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AAV-mediated miRNA Delivery and Therapeutics

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Abstract

MicroRNAs are 20-24 nt long, single-stranded RNAs that repress gene expression. Dysregulation of miRNA expression is associated with many human diseases. Modulating the level of endogenous miRNA alters gene profiling and can achieve therapeutic benefits. Here, we reviewed currently used methods of altering miRNA activity *in vivo. We* focus on the delivery of miRNAs and miRNA inhibitors using recombinant adeno-associated virus (rAAV). In general, rAAV-mediated miRNA inhibition or overexpression provides a simple, efficient and informative way to study miRNA function in mammals. This method also provides the opportunity to explore potential miRNA therapeutics for many diseases.

Keywords

AAV; *in vivo* gene delivery; miRNA inhibition; miRNA overexpression

MicroRNAs (miRNAs) are small 20–24 nt RNAs that repress the expression of mRNAs by binding to the 3'UTR of the targeted mRNA. As a whole, miRNAs are predicted to regulate more than half of all mammalian protein-coding genes[1]. Based on their location in the genome, the genes that code for miRNA can be categorized into three groups: exonic miRNAs, intronic miRNAs and miRNAs embedded into protein-coding transcripts[2].Most miRNAs are transcribed as primary miRNA (pri-miRNAs) by RNA polymerase II[3], though some are transcribed by RNA polyIII[4]. A pri-miRNA contains a 7-methylguanosine cap at its 5′ end and a poly (A) tail at its 3′ end. It is cleaved by an intranuclear ribonuclease III (RNase III) enzyme, referred to as Drosha, to generate a precursor miRNA (pre-miRNA), which is a stem-loop molecule approximately 70 nt in length. Subsequently, Exportin-5 binds to the pre-miRNA and transports it into the cytoplasm. It is here that another RNase

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Disclosure

G.Gao is a founder of Voyager Therapeutics and holds equity in the company. G.Gao is an inventor on patents with potential royalties licensed to Voyager Therapeutics and other biopharmaceutical companies. J.X. and D.R.B have nothing to disclose.

III, Dicer, processes the pre-miRNA into a mature miRNA. This miRNA is loaded into an RNA-induced silencing complex (RISC). Upon amalgamation this fully active protein-RNA aggregate is capable of repressing gene expression through the cleavage and/or degradation of mRNAs.

miRNA dysregulation in human diseases and miRNA therapeutics

In 1993, Ambros and his colleagues discovered the first miRNA, Lin-4, in *Caenorhabditis elegans. Since this event*, thousands of miRNA have been found and submitted to the miRNA database (http://www.mirbase.org). These miRNAs have been isolated from mammals and non-mammals; more than 2500 of which have been isolated from human[5]. The correlation between miRNA dysregulation and human disease was first reported by Calin *et al*. This pioneering study revealed that the loci for miR-15 and miR-16 were deleted in the majority of patients suffering from B cell chronic lymphocytic leukemia[6]. Accumulated data have demonstrated that miRNAs play important roles in almost all classes of human disease; including cancer, cardiovascular disease, diabetes, *etc.* For example, more than 50% of human miRNA-encoding genes are located in chromosomal locations associated with cancer or fragile sites on a genome-wide base [7]. *Let-7* is the first miRNA that was found to regulate the oncogene *RAS* expression by directly targeting its 3'UTR[8]. Further studies have shown that in non-small-cell lung cancer (NSCLC) mouse models, intratumoral injection of synthetically produced let-7 molecular mimics significantly reduces tumor burden[9]. In a cohort of 241 patients with hepatocellular carcinoma (HCC), it was shown that tumor tissues have reduced expression of miR-26 compared with noncancerous liver tissue from the same patient. Furthermore, in patients whose tumors have decreased miR-26 expression, lower levels of miR-26 correlate with shorter overall survival[10]. Subsequently, systemic delivery of miR-26a via adeno-associated virus vector 8 (AAV8)[11], a vector known for its high liver tropism, dramatically suppresses the tumor progression in a murine liver cancer model[12]. In addition to the miRNA studies in cancer, Olson and his colleagues reported that they had found a signature pattern of miRNAs in cardiac hypertrophy and heart failure which initiated a wave of research focused on miRNA function in heart disease[13]. In a failing heart, miR-21 level is specifically increased in fibroblasts through the suppression of ERK-MAP kinase signaling pathway which triggers fibroblast motility and initiates the process of cardiac scarring. Scarring, or fibrosis, of the heart is an inappropriate physiological response that oftentimes is severely deleterious to the individual. *In vivo* silencing of miR-21 by antisense oligonucleotide inhibits interstitial fibrosis and corrects cardiac dysfunction in a TAC (Transverse aortic constriction) mouse model[14]. Genetic knockout (KO) of the cardiac-specific miRNA, miR-208a, can prevent pathological cardiac remodeling. Similarly, the anti-miR-208a oligonucleotide improved cardiac function and survival in a rat hypertension-induced heart failure model[15,16]. Another study found that mice who received anti-miR-208a oligonucleotide therapy confer resistance to diet-induced obesity and improved insulin responsiveness[17]. MiRNAs are also associated with metabolic diseases. MiR-375 is highly expressed in pancreatic islets and miR-375 KO mice are hyperglycemic[18]. MiR-33, an intronic miRNA located in the intron of SREBF-2 gene, cooperates with its SREBF-2 host gene to control cholesterol homeostasis[19]. Moreover, administration of anti-miR-33 oligonucleotide raises the plasma HDL level and

