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REVIEW

MicroRNA-regulated viral vectors for gene therapy

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Abstract

Safe and effective gene therapy approaches require targeted tissue-specific transfer of a therapeutic transgene. Besides traditional approaches, such as transcriptional and transductional targeting, microRNA-dependent post-transcriptional suppression of transgene expression has been emerging as powerful new technology to increase the specificity of vector-mediated transgene expression. MicroRNAs are small non-coding RNAs and often expressed in a tissue-, lineage-, activation- or differentiation-specific pattern. They typically regulate gene expression by binding to imperfectly complementary sequences in the 3′ untranslated region (UTR) of the mRNA. To control exogenous transgene expression, tandem repeats of artificial microRNA target sites are usually incorporated into the 3′ UTR of the transgene expression cassette, leading to subsequent degradation of transgene mRNA in cells expressing the corresponding microRNA. This targeting strategy, first shown for lentiviral vectors in antigen presenting cells, has now been used for tissue-specific expression of vector-encoded therapeutic transgenes, to reduce immune response against the transgene, to control virus tropism for oncolytic virotherapy, to increase safety of live attenuated virus vaccines and to identify and select cell subsets for pluripotent stem cell therapies, respectively. This review provides an introduction into the technical mechanism underlying microRNA-regulation, highlights new developments in this field and gives an overview of applications of microRNA-regulated viral vectors for cardiac, suicide gene cancer and hematopoietic stem cell therapy, as well as for treatment of neurological and eye diseases.

Key words: MicroRNA; MicroRNA regulation; MicroRNA target sites; Viral vectors; Adeno-associated virus; RNA interference; Gene therapy; Vector targeting

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Core tip: Post-transcriptional microRNA-induced suppression of gene expression is a simple new, highly efficient technology to restrict transgene expression to a specific tissue. It is based on the insertion of a target sequence for a cell-specifically expressed microRNA, typically into the 3′ untranslated region of a transgene expression

cassette. MicroRNA-induced regulation can result in an up to 100-fold reduction of transgene expression in tissues where expression is not desired. This targeting strategy can be used in combination with other targeting strategies to further improve vector specificity for gene therapeutic approaches.

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INTRODUCTION

Tissue-specific targeting of viral vectors is a key requirement for safe and efficient gene therapy. However, many viral vectors transduce a broad range of cell types. Improvement of specificity can be achieved by direct vector application into a defined location within the organ. But in general, transgene delivery by this means remains near the injection site and the application can lead to tissue injury, making this procedure unsuitable for many applications[1,2]. Other targeting strategies focus on improvement of target specificity after systemic vector application. In this regard, several approaches of transductional targeting have been successfully employed, including switching the virus serotype and capsid engineering *via* directed evolution, creation of vectors with chimeric or mosaic structures and insertion of antibodies or bi-specific fusion proteins containing the targeting ligands[3-5]. However, being based on the various natural entry mechanisms of infection of many viruses, this strategy has limitations. Transcriptional target-specific expression of the transgene using tissue-specific promoters represents another frequently used approach to improve vector specificity. Although this targeting strategy has been used successfully in many applications, it is limited by the small number of promoters whose activity is exclusively restricted to the target tissue and sufficiently strong to induce transgene expression at therapeutic levels[6,7]. Based on discovery of the RNA interference mechanism enabling post-transcriptional suppression of gene expression[8], microRNA-dependent suppression of transgene expression has been emerging as a promising new approach to improve vector targeting. Since its initial application for reduction of off-target transgene expression in antigen presenting cells (APC)[9], microRNA-dependent control of transgene expression has been used to restrict transgene expression in gene therapeutic approaches, to control oncolytic viruses, for live attenuated virus vaccine development and basic research. In this review we will provide an overview of this technology, its application in gene therapy and discuss perspectives for its further development.

MICRORNAS - IMPORTANT PLAYERS IN CELLULAR GENE REGULATION

MicroRNAs are small endogenous non-coding RNA sequences of approximately 21 bp that post-transcriptionally regulate gene expression of about more than 60% of human protein-coding genes[10,11]. They are involved in most of cellular processes, including development, differentiation, proliferation and apoptosis[12-16]. MicroRNAs are highly conserved between species and expressed specifically and at certain levels dependent on tissue, lineage or differentiation state[17]. More than 2500 unique mature human microRNAs have been identified so far (http://www.mirbase.org/). Individual microRNA species can vary widely in copy number ranging from less than 10 to more than 30000 copies per cell[18-20]. Beside the tissue-specific expression profile, several microRNAs are dysregulated in cancer[21,22], infectious diseases[23] or diseases of the heart[24] and liver[25], which gives them potential as targets for new therapies.

MicroRNAs are usually processed from a precursor molecule (pri-microRNA) that folds into hairpin structures with imperfectly base-paired stems. Pri-microRNAs are further processed by nuclear and cytoplasmatic cleavage proteins, resulting in a short RNA duplex (see references for greater detail[10,26]). One strand of the duplex, the guide strand (microRNA), is selected based on the relative free energies of the microRNA duplex ends[27,28] and is loaded into a multi enzyme complex, the RNA-induced silencing complex (RISC). The less common product is defined as the passenger strand (microRNA*), and is assumed to be degraded[26]. Alternatively both strands of the RNA duplex, namely the 5' strand (miR-5p) and the 3' strand (miR-3p) become mature functional microRNAs[29-33]. The mature microRNA is associated with an Argonaute (AGO) family protein, that constitutes the core of the RISC, and functions by base-paired binding to the corresponding target site located in the mRNA, resulting in repression of protein synthesis[10]. However, some microRNAs are able to activate mRNA translation[34-38]. Complete complementarity of microRNA and its target site leads to endonucleolytical central cleavage of the microRNA/mRNA-duplex by AGO2[26,39-43], using a mechanism similar to RNA-interference mediated by siRNAs. In plants, this mechanism is the predominant one[44], while the prevalent mechanism in animals involves binding with incomplete complementarity[12], resulting in inhibition of translation and/or initiation of mRNA degradation[10,39,45-50]. In contrast to plants, whose microRNA target sites are mostly located in protein-coding regions, target sites in animals are often found as repeats in the 3' UTR of the mRNA[51-54].

Some important rules relating to the interaction between microRNA and its target site were determined by experimental and bioinformatic analysis. The mRNA targeting specificity of a microRNA is determined by perfect match between the seed sequence, a conserved

sequence which is usually situated at positions 2-7 or 2-8 of the 5'-end of the microRNA, with a corresponding sequence in the mRNA[12,51-54]. An adenine opposite microRNA position 1 and an adenine or uracil opposite microRNA position 9 are not essential, but increase the efficacy of binding[54]. MicroRNAs exhibiting the same seed sequence belong to the same microRNA family and can regulate the same mRNA targets[51]. A single microRNA species can regulate the production of hundreds of proteins, most likely by recognizing the same seed-matched sequence in the mRNA[55]. A second common characteristic of endogenous microRNA-mRNA interaction comprises nucleotide mismatches in microRNA positions 9-12, thereby most likely preventing an AGO2-mediated cleavage of the target mRNA[10,56]. A third characteristic is that matches within the seed region alone are not always sufficient to induce gene repression; stabilization of microRNA binding may need additional complementarity in the 3' part of the microRNA. In particular if seed matching is suboptimal, nucleotides at microRNA positions 13-16 become important[51,57]. Thus the 3' portion can help to compensate for a single nucleotide mismatch in the seed region, as experimentally confirmed for let-7 sites in Caenorhabditis elegans lin-4[58] and for miR-196 site in mammalian Hoxb8[43] mRNA. Additional factors can influence binding stability and thereby the efficacy of microRNA-mediated gene regulation[54,57,59,60]. Thus a more effective repression can result from an AU-rich nucleotide composition near the target site[57]. Additionally, more than 15 nucleotides between miR-TS and the stop codon may reduce competition between proteins involved in translation and microRNA-mediated silencing, respectively[57].

