of miRNA targeting for replication-competent viruses.[57] Clearly, a miRNA-targeted virus whose replication is restricted in

a specific tissue should be capable of gaining a replication advantage through deletion or mutation of the miRT. Thus, the mutation rate of the viral or cellular polymerase becomes the limiting factor. In general terms, the mutation rate of viruses is such that ssRNA virus>retrovirus>single-stranded DNA virus>double-stranded DNA virus, ranging from ~10^{-3} to 10^{-8} mutations/nucleotide/genomic replication.[58]

It is hypothesized that viruses may escape the intrinsic miRNA defense mechanism found in plants by having evolved to avoid homology to miRNAs that are present within those cells in which replication occurs.[22] It is clear that viruses can escape siRNA-mediated silencing, and it has now been demonstrated that even in the absence of selective pressure viruses can escape miRNA targeting as well.[38] While increasing miRT number and sites could potentially delay the emergence of escape mutants, this might also act to form miRNA sponges that could increase toxicity and limit performance. With both attenuated viral vaccines and oncolytics, however, an immune response against the virus will often clear viremia such that escape mutants may never become apparent.

Replicating viruses present additional, alternative, and in some cases more complex obstacles for the success of miRNA-mediated targeting. However, miRNAs can provide methods for the creation of safer, improved vaccines and increase the number of oncolytic viruses that can be safely employed for cancer gene therapy.

VIRALLY ENCODED miRNAS

The utilization of cellular miRNAs for targeting the tissue tropism of gene therapy vehicles has lately garnered much attention. A large database of cellular miRNAs exists,[17] and the tissue distribution, abundance, and function of these miRNAs have been extensively investigated. Perhaps equally important, however, are virally encoded miRNAs.

Viral miRNAs identified thus far are very similar to cellular miRNAs in terms of biogenesis and function. Like cellular miRNAs, they are thought to recognize targets through imperfect homology and most often inhibit gene expression by translational suppression. However, they are more likely than cellular miRNAs to be derived from open-reading frames and intronic regions. Because all identified viral miRNAs are transcribed in the nucleus, they have all, thus far, come from double-stranded DNA viruses. Adenoviruses and polyomaviruses encode viral miRNAs, and the herpesvirus family encodes upward of 100 viral miRNAs.[59] It is improbable that RNA viruses would encode miRNAs. Many RNA viruses replicate exclusively in the cytoplasm, necessitating a different biogenesis pathway were a miRNA to be encoded. In addition, an encoded miRNA could act in an inhibitory fashion against the complementary viral RNA strand.

Viral miRNAs can mediate the regulation of both cellular and viral gene expression, to different ends. To mediate latency, viral miRNAs have been shown to downregulate the expression of specific viral proteins. SV40, for example, downregulates the expression of the large T antigen to avoid the adaptive immune response.[60] By reducing expression of large T antigen, presentation to cytotoxic T lymphocytes is reduced, as is the release of cytokines such as gamma IFN. Similarly, herpes simplex virus-1 has recently been shown to downregulate the expression of the ICP0 and is thought to act to establish viral latency through this means.[61] In addition, it is thought that viral miRNAs can act in tumorigenesis and may influence the immune response to viral infection.[62]

What the viral miRNAs are theoretically engineered to encode is as important or more important than what they do encode. Just like cellular miRNAs, viral miRNAs have the potential for acting on cellular gene expression, as well as viral gene expression. Viruses have evolved proteins that act to inhibit or evade the host immune response or to prevent the infected cell from activating its apoptotic machinery. In many cases, these innate immune modulatory and apoptosis inhibitory proteins represent targets for viral engineering to generate recombinants that are unable to suppress innate immunity or apoptosis and which, therefore, replicate exclusively or selectively in tumor cells.

It seems likely that viral miRNAs could be designed against many targets (both cellular and viral) to enhance the performance of oncolytic viruses. Potential targets that would limit natural killer–mediated recognition limit viral antigen presentation, control the IFN response, suppress inflammatory cytokine release, and inhibit cellular apoptosis, could prove to be of particular importance (**Figure 3**).

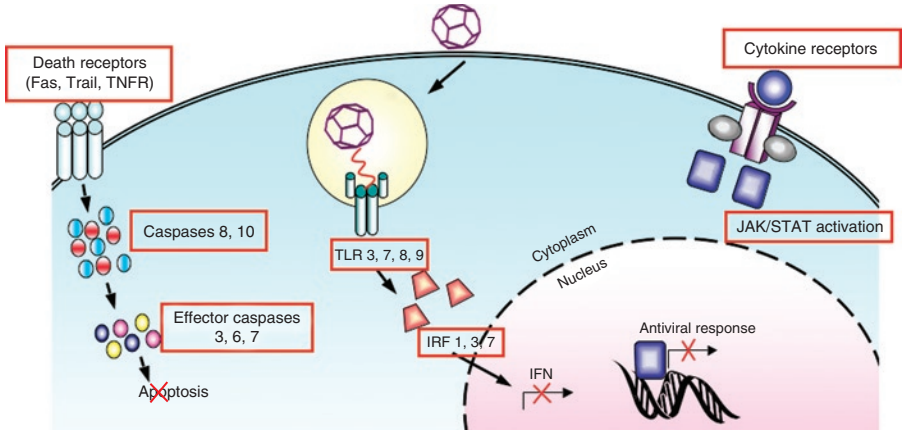

Figure 3 Potential targets of viral microRNAs. Red boxes represent potential targets. IFN, interferon; TLR, Toll-like receptor; TNFR, tumor necrosis factor receptor; TRAIL, tumor necrosis factor–related apoptosis-inducing ligand.

CONCLUSIONS

miRNA targeting is an effective approach for controlling the stability of vector-encoded mRNAs and of certain RNA viral genomes and is, therefore, rapidly emerging as a versatile new targeting strategy. The approach requires <100 bases of new vector sequence and, in contrast to previously developed targeting strategies, is potentially transferrable to all known viral and nonviral expression vectors as well as replication-competent viruses. Therefore, it is expected to be of major utility for the generation of targeted expression vectors, tumor-specific oncolytic viruses, and attenuated viral vaccines derived from serious pathogens. Because cellular miRNAs are not all equally abundant, and because flanking sequences influence the efficiency of target destruction, the precise molecular approach to miRNA targeting must be subtly different for each new vector or virus. Trial and error is, therefore, a necessary step at the current time in order to obtain optimal control of expression. Numerous miRTs exist, encompassing oncogenic (tumor-selective), oncosuppressive (selectively expressed in nontumor tissues), and tissue-specific miRNAs. These targets are already well characterized and most are highly conserved, even across species barriers. Therefore, it seems realistic to expect this targeting approach can be validated in disparate species, a distinct advantage over transductional targeting where targeting moieties against human cells are unlikely to function in nonhomologous species. One concern with the approach is that viruses can revert to their original tropism by acquiring mutations in their miRT sites, but such revertants are considered unlikely to be of clinical significance because they emerge late after the initial virus challenge by which time they must contend with a fully developed antiviral immune response.

In the future, exploitation of both cellular and viral miRNAs will likely be of great benefit for targeting gene therapy viruses and vectors, generation of new and safer vaccines, and, in addition, could provide a modality for increasing the persistence of nonintegrating gene therapy vehicles and the evasion of innate immunity.

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