represses the atherosclerosis in a hypercholesterolemia mouse model[20]. Using a similar approach, inhibition of the miR-33 family in non-human primates also raised plasma HDL and lower VLDL triglyceride levels[21]. MiR-122 antagomiR could be the first miRNA-target drug to treat human disease. MiR-122 is liver specific and highly expressed, constituting 70% of the total liver miRNA population[22]. The binding between miR-122 and the conserved 5' untranslated region of the hepatitis C virus (HCV) genome protects the HCV from nucleolytic degradation and host innate immune response[23,24]. HCV load was dramatically reduced with the therapeutic use of miR-122 antagomiR to competitively bind endogenous miR-122. The clinical trial using miR-122 antagomiR to treat HCV patients has completed the Phase 2a stage, showing prolonged dose-dependent reductions in HCV RNA levels without evidence of viral resistance [25]. All of these researches prove that modulation of miRNA is providing a new route of treatment against human diseases.

Strategies to investigate miRNA function *in vivo*

Though thousands of miRNAs have been discovered, few miRNA-target interactions have been experimentally validated, especially *in vivo*. To understand the complete spectrum of miRNA function, we need approaches to modulate miRNA activities in order to perform gain-and-loss of function studies. Genetic disruption of a miRNA gene provides a powerful strategy to study miRNA function, but many miRNAs share the same seed sequence, the 6–8 nt miRNA region that defines the target repertoire of a miRNA. Consequently, one member of a miRNA family may compensate for loss of another. Creating an animal model in which all members of a miRNA family are deleted is time consuming and expensive. In some cases, miRNA genetic ablation in mice are embryologically lethal, such as miR-17-19 cluster KO leads to 100% postnatal death with cardiac and lung defects[26] and miR-126 KO results in around 50% embryonic death due to vascular ruptures and subsequent hemorrhage[27].

Chemically modified miRNA mimics or anti-miRNA oligonucleotides (AMOs) complementary to mature miRNAs can be used to increase or decrease the activities of miRNAs respectively, *in vitro* and *in vivo*. Though effective, these oligonucleotides therapies are typically expensive and/or require proprietary modifications such as 2′-*O*-methyl, 2′-*O*-methoxyethyl, or 2′, 4′-methylene (LNA)[28-30]. Unfortunately, due to their transient lifespan of miRNA mimics and AMOs, treatment requires repeated administration to effectively express or suppress the cognate miRNAs. Current chemistries and formulations are limited in their success by the delivery of oligonucleotides to many tissues or organs, such as pancreas, muscle and brain.