DISCUSSION

Engineering, optimization and limits of artificial target sites for microRNA-mediated post-transcriptional transgene silencing

Endogenously expressed microRNAs can also be used to specifically modulate the expression of an exogenously applied nucleic acid as a therapeutic cDNA. Therefore artificial microRNA target sites, referred to as miR-TS in this review, serve as targets for a specific microRNA, resulting in post-transcriptional silencing of the transcript[9] (Figure 1). In contrast to transcriptional targeting using tissue-specific promoters that positively regulate transgene expression, expression is negatively controlled by tissue-specifically expressed microRNAs. It was shown that insertion of miR-TS completely complementary to the microRNA in an arrangement of multiple tandem repeats of miR-TS results in strong repression of transgene expression in cells with corresponding microRNA expression[9,17,61]. In this case the transcript is endonucleolytically cleaved, similar to degradation by siRNAs, and the microRNA-RISC is rapidly recycled[39]. Usually the microRNA guide strand mediates transgene repression, thus a corresponding sequence representing the miR-TS is inserted into the transgene expression cassette. However, recently Kim *et al*[62] discovered that expression of a therapeutic transgene can also be regulated by the passenger strand of a microRNA (miR-122) linked to the transgene, thereby eliminating the risk of affecting expression of endogenous microRNA guide strand-regulated genes.

There are several factors influencing the efficacy of microRNA-regulated transgene expression systems. First the cell type- and tissue-specific expression of a chosen microRNA candidate is an important factor that must be taken into account for microRNA-regulated transgene expression systems. Many suitable microRNAs fulfilling this requirement have already been defined (please see reference[63,64]). Among them miR-122, a microRNA, almost exclusively expressed in liver tissue, represents the best candidate for a specific hepatocyte-de-targeting[20,65,66]. Indeed, successful miR-122-mediated suppression of transgene expression in liver was found in several studies[67-69]. However, specific microRNA expression may not be sufficient to achieve a stringent microRNA-dependent transgene repression.

Second, the expression level of a microRNA is crucial for microRNA-mediated regulation. High-throughput assays revealed that only the most abundant microRNAs within a cell mediate significant target suppression[70]. Consequently a fraction of microRNAs that is highly and specifically expressed in certain tissues, possibly involved in defining and maintaining tissue identity[71], are the most potent to de-target transgene expression. In general more highly expressed microRNAs repress their targets to a greater degree[67]. However, the relationship between microRNA expression and its repression activity is nonlinear; in one case it was shown that a 10-fold increase of microRNA concentration results only in a 10% increase of repression[72]. In addition, microRNA expression level is not the only determinant of repressive activity and repression capacity can differ among microRNAs. Kozomara *et al*[72] showed that although some microRNAs exhibit similar expression levels, they mediate very different repression levels. Conversely some microRNAs repress their targets to similar degrees despite of large difference in expression levels. Another key factor for microRNA-mediated repression of a miR-TS-containing transcript is the need for a certain minimal microRNA expression threshold, quantified as 100 copies/pg small RNA by Brown *et al*[17]. The value is not only important because it defines the minimal amount which is necessary to suppress transgene repression but it also indicates that under a certain level, microRNAs are unable to induce repression. This consideration becomes important if the chosen microRNA is expressed in both the tissue where transgene expression is intended as well as in the tissue where it is not. Therefore microRNAs expressed under the minimal expression limit may not affect transgene expression in the targeted tissue.

Third, the configuration of the miR-TS is another important factor affecting repression efficacy. Corre-

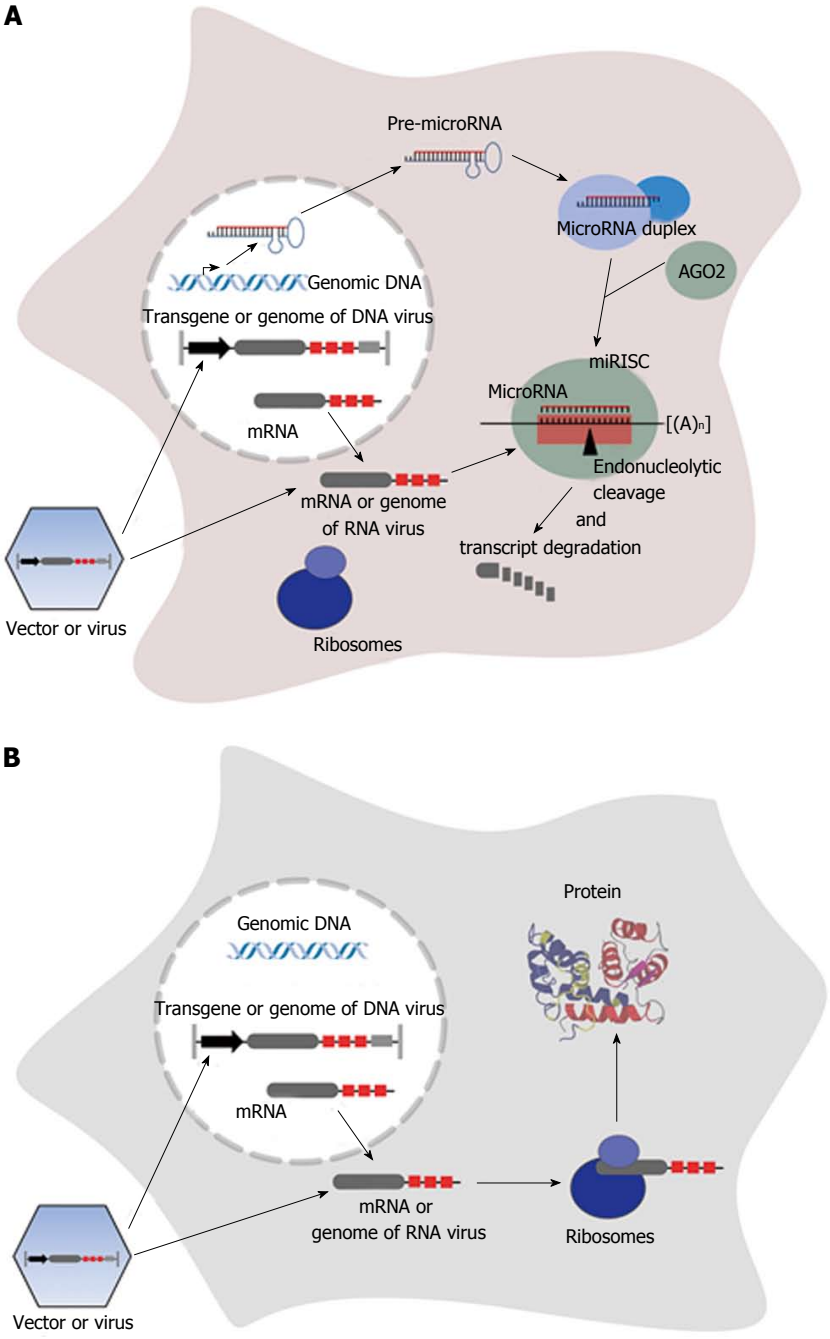

Figure 1 Principle of microRNA-mediated suppression of transgene expression and viral replication. After transduction/infection of a cell, vector DNA/viral DNA is transcribed in the nucleus (for lentiviral vectors integrated into host DNA). The transcript/viral RNA containing artificial miR-TS (red boxes) is transported into the cytoplasm. If corresponding microRNA is expressed (A), it binds to miR-TS and the target RNA is endonucleolytically cleaved and degraded. If certain microRNAs are not expressed (B), the RNA is translated into protein. The protein shown in the three-dimensional structure is dimeric S100A1[180]. AGO: Argonaute; miRISC: MicroRNA-induced silencing complex.

sponding to the different mechanism of microRNA-induced gene silencing, complete complementary miR-TS allow *per se* higher suppression of the transgene than imperfectly complementary sequences[33,61,73-75]. As perfectly complementary target sequences are endonucleolytically cleaved between microRNA positions 10/11, the microRNA is rapidly recycled[39,69,76-78]. Consequently complete complementarity reduces the risk of microRNA saturation and thus induction of undesirable side effects because the bioavailability of the cognate microRNA to regulate its natural targets is maintained[79].

In addition, the number of miR-TS repeats affects microRNA-mediated suppression of transgene expression. In general an increased number of miR-TS enhances microRNA-dependent transgene repression. But repression efficacy does not linearly correlate with an increasing number of miR-TS, and above a certain number of miR-TS, only a relatively modest increase of repression of transgene expression is observed[67,73,80]. Thus maximal suppression of transgene expression of

viral vectors, even under optimal conditions (highly and specifically expressed microRNA, use of tandem miR-TS repeats), rarely exceeds 100-fold[9,61,67,68,81]. The reason for this limitation is currently not fully understood, but different factors may be involved. It seems that the rate-limiting steps in microRNA activity are the level and dynamics of association of the target mRNA with the microRNA-loaded RISC, rather than level of target transgene mRNA[17,72,82], which is directly dependent of vector and expression systems. We and others have shown that 3-4 identical repeats of miR-TS are sufficient for efficient transgene repression (Table 1). An increase to 6 or even 12 copies is possible, but may only have a marginal effect on repression efficacy[67,80,83]. Moreover, an increase of miR-TS repeats can induce saturation of the respective microRNA, as more target sites means more substrate for the microRNA to bind, resulting in potentially undesirable off-target effects[76]. As shown by several studies, different miR-TS can be inserted in series in order to de-target various cell types with different microRNA expression profiles[17,61,69,84]. On the other hand, a combination of miR-TS corresponding to different microRNAs for a given cell type or tissue can enhance microRNA-mediated transgene suppression, especially if the microRNAs are only expressed at moderate levels[85]. Moreover, employing cooperative microRNAs may reduce the risk saturating the function of one microRNA[67]. An enhancement of microRNA-mediated suppression of transgene expression can also be achieved by combination of microRNA regulation with other regulation systems. Thus, Bennett *et al*[86] developed an adenoviral vector (AdV) system utilizing both microRNA-mediated regulation and the Cre recombinase (Cre)-loxP recombination system to further reduce hepatic transgene expression and to achieve relatively pure spleen-/dendritic cell-specific expression thereby improving AdV-based vaccination against transgene products. Although insertion of miR-122TS into the transgene expression cassette of AdV suppressed liver expression by about 100-fold, significant transgene expression levels were still found in the liver[86,87]. Therefore they engineered an AdV carrying the transgene flanked by loxP sites and containing miR-122TS, while another AdV expressed Cre equipped with miR-TS for miR-142-3p, which is highly expressed in the spleen. Intravenous co-administration of the AdVs resulted in an approximately 3800-fold reduction of hepatic transgene expression and a slightly reduced, but substantial, transgene expression in the marginal zone of the spleen, which was sufficient to exhibit therapeutic value[86]. In most studies, small 4 to 6 nucleotide long spacer sequences have been used to separate miR-TS repeats[9,61,67,68]. Introduction of spacers may, in general, reduce steric hindrance of enzyme complexes binding to microRNA/miR-TS duplexes and thus facilitate better repression. Nevertheless spacer-free multimeric miR-TS were also successfully applied for transgene repression[69]. Furthermore it is thought that, compared to longer spacers, insertion of shorter spacers might reduce the risk of formation of secondary structures around the miR-TS that might disturb base-paring of microRNA with the miR-TS[88,89]. Thus whether and to what degree spacer sequences affect efficacy of designed microRNA-regulated suppression systems remains to be elucidated. Besides the functional importance of applied miR-TS, copy number of miR-TS, as well as spacing between miR-TS, are relevant to viral vector construction, as it directly enlarges the transgene expression cassette inserted into the vector genome. As miR-TS are small in size (about 22 bp), insertion of tandem repeats of miR-TS, including spacer sequences, generally does not constitute a capacity problem for most viral vectors. However, for some viral vectors with low packaging capacity, such as self-complementary adeno-associated virus (AAV) vectors[90], keeping down the total length of miR-TS could be an essential requirement. In this regard, our group investigated whether shortening of miR-TS corresponding to miR-122 affects the miR-122-mediated repression function. Indeed, we found that a deletion of up to 5 nucleotides from the 5′ end of the miR-122TS was well tolerated and did not influence transgene suppression[67]. However, more studies are necessary to confirm our results for other miR-TS.

Although a systematic investigation defining common standards for insertion of miR-TS into a transgene expression system is currently not available, location of the miR-TS in the mRNA is obviously important for repression efficacy. Based on knowledge of microRNA target recognition and microRNA-induced cleavage of cellular targets, it becomes obvious that secondary structures of the mRNA itself or structures formed by miR-TS insertion can also affect transgene suppression as accessibility to the target mRNA is hindered[78,91]. Since the 3′ UTR of mRNAs almost always lack secondary RNA structure[92], miR-TS can be placed there, usually near the stop codon, but insertion within the 5′ UTR or the open reading frame is also possible[93-96]. Other factors influencing repression capacity of a given microRNA include increased nuclear localization of a microRNA, its stability[97] and the stability of the microRNA/miR-TS duplex[70,72]. Concerning the latter, analysis of the free energy of multiple duplexes revealed a weak negative correlation of duplex stability and microRNA-mediated regression. Therefore lower stability seems to favor transient interaction with target mRNAs and efficient down-regulation of multiple targets[39,72].