An additional strategy to lower miRNA activities is to use transcribed miRNA inhibitors referred to as miRNA "sponges"[31] and "tough decoy RNAs (TuD RNAs)"[32]. The first alternative to AMOs are "sponges," which are miRNA inhibitors that can be expressed in cells (Fig. 1a). They oftentimes contain multiple binding sites for a specific miRNA of interest and soak up endogenous miRNA like "sponges"[31]. To further improve the miRNA inhibition efficacy, Hideo IBA and his colleagues invented a more potent miRNA inhibitor known as TuD RNA (Fig. 1b). They did this by replacing the Pol II promoter in the "sponge" with the more robust Pol III promoter. Additionally, they optimized the secondary

structures and the sequence of the miRNA-binding site[32]. Depletion of miR-223 using a sponge-expressing lentiviral vector to stably modify hematopoietic stem cells *ex vivo*, followed by bone marrow reconstitution in mice, produced a phenotype similar to that observed in a genetic miRNA knockout mouse[33]. This work successfully demonstrated that viral-vector delivered miRNA inhibitors can functionally knock down miRNAs in mice. However, the use of lentiviral vector-based miRNA inhibition for functional genomic studies and human therapy is limited due to the risk of insertional mutagenesis and the requirement for *ex vivo* manipulation. To create an efficient and simple way to study miRNA function in mice, Gao and his colleagues combined rAAV9 vector, which exhibits high tropism for mouse liver and heart[11] with miRNA TuDs to inhibit specific miRNAs. One single dose of rAAV9 expressing anti-miR-122 or anti-let-7 TuD depleted the corresponding miRNA and thereby increased the expression of its mRNA targets in the liver and heart of adult mice. This miRNA inhibition lasted through the 25 weeks of monitoring without adverse side effects or any drop in efficacy overtime. High throughput sequencing of liver miRNAs from the treated mice demonstrated that the targeted miRNAs, but no other miRNAs, were depleted [34], indicating the specificity of target miRNA inhibition. Thus rAAV-mediated miRNA modulation is holding great promise on miRNA function study *in vivo* and potential therapeutics.

RAAV vector: a powerful *in vivo* gene delivery platform

The first human AAV (i.e. AAV serotype 2) was found as a contaminant of adenovirus preparation. Although 80-90% of the human population is AAV seropositive, infection has not been associated with any human disease. The wild-type AAV (wtAAV) contains a small non-enveloped capsid with a diameter of 26 nm and a 4.7 kb single-stranded (ss) genome. This genome contains two 145-nt inverted terminal repeats (ITRs) at each end and two open reading frames (ORF; *Rep* and *Cap*). The ITRs form two T-shaped structures at each end of the genome and contribute to AAV genome replication, packaging, integration and rescue from host genome [35,36]. The *Rep* gene is involved in viral genome replication and encapsidation during AAV replication [37,38]in the presence of a helper virus, such as adenovirus[39] and herpes simplex virus[40]. The cap ORF provides structural proteins for the viral capsid which determine the tissue tropism and immune biology in viral infection[41].

AAV can transduce dividing and non-dividing cells without causing known pathogenic consequence, which has made it a popular *in vivo* gene delivery tool. Long-term transgene expression is the major advantage of AAV vectors[42]. So far there are 12 AAV serotypes and more than 100 variants available to transfer foreign genes into the liver, pancreas, heart, lung, skeletal muscle and CNS efficiently[43]. The major barrier of translating AAV into human clinical trials is the host immune response which is relatively low however persistent though out preclinical models and clinical trials[44], even though the immune responses to AAV vectors in mice is minimal.