In summary, optimal microRNA-mediated repression of the vector-encoded transgene needs high expression of the microRNA, insertion of 3-4 copies of miR-TS with complete complementarity, uniqueness with regard to regulation by other microRNA candidates and insertion within a site with low secondary structure. All these aspects need to be pre-evaluated for optimal microRNA-mediated regulation of transgene expression of viral vectors.

Although this technology displays many advantages compared to other methods, it is not without concerns. Given that endogenous microRNAs are involved in target regulation, miR-TS containing transgene mRNAs

Table 1 Application of microRNA-regulated vectors for gene therapy

Target cell/tissue for therapy	miR-TS for	Number miR-TS	Vector	MicroRNA-regulated transgene	De-targeted cell/tissue	Ref.
Heart						
Heart	miR-122	3, 5	AAV9	EGFP, lacZ, luciferase	Liver	[67,68]
Heart	miR-122	3	AAV9	Human S100A1	Liver, skeletal muscle	[61]
	miR-206	3				
Cancer						
PyMT mouse breast cancer	miR-1	4	AAV9-ESGLSQS	Herpes simplex virus thymidinkinase (and ganciclovir), luciferase	Heart	[129]
Metastatic hepatocellular carcinoma model	miR-122	4	AAV8	Herpes simplex virus thymidinkinase (and ganciclovir), luciferase	Hepatocytes/liver	[128]
Glioblastoma cells *in vitro*	miR-128	2	Lentiviral	Herpes simplex virus thymidinkinase (and ganciclovir)	Neuronal differentiated cells	[127]
Subcutaneous fibrosarcoma xenografts	miR-122	4	Adenoviral	Luciferase	Hepatocytes/liver	[181]
Intradermal melanoma tumor	miR-122	4	Adenoviral	Herpes simplex virus thymidinkinase	Hepatocytes/liver	[87]
Human glioma xenografts	miR-31	3	Baculoviral	Herpes simplex virus thymidinkinase (and ganciclovir)	Astrocytes/brain	[85]
	miR-127	3				
	miR-143	3				
Hepatocellular carcinoma model	miR-181	2	Adenoviral	Human telomerase reverse transcriptase RNA-targeting ribozyme	Hematopoietic stem cell- and progenitor-derived blood cells	[130]
	miR-122	3			Hepatocytes/liver	[131]
HSC and iPSC						
Tumor-infiltrating monocytes/macrophages	miR-126	2	Lentiviral	Interferon-α	Hematopoietic stem and progenitor cells	[134]
	miR-130a	2				
Differentiated myeloid cells	miR-126	2	Lentiviral	$Gp91^{phox}$	Hematopoietic stem and progenitor cells	[135]
Differentiated hematopoietic cells	miR-126	4	Lentiviral	Galactocerebrosidase	Hematopoietic stem and progenitor cells	[133]
	miR-130a	4				
Lymphoid progenitors, myeloid cells	miR-150	4	Lentiviral	GFP	Differentiated T and B lymphocytes	[132]
Post-thymic T cells	miR-181a	4	Lentiviral	GFP, inhibition of negative thymic selection	Developing T cells	[182]
Myeloid cells	miR-223	4	Lentiviral	EGFP, NGFR	Granulocytes and monocytes, mature dendritic cells	[17]
Immature dendritic cells	miR-155	4				
Human fetal fibroblasts	miR-302a, d	4	Lentiviral	EGFP, mCherry	Human ES, neural progenitors	[137]
	miR-142-3p	4				
	miR-155	4				
	miR-223	4				
ES, iPSC	miR-292-3p	4	Lentiviral	GFP, differentiation of ES to neural progenitors	Pluripotent cells	[138]
ES, iPSC	let-7a	4	Lentiviral	GFP	Non pluripotent cells	[136]
CNS and eye						
CNS	miR-1	3	AAV9	lacZ, EGFP	Heart, liver	[69]
	miR-122	3				
CNS/astrocytes	miR-124	4	Lentiviral	lacZ, glial glutamate transporter	Neurons	[141]
CNS/cortical excitatory neurons	miR-128	4, 8, 12	Lentiviral	EGFP	Cortical inhibitory neurons	[83]
	miR-221	4, 8				
Retina/eye	miR-124	4	AAV5	EGFP	Photoreceptors/retinal pigment epithelium	[143]
	miR-204	4	AAV8			
Photoreceptor/eye	miR-181c	4	AAV2 (quadY-F+T-V)	GFP	Ganglion cells and inner retina	[144]
Immune system and other applications						
Skeletal muscle	miR-142-3p	2, 4, 8	AAV1	Ovalbumin human sarcoglycan	Antigen presenting cells	[152,153]
Hepatocytes/liver	miR-142-3p	4	Lentiviral	Factor Ⅸ, Ⅷ, induction immunologic tolerance	Antigen presenting cells	[9,145,147,183]
Dendritic cells/spleen	miR-142-3p	4	Adenoviral	Luciferase, lacZ, combination with Cre-loxP system	Hepatocytes/liver	[86]
	miR-122	4				
Neural stem cells	miR-124a	4	Lentiviral	GFP, identification of neurons	Neurons	[184]
Adipose tissue	miR-122	3, 8	AAV8	Human leptin	Hepatocytes/liver	[185]
Intramuscular delivery	miR-122	6	AAV8	201Ig immunoadhesin, luciferase	Liver, skeletal muscle	[186]
	miR-206	6				

Human fibroblasts HFL1	miR-124	4	Lentiviral, replication deficient	Neural conversion genes *Ascl1, Brn2* and *Myt1L*	Human induced neurons	[179]
Skeletal muscle	miR-208a	2	AAV9	Human calpain3	Heart	[81]

miR-TS: Artificial microRNA target sites; HSC: Hematopoietic stem cell; CNS: Central nervous system; AAV: Adeno-associated virus; GFP: Green fluorescent protein; EGFP: Enhanced green fluorescent protein; iPSC: Induced pluripotent stem cells; NGFR: Nerve growth factor receptor; ES: Embryonic stem cells; HFL1: Human fetal lung fibroblast 1.

are in competition with cellular target mRNAs. By the use of complete complementary miR-TS, enabling rapid microRNA recycling, and defining an optimal target site number, the risk of microRNA saturation can be minimized. We and others showed that endogenous microRNA profile or microRNA-regulated genes were not altered after administration of optimized miR-TS-bearing vectors[61,67,69,81].

Application of microRNA-regulated transgene expression systems

Several promising results using microRNA-dependent regulation have been obtained for different disease models and vector systems (Table 1).

Application to cardiac gene therapy

AAV vectors have been established as the leading vector type for myocardial gene transfer in preclinical and clinical applications[98]. Their main advantage is their ability to transduce cardiomyocytes *in vivo* at high efficiency. In addition, AAV vectors allow long lasting expression of encoded transgenes and do not trigger a strong immune response or inflammation[99-102]. Importantly AAV vector-driven expression is performed without integration of the vector genome into the host genome[103] which, in consequence, reduces the risk of long term, irreversible side effects[104-106]. As conventional AAV vectors containing capsid proteins of serotype 2 (AAV2) revealed a natural cardiac tropism, their transduction efficiency was limited. Therefore discovery of naturally occurring AAV serotypes carrying variations in the amino acid sequence of the capsid protein[107,108] enabled the development of pseudotyped AAV vectors and led to substantial improvement of cardiac gene transfer. AAV9 vectors are the most cardiotropic serotype upon systemic vector administration for transgene delivery into the heart of rodents[109-115], whereas AAV6 vectors seems to be superior to other serotypes in large animals[116]. In humans, a clinical phase 2b trial showed promising results upon intracoronary administration of an AAV1 vector expressing the calcium regulatory protein SERCA2a in patients with advanced heart failure[117]. However, improved cardiac transduction by serotype switch did not abrogate the intrinsic property of AAV resulting in sustained transduction of a broad range of tissues, in particular liver and skeletal muscle.

Transcriptional targeting represents another approach to increase cardiac specificity of AAV vectors. To restrict AAV vector-mediated transgene expression to the heart upon intravenous injection, a variety of cardiac-specific promoters have been investigated. Promoters of the heavy chain (MHC)[118-120] and the light chain (MLC)[121,122] of cardiac protein myosin are most potent to transcriptionally mediate heart-specific transgene expression. However, endogenous cardiac-specific promoters are too large to be packaged into AAV vectors and their core elements alone provide only weak transgene expression. Smaller hybrid promoters consisting of a cardiac core element and another strong enhancer element increased cardiac expression[109,122] but transgene expression was also detected in other tissues, especially in the liver, skeletal muscle and pancreas[61,122]. At this point it seems unlikely that promoter-only approaches will sufficiently target the vectors, therefore microRNA-dependent regulation of AAV vector-mediated transgene expression has shifted into focus to further improve cardiac specificity of AAV vectors (Figure 2). Two studies carried out by Qiao *et al*[68] and Geisler *et al*[67] addressed microRNA-mediated suppression of transgene expression in liver after systemic AAV9 vector application. Among three microRNA candidates (miR-122, miR-192 and miR-148a) that have been described to be selectively expressed in liver[65,123], we found that miR-122 was most abundantly expressed in murine liver, whereas expression in murine heart was approximately 5 orders of magnitude lower. Systemic application of AAV9 vectors containing miR-122TS in the 3' UTR of an EGFP reporter confirmed effectiveness of miR-122-mediated suppression in the liver, whereas cardiac expression remained unaffected. Interestingly, microRNA-mediated transgene suppression in the liver was by far more efficient than transcriptional control by the en-CMV-MLC0.26 cardiac hybrid promoter[67]. The other study carried out by Qiao *et al*[68] confirmed improved specificity of miR-122-regulated AAV9 vectors. Similar to our study, they reported that microRNA-mediated increase of cardiac specific expression was even more efficient than transcriptional control of transgene expression by an en-MHC-TNT hybrid promoter.

Abundant transgene expression upon systemic AAV9 vector application can also be found in the skeletal muscle. Therefore transgene suppression in this tissue is necessary to further enhance the cardio-specificity of AAV vector-mediated gene transfer. According to the guidelines for microRNA selection (see above), only miR-206 fulfills the major requirements of microRNA-mediated repression: It is highly expressed in the skeletal muscle and absent in the heart. However, we found that a transgene containing miR-206TS was strongly suppressed in both the skeletal muscle and heart of mice

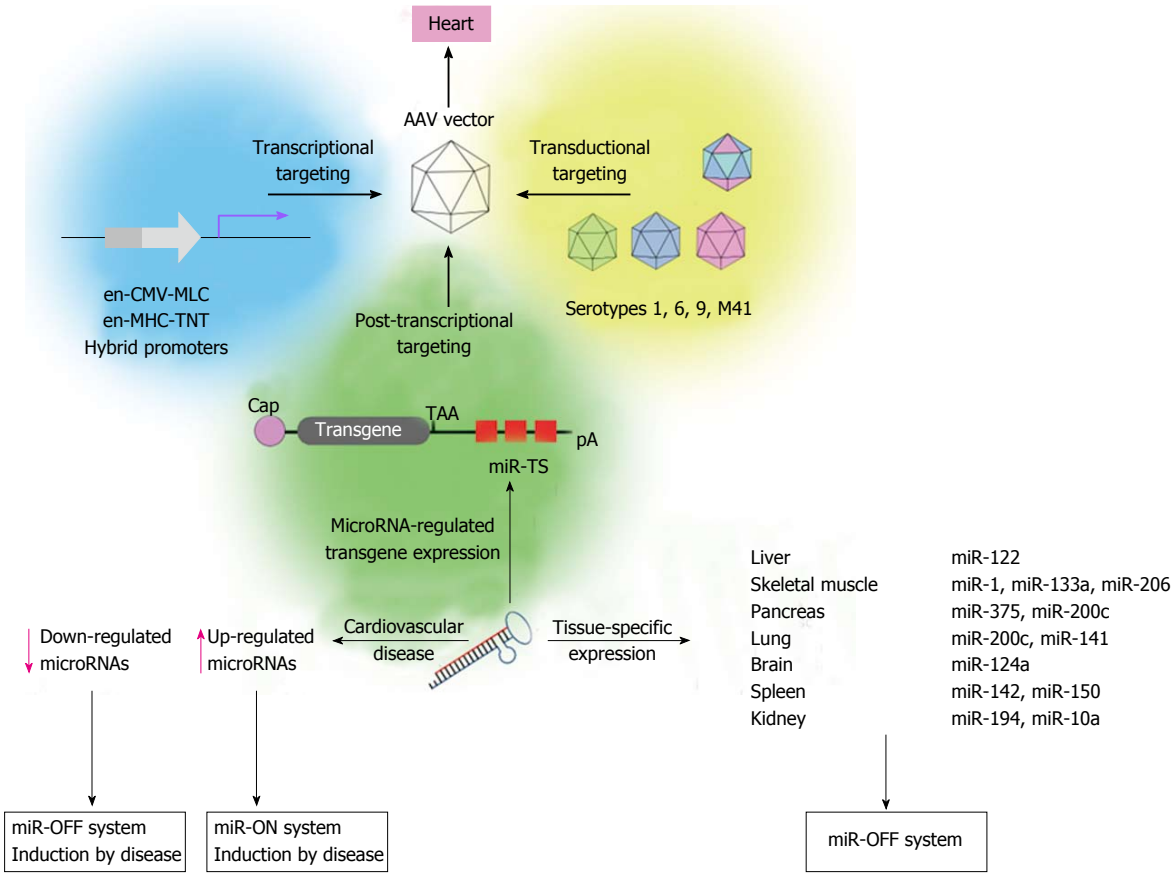

Figure 2 Adeno-associated virus vector-based microRNA targeting strategies to improve cardiac - specific gene therapy. Cardiac-specific gene transfer of AAV vectors can be obtained by different targeting strategies, including transcriptional targeting using cardiac hybrid promoters[68,122], transductional targeting[109-113,187,188] or microRNA-regulated post-transcriptional transgene suppression. miR-OFF system (right site): Expression of miR-TS-bearing transgenes can be selectively suppressed in non-cardiac tissue by microRNAs, which are expressed in these tissues, but not in the heart. For the miR-OFF system (left site): If microRNAs are selectively down-regulated in cardiovascular diseases, transgene expression may be restricted to these cells by use of miR-TS corresponding to down-regulated microRNAs. If microRNAs are up-regulated in cardiovascular diseases, corresponding miR-TS can be used in the miR-ON system[178] for regulation of a repressor protein to restrict transgene expression to respective cells. Shown microRNAs are specifically expressed in mouse tissues (unpublished). AAV: Adeno-associated virus; pA: Polyadenylation site; CMV: Cytomegalovirus; MLC: Myosin light chain; MHC: Myosin heavy chain; TNT: Troponin T.