A modified AAV, referred to as recombinant AAV (rAAV), which is made by taking the most useful aspects that wtAAV has to offer and removing the rest, has become the standard AAV vector. RAAV is designed using wtAAV as a template, but it is crafted brilliantly so that it is castrated, less risky and more predictable to work with. Though the two ITRs from

the wild-type AAV remain in place, the original *Rep* and *Cap* genes of wtAAV are completely removed and are replaced with genes of interest[45]. WtAAV can integrate into the chromosome 19q13 in cultured human cell lines [46,47], but unlike wtAAV, rAAV genomes, due to their incapability to produce rep, do not integrate into this specific chromosomal site in cultured cells or infected mammals. Most rAAV genomes persist as episomes in cells[48]. Less than 0.1 to 0.5% of the genome randomly integrates into the host genome of mice and humans[49-51]. Currently rAAVs are used in many human gene therapy clinical trials, such as cystic fibrosis, muscular dystrophy, Parkinson's, hemophilia B, and Leber's congenital amaurosis[44]. The first rAAV-based commercial drug, Glybera, has been approved to treat lipoprotein lipase deficiency in Europe [52]. RAAV vectors are regarded as the most efficient, long-lasting and safe somatic gene transfer vehicle *in vivo*.

RAAV-based *in vivo* toolkits for miRNA functional genomics and miRNA therapeutics

Though rAAV vectors have many advantages in *in vivo* gene delivery comparing with other viral vectors, the small size of AAV genome (< 4.7 kb) limits its applications on large gene replacement. Furthermore, to improve the *in vivo* efficacy of rAAVs transduction, the capability of rAAV genome is shortened even more to create what is known as self-complimentary rAAV vectors (scAAV). These vectors are essentially constructed by mutating one ITR. The mutated ITR is lack of the terminal resolution site for Rep endonuclease nicking during virus genome replication and enable continuing the synthesis of second strand. The failure of Rep nicking the mutated ITR generated two complimentary single stranded AAV genome and folds upon itself upon uncoating, creating a double stranded genome which is transcriptionally active in cells. When the virus genome released from scAAV vector in host cells, it bypasses the conversion of single-stranded genome to double-stranded genome to make the transgene expression more rapidly and efficiently. This optimization greatly enhanced the transduction efficacy of AAV *in vivo* [53,54]. This mutation further reduces the AAV package capability to 2.5 kb. However, the small size of transcribed miRNA inhibitors (sponges < 0.2 kb; TuD RNAs including Pol III promoter< 0.5 kb) is well suited to scAAV delivery.

Based on the scAAV vectors, we can generate a library of rAAV vectors expressing miRNA inhibitors as well as companion vectors, over-expressing the corresponding miRNA. This toolkit will enable researchers to test the phenotypic effects of gain-or-loss of function *in vivo*. Before murine model study, these constructs can be validated in HEK 293 cells. Each miRNA toolkit contains 3 components: miRNA sensor plasmid, miRNA over-expression plasmid and miRNA inhibitor plasmid. The miRNA sensor plasmid is used to monitor the miRNA activity in cells. It contains two reporter genes. One reporter gene carries multiple sites complementary to miRNA, which allows the binding of cognate miRNA to abolish the reporter gene expression. The more miRNA in the target cells, the lower the expression of the reporter gene can be detected. The other reporter gene is served as a transfection control (Fig. 2a).

In the standard practice of production of mature miRNAs, researchers integrate the pri-miRNA fragments into the 3'UTR of transgene or into the intron before the transgene (Fig.

2b).Basically, pri-miRNA fragments are isolated by polymerase chain reaction (PCR) from genomic DNA. The amplicons include the pre-miRNA and ~ 100 bp flanking sequence at both ends (Fig. 2b). We do not recommend embedding pri-miRNA fragments into the 3'UTR because the co-transcribed pri-miRNA will be processed by Drosha, resulting in the loss of reporter gene poly (A) and the consequent loss of its expression (Xie, et al., unpublished data).

In addition, the pri-miRNA fragments with the same design can be harbored by lentiviral vectors to suppress the development of non-small cell lung tumor by expressing the *let-7* family [55]or used to reduce hyperlipidemia and atherosclerosis by expressing miR-30c[56].

We prefer the TuD RNAs in the miRNA inhibitor constructs because of their supremacy in potency among currently available plasmid-based miRNA inhibitors (Fig. 2c)[34,57]. In brief, miRNA over-expression constructs will be co-transfected with the corresponding miRNA sensor plasmid into HEK293 cells. The reduction of reporter gene will reflect the level of functional miRNA produced by both 293 cells endogenously and the miRNA over-expression construct. In the presence of miRNA inhibitor in the transfected HEK293 cells, the reemergence of reporter gene activity indicates the repression of miRNA activity. This is a result illustrating the repression of reporter gene from construct expressing miRNA (Fig. 2d top) and de-repression of reporter gene from miRNA inhibitor in the presence of ectopic miRNA (Fig. 2d bottom). Theoretically this strategy is able to cross-validate all the miRNA expression and inhibition constructs in HEK293 cells. For *in vivo* studies, *in vitro* validated miRNA expression and inhibition constructs can be packaged into different serotypes of AAV vectors by the conventional " triple-transfection" method[58]. In brief, validated rAAV plasmid (Fig, 2 b and c), packaging plasmid which contains Rep and Cap genes, and adenovirus helper plasmid were co-transfected into HEK293 cells. After 2-3 days, cells were harvested for vector purification by ultracentrifugation on CsCl or iodixanol gradient and column chromatography[59,60]. In the packaging process, the vector genome flanked with 2 ITRs will be excised from the rAAV vector plasmid, replicated and packaged into AAV virions. Rep and Cap proteins expressed from the packaging plasmid and the adenovirus helper plasmid provides helper functions essential for rAAV rescue, replication and packaging. The serotype of AAV capsid determines tissue tropism, efficiency of transduction and immune biology of a rAAV vector.

In vivo delivery of ectopic miRNAs or their inhibitors is essential for the study of miRNA functional genomics and therapeutics. As we reviewed above, the advantages of tissue specific tropism, long-term expression and high transduction efficacy make AAV vectors ideal *in vivo* gene delivery tools. Even the TuD RNA expression driven by Pol III promoter cannot be regulated. By choosing different AAV serotype and by modifying the route of vector administration, manipulation of miRNA levels in target tissues can be achieved (Table 1).

Overall the combination of rAAV, a highly efficient *in vivo* gene delivery vehicle with optimized miRNA expression cassettes or inhibitors, will be valuable tools for miRNA functional genomic study and potential therapeutics.

RAAV-mediated miRNA Delivery and miRNA Therapeutics: case studies

The first remarkable breakthrough in miRNA-based therapy is using AAV vector delivered miR-26a to suppress liver cancer in an inducible-cMYC mouse model[12]. In the study, Kota et al found that miR-26a was the most down-regulated miRNA in the liver tumor resulting from the specific activation of cMYC oncogene in hepatocytes. They then built a scAAV construct carrying the pri-miR-26a fragment and packaged it into AAV8 vector, an AAV serotype so efficient in hepatic transfection that it can transduce nearly every hepatocyte in an individual mouse. After a single tail vein injection, there was a dramatic decrease of tumor burden, induced by a massive tumor-cell specific apoptosis. By putting back one miRNA into tumor cells, Kota et al achieved noticeable therapeutic benefit, leading the way in a novel and exciting therapeutic strategy for the treatment of liver cancer and the other diseases.

Another successful example of rAAV delivered miRNA therapy was accomplished through the use of rAAV9, which can cross blood brain barrier [61-63] . RAAV9 was used to deliver miR-196a to treat spinal-bulbar muscular atrophy (SBMA) [64]. In this study, the authors compared the miRNA expression profile in the spinal cords of the diseased mice with those of wild type mice and found that miR-196a, along with 4 other miRNAs, were up-regulated more than two-fold during the advanced stage of this mouse model disease. The researchers found evidence that miR-196a was up-regulated in the diseased mice as a protective mechanism against the progression of the disease and decided that they would attempt to treat the disease by aiding the natural up-regulation of miR-196a by bolstering the amount of this miRNA with a delivery of exogenous miR-196a. After miR-196a was successfully delivered via rAAV9, they found the disease related gene expression was down-regulated, resulting in the improvement of not only mouse behavior but of body weight and mouse survival. The benefit from miR-196a over-expression also indicates endogenous miRNAs can be protective factors in the disease progress.