following systemic application of AAV9 vector. The reason for this is binding of the miR-1, which shows high sequence homology to miR-206 and is highly expressed in heart tissue. By introducing single nucleotide substitutions into the seed region of the microRNA/miR-206TS duplex, we generated mutated miR-206TS that became resistant to miR-1 regulation but remained fully sensitive to miR-206[61] (Figure 3). This was a result of compensatory effects induced by perfect complementarity of the 3' portion of miR-206 to mutated miR-206TS. *In vivo* transgene expression of AAV9 vectors bearing mutated miR-206TS and miR-122TS was strongly suppressed in both skeletal muscle and the liver, whereas expression in heart remained unaffected[61]. Interestingly, contrary to the generally applicable concept of miR-TS recognition and repression by microRNAs, we observed that insertion of single nucleotide mutations within miR-206TS can even enhance miR-206-mediated transgene repression. Although the underlying mechanism remains to be elucidated, we observed similar results for miR-122TS repression but not for other modified miR-TS (data not published).

Application to suicide gene cancer therapy

Regulation of transgene expression by endogenous microRNAs has been exploited for vector-mediated suicide gene therapy with Herpes Simplex Virus thymidine kinase (HSV-tk). Expression of the HSV-tk mediates phosphorylation of the prodrug ganciclovir (GCV), thus inhibiting DNA replication in rapidly dividing cancer cells. As phosphorylated GCV is also toxic in normal cells[124], tumor cell-specific expression of HSV-tk is required. AdVs are widely used in cancer gene therapy. However, they exhibit strong hepatotropism after systemic application and can induce severe hepatotoxicity after HSV-tk delivery[125,126]. Repression of HSV-tk containing miR-122TS induced by miR-122 distinctly reduced hepatotoxicity upon local delivery by AdV, without altering the antitumor effects, as miR-122 is weakly expressed in transduced melanoma tumor cells but highly expressed in hepatocytes[87]. In another approach, a lentiviral vector (LV) expressing HSV-tk in combination with ganciclovir showed improved tumor specificity *in vitro* by miR-128-mediated transgene regulation, a microRNA that is differentially expressed

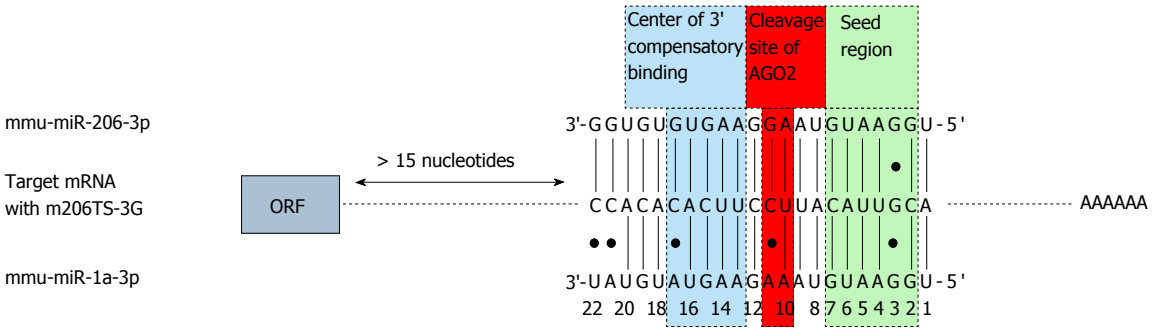

Figure 3 Sequence alignment of murine miR-206, miR-1 and mutated miR-206TS, referred to as m206TS-3G. The last of these sequences contains a C to G transition compared to miR-206TS at microRNA nucleotide position 3. Vertical dashes, Watson-Crick pairing; dots, mismatches; green boxed capital letters, seed region. Potential cleavage site of AGO2 between nucleotide 10 and 11 is boxed in red. The center of the potential 3' compensatory microRNA binding is boxed in blue. Nucleotide positions are numbered according to microRNA sequence. ORF: Open reading frame; AGO: Argonaute.

between glioblastoma cells and normal brain tissue[127]. Other studies confirmed the reduction of side effects by microRNA-dependent suppression of suicide genes in cancer gene therapy approaches. Wu *et al*[85] found that in addition to transcriptional control by a glioma-specific promoter, insertion of miR-TS for the miR-31, miR-127 and miR-143 into baculoviral vectors led to repression of HSV-tk in normal neural cells. As these microRNAs are more highly expressed in these cells than in glioma cells, this approach led to reduced HSV-tk/GCV induced cytotoxicity. More recently, Della Peruta *et al*[128] improved tumor specificity of systemically applied AAV8 vectors expressing HSV-tk linked with miR-122TS in combination with a liver-specific promoter in a syngeneic metastatic murine hepatocellular carcinoma (HCC) model. Administration of the vector and GCV treatment resulted in a reduction of cancer growth and number of metastases without liver toxicity. Moreover Trepel *et al*[129] used miR-TS of the miR-1 to inhibit cytotoxicity in heart after HSV-tk delivery with an AAV9 vector capsid variant. Animals treated with the vector were protected from cardiac HSV-tk expression and drug-induced development of dilative cardiomyopathy, whereas delivery of the suicide gene to tumors significantly inhibited tumor growth after GCV treatment. Another study recently carried out by Won *et al*[130] improved specificity of cancer gene therapy by developing microRNA-regulated trans-splicing ribozyme that targets human telomerase reverse transcriptase (hTERT) RNA in cancer cells. The group I intron-based ribozyme targets and cleaves its substrate RNA and trans-splices an exon attached at its 3′ end (*e.g.*, a therapeutic RNA) onto the cleaved target RNA, resulting in expression of the therapeutic RNA and repression of substrate RNA. As the ribozyme specifically targets hTERT RNA positive cancer cells, but also hematopoietic stem cell-derived blood cells, the ribozyme was modified by inserting target sites for the blood cell-specific miR-181a downstream of its 3′ exon. Analysis of AdV-mediated expression of the hTERT-targeting trans-splicing ribozyme harboring miR-181aTS with HSV-tk gene as a 3′ exon under control of a liver-specific promoter and GCV treatment resulted in specific anticancer effects. Moreover systemic vector application in an orthotopic multifocal HCC mouse model demonstrated a regression of liver tumor nodules and tumor volume, with minimal hepatotoxicity[130]. In a similar approach, AdV-mediated administration of the hTERT-targeting trans-splicing ribozyme containing miR-122TS resulted in efficient anti-cancer effects and reduced hepatotoxicity[131].

Application to hematopoietic and pluripotent stem cell therapy

For hematopoietic stem cell (HSC) therapy, lineage- and differentiation stage-specific microRNA expression can be used to specifically express a transgene in a discrete subset of progeny. Although integrating vectors, such as LVs, mediate long-term expression in progenitor cells, expression needs to be often restricted to their differentiated progeny. Therefore Brown *et al*[17] showed that miR-223 de-targets transgene expression in the myeloid progeny of HSC (granulocytes and monocytes), thus restricting expression to the lymphoid progeny. Similarly, transgenes were equipped with miR-TS for miR-150 and miR-155, thus repressing their expression in mature T and B cells and mature dendritic cells, but not in lymphoid progenitors or immature dendritic cells, respectively[17,132]. On the other hand, microRNA-regulation technology also worked if the expression of a toxic transgene needs to be avoided in hematopoietic pluripotent stem/progenitors cells (HSPC). In this regard it has been shown that miR-126 and miR-130a, both expressed in the HSPC compartment, but not in mature blood cells, suppressed LV-mediated transgene expression of galactocerebrosidase containing the corresponding miR-TS in HSC, allowing long-term hematopoiesis after vector transduction and a stable bone marrow graft in mice. Moreover a robust expression of galactocerebrosidase in mature hematopoietic cells enabled successful treatment of globoid cell leukodystrophy in a mouse model[133]. In a similar approach Escobar *et al*[134] targeted interferon-α expression to tumor-infiltrating monocytes/macrophages using a combination of transcriptional and miR-126-/miR-130a-mediated control during post-transplant hematopoiesis to limit HSC exposure to the transgene thereby inhi-

biting breast cancer progression. Furthermore in an approach using therapeutic LV encoding gp91^{phox} linked with miR-126TS under the control of a myeloid-specific promoter, high levels of myeloid-specific transgene expression was achieved for X-linked chronic granulomatous disease therapy, entirely sparing the miR-126 positive CD34^{+} HSC compartment. As ectopic gp91^{phox} expression in HSC could cause production of reactive oxygen species that may damage DNA, alter cell growth or induce apoptosis, the transcriptionally and post-transcriptionally regulated LV reduced off-target expression and effectively corrected the X-phenotype in gp91^{phox}-deficient mice[135].

MicroRNA-regulated reporter expression systems can also be used to track the differentiation of stem cells and have been widely disseminated in the field of pluripotent stem cell technology (induced pluripotent stem cells, embryonic stem cells)[17,136-138]. A big challenge in this field is the *in vitro* generation of a homogenous cell population of a pre-defined lineage which can be successfully transplanted. Therefore cell type- and differentiation-specific microRNAs are helpful to identify and select cells with desired differentiation fates. Upon differentiation, cells can be selected either by combining miR-TS with a positive or negative regulator such as neomycin or thymidine kinase[17,136] or by combining with a reporter, such as GFP, and sorting by fluorescent activated cell sorting (FACS)[138], respectively.

Application to central nervous system and eye

Gene therapy holds promise for treating of genetic as well as metabolic disorders of the central nervous system (CNS) and in particular AAVs have received growing interest as viral vector systems for gene delivery in the last few years. Besides the well-known generally favorable properties of AAV vectors, several AAV serotypes are able to cross the blood-brain barrier and thus effect an efficient transduction of cells of the CNS with systemic vector application[139]. Based on the broad range of tissue targeting by AAV9 vectors, Xie *et al*[69] exploited miR-TS technology to restrict AAV9 vectors to the CNS. Therefore they constructed AAV9 vectors with miR-TS completely complementary to hepatocyte-specific miR-122 and muscle-specific miR-1 in order to repress reporter gene expression in liver, heart and the skeletal muscle. *In vivo* comparison of vectors, each bearing one or three miR-122TS or/and miR-1TS, found that the number of miR-TS repeats inversely correlated with reporter expression levels in tissues where the corresponding microRNA is expressed. Moreover systemic application of the microRNA-regulated AAV9 vector resulted in distinct suppression of the transgene in the liver and muscle, whereas it was not altered in the brain. Most vector systems transducing the CNS have a strong neurotropism. However, for several neurodegenerative disorders, certain cell types of the CNS, such as astrocytes, need to be specifically targeted, as they exhibit important physiological functions and may display altered functions. Among CNS-specific microRNAs, miR-124 is specifically expressed in neurons but absent in astrocytes[140]. Accordingly, Colin *et al*[141] demonstrated that insertion of miR-124TS into a LV is sufficient to repress transgene expression in neuronal cells *in vitro* and *in vivo*, whereas expression remained unaffected in astrocytes. Moreover, exploiting microRNA regulation enables more than targeting transgene expression to neurons or supporting cells. Thus Sayeg *et al*[83] showed that a combinatory regulation of a locally applied LV by miR-128 and miR-221 targeted transgene expression between neuronal subtypes in the murine neocortex as expression was selectively inhibited in excitatory neurons but not in inhibitory cells.

Inherent retinal degeneration of the eye results from impaired function of the retinal pigment epithelium (RPE) or photoreceptor (PR) cells and leads to severe visual deficits and blindness[142]. In an approach to restrict AAV-vector-mediated EGFP reporter gene expression to either RPE or PRs, Karali *et al*[143] subretinally applied miR-TS-bearing AAV5 vectors into the eye of mice and pigs. AAV vectors containing miR-204TS were selectively repressed in RPE, which was in agreement with the miR-204 expression in RPE. In contrast the EGFP reporter was repressed in PRs but not in RPE if the vector was equipped with miR-TS for miR-124, a microRNA that is expressed in PRs but absent in RPE. For advanced retinal gene therapy, Kay *et al*[144] recently developed an AAV2 vector capsid mutant with a strongly increased transduction efficacy for PRs upon intravitreal vector delivery. Furthermore, transgene expression was restricted to PRs only by use of a combinatorial approach of a PR-specific promoter and incorporation of multiple target sites for miR-181c, a microRNA which is expressed in retinal ganglion cells and the inner retina.

Application to reduce transgene-directed immunogenicity

The concept of microRNA-mediated negative regulation of transgene expression was first shown by Brown *et al*[9] in order to reduce off-target transgene expression in APC upon systemic injection of LV. By combining a hepatocytic-specific promoter and target sites for the hematopoietic-lineage specific miR-142-3p, a reduced transgene expression in APCs allowed a long-term expression of clotting factor F.Ⅸ for stable correction of hemophilia B mice upon systemic LV administration[145,146]. Furthermore it was shown that this combined approach induced an immunological tolerance for the transgene[147-149] resulting from hepatocyte-directed expression[147,150]. Further studies confirmed these results for other vector systems. Intramuscular application of AAV vectors leads to appearance of immune responses against the transgene and rapid decrease of transgene expression, possibly as a result of vector transduction of APC[151]. Thus Majowicz *et al*[152] and Boisgerault *et al*[153] inserted target sites for miR-142-3p into the transgene expression cassette of AAV1 vectors. Upon intramuscular application they observed prolonged expression of the transgene in the skeletal muscle, which correlated with