To understand the function of nearly 300 conserved miRNAs between humans and mice, we can continue to develop the field by producing hundreds of miRNA-KO mouse strains for future research or we can produce mouse miRNA toolkits as described above, applicable and adaptive for use in many animal and cell models. To accelerate analysis of miRNA function in mammals, Xie, *et al*., achieved similar phenotypes as miR-122 KO mice [65,66] by combining the advantages of rAAV vectors and TuD RNAs [34]. Using the same approach, AAV delivered anti-miR-26a TuD delays the differentiation from myoblasts to myotubes[67]. It is likely that the desired results from the development of miRNA somatic KO mice can be obtained more quickly and easily by means of scAAV-delivered TuDs. There is also much to be learned from the up-regulation of miRNA; a goal that is difficult to be addressed by the creation of transgenic overexpression mouse libraries, but can be quite robustly accomplished with the construction of scAAV-delivered pri-miRNAs with a single bonus of rAAV injection.

Prospects and challenges

Over the past few years, miRNA-based therapeutics have achieved great success in many preclinical animal models, such as HCV[29], HCC[12], metabolic disorders[21,56], and cardiovascular disease[68]. The first miRNA-targeted drug, miravirsen (miR-122 antisense oligonucleotide), completed its Phase 2a study recently[25]. The clinical data showed the patients were well tolerated, 2 to 3 logs reduction of HCV RNA and no signs of viral resistance. Miravirsen provides an additional option for the patients who are not responding to interferon therapy and avoids virus mutation because it targets the host gene miR-122 on which the virus relies, not on the virus itself. The clinical results are very attractive, but there are still concerns about the long term safety of miR-122 inhibition in patients. Every single miRNA regulates hundreds of target genes involved in multiple pathways. Modulation of miRNA may easily lead to unwanted outcomes. Indeed, miR-122 inhibition by AMOs or scAAV-delivered TuDs lowered high-density lipoprotein (HDL) and low-density lipoprotein (LDL) simultaneously[28-30,34]. However HDL is regarded as good cholesterol. The most severe concern is the correlation between low miR-122 level and HCC development in patients, although no direct causal link has been established [69-71]. Furthermore, aged miR-122 KO mice developed HCC [65,66]. This germ line depletion of miR-122 may not accurately reflect the real risk of liver cancer in adult HCV patients who only lose miR-122 during the miR-122 antisense oligonucleotide treatment, but it does warn of potential threats. AAV vector expression is stable for years in mice and humans. AAV delivered anti-miR-122 TuD may be used to evaluate the HCC risk of long-term miR-122 inhibition and other side effects.

MiR-26a based liver cancer therapy also confronts the same concern. AAV8 delivered miR-26a can result in tumor suppression in the mouse liver cancer model driven by *cMYC* gene[12], but miR-26a also promotes cholangiocarcinoma growth by activating β-catenin[72]. The activation of Wnt/β-catenin is one of the major pathways involved in HCC[73]. In glioma, miR-26 was also reported as an oncomiR (miRNA associated with cancer) by directly repressing a well-known tumor suppressor[74], *PTEN*. It is a legitimate concern that bolstering levels of miR-26a may worsen the pathogenesis in certain populations of HCC patients.

High unregulated expression of shRNAs delivered by rAAV has been reported to saturate endogenous cellular miRNA machinery and cause fatal effects in mice[75,76]. A surplus of rAAV-delivered shRNAs diminishes two crucial RNAi machineries, Exportin-5 and Argonaut-2 protein [76,77]. To improve the safety, shRNAs were engineered into the position where the mature miRNA duplex is and flanked with native sequence to direct correct processing. By producing less unprocessed precursors and by increasing the effectiveness of processing, this procedure optimization reduced shRNA-mediated toxicity delivered by AAV in the CNS [78,79]. However, this strategy also raised another risk from