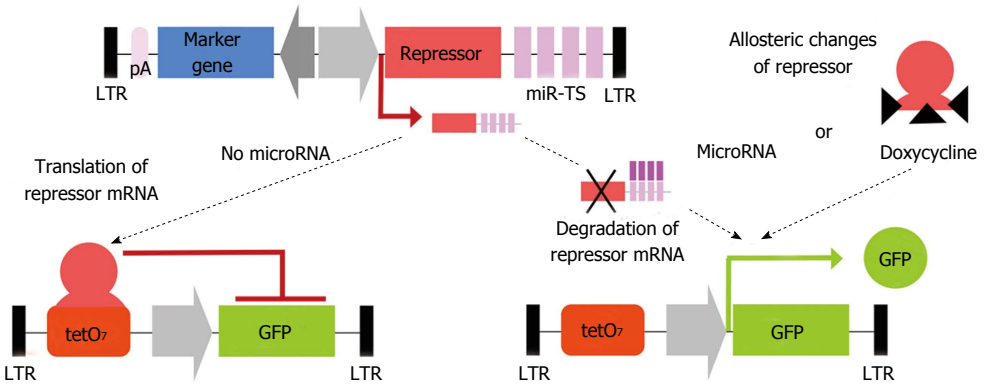

Figure 4 Schematic of the miR-ON system using lentiviral vectors in the indicated conditions. Two independent lentiviral vectors containing a repressor protein and a GFP reporter with operator sequences for the repressor protein, respectively. The repressor protein, either tetracycline repressor (tTR) or tTR-Kruppel-associated box, constitutively binds to the tetO sequence, thereby suppressing reporter gene expression. If the repressor mRNA containing miR-TS is not translated into native protein due to microRNA-mediated degradation of the repressor mRNA or is unable to bind tetO sequence because of doxycycline-induced allosteric changes of the repressor, GFP expression is switched on. GFP expression is inhibited if the repressor is translated due to lack of an inhibiting microRNA, thereby allowing the repressor to bind to the operator sequence. Arrows indicate orientation of transcription. Modified according to[178]. LTR: Long terminal repeats; miR-TS: Artificial microRNA target sites; pA: Polyadenylation site; tetO7: Seven tandemly arranged tetracycline operator sequences; GFP: Green fluorescent protein.

reduced immunogenicity to the transgene.

Oncovirotherapy and vaccines

Besides the development of microRNA-regulated viral vectors in order to de-target transgene expression in certain tissues, lineages or differentiation states or to identify cell subsets, modification of tropism of replication competent oncolytic viruses (OV) has gained growing interest. Although OVs preferentially replicate in tumor cells, application to cancer gene therapy is limited by their replication and corresponding induction of toxicity in normal tissues. By insertion of miR-TS for tissue-specific microRNAs into the viral genome, microRNAs have been used to reduce replication in normal cells, while maintaining their oncolytic potential in tumor cells. This was initially shown by Kelly *et al*[154], who engineered a coxsackie A21 OV with miR-TS for the skeletal muscle-specific miR-206 and muscle-specific miR-133a, thus reducing myotoxicity while maintaining oncolytic potential. MicroRNA-regulation of OVs to increase tumor specificity has been successfully applied for several RNA[84,155-158] and DNA[80,93,159-167] viruses in the meantime. For detailed information the reader is referred to Ruiz *et al*[168].

Another aspect that needs to be addressed is the enhancement of safety of live attenuated virus vaccines by microRNA-regulation[169]. Insertion of miR-TS into the viral genome leads to reduced primary replication in the viral target organ, whereas weaker replication in other tissues induces protective immunity of the host. This was successfully shown for poliovirus[170], influenza virus[171], flaviviruses[172-175] and alphavirus[176].

Other applications

Activity of a given microRNA can be easily identified by coupling corresponding miR-TS to a reporter gene such as lacZ, GFP or luciferase and analysis of reporter gene expression. This allows dynamic measurement of microRNA expression over time on a single cell basis. Because reporter gene expression is observed as microRNA-mediated shut down of expression, the absence of signal might be not distinguishable from false negative results of failed transduction or transgene silencing. By LV-mediated co-expression of the microRNA-regulated sensor reporter and a second non-microRNA-regulated reporter, both under the control of a bi-directional promoter, microRNA activity can be measured independently from vector dose, transduction level and promoter activity[9,17,133]. This principle has been applied to construct a microRNA reporter library for analysis of microRNA activity in FACS-sorted cells. This technique has many advantages, as it allows the measurement of microRNA bioactivity in heterogeneous cell populations such as HSC[133] or neural stem cells[177]. However, for microRNAs with expression levels close to the detection threshold of the reporter system, its reliability is limited and other methods are preferred. Thus recently a miR-ON system (Figure 4) was generated to identify small subpopulations of microRNA-expressing cells and to select them directly. This system includes transduction of a repressor containing miR-TS, together with a reporter under its regulation. Reporter activity is observed only if the upstream repressor mRNA is degraded and cells expressing corresponding microRNA become selectable by reporter gene expression[178].

Furthermore microRNA-mediated regulation of transgene expression can help to improve direct cell conversion as shown for conversion of human fibroblasts into functional neurons (human induced neurons) by a self-regulating vector. Insertion of miR-TS for the neuron-specific miR-124 linked to the neural reprogramming genes *Ascl1*, *Brn2* and *Myt1L* of an integration deficient LV facilitated a down-regulation of conversion gene expression once the cell has reached a stable neuronal fate, thereby allowing for a more complete functional maturation of the cells in culture[179].

For additional applications in further fields, the reader is referred to Table 1.

CONCLUSION

In this review we have described the features of microRNA-regulated transgene expression, including its technical mechanisms and its application to transgene delivery by viral vectors. The variety of examples showed how microRNA regulation can be diversely exploited in different contexts as vectors, routes of application or fields of research in general. Compared to other targeting strategies, including transcriptional and transductional targeting, microRNA-regulation displays many advantages. Insertion of approximately 100 bp sequence of miR-TS can be easily accomplished by conventional cloning techniques. In general the small size of miR-TS prevents interference with the packaging capacity for therapeutic genes or foreign genes, thus making it applicable for both viral vectors and viruses. This system has now become a major technique for specific application of therapeutic transgenes, for increased tumor specificity of oncolytic viruses and enhanced safety of recombinant live attenuated virus vaccines. Nevertheless each miR-TS needs to be carefully evaluated with regard to its optimal effectiveness. Moreover, as many viral vectors have a broad tissue tropism, which in general requires the use of several target sites to restrict a transgene to a specific tissue or cell type, target site optimization is required. The miR-ON system may be an alternative to overcome this disadvantage. Importantly, it may also be a platform to develop microRNA-regulated shRNA and artificial microRNA expression systems, which are currently not available.

Based on the results of the overall studies investigating potential side effects induced by microRNA-mediated transgene regulation, no major side effects have been observed to date, suggesting the mechanism is safe for therapeutic use. But there is a need to consider specific guidelines concerning the complementary between target sites and its corresponding microRNA, the number of target sites and the strength of transgene expression. In summary, microRNA regulation has been emerged as a powerful new technology to improve vector-mediated transgene expression *in vitro* and *in vivo*. It has the potential to be translated into clinical applications, thereby improving efficacy and safety of gene- and virotherapeutic approaches to the degree that their use can become more common and be applied to additional indications.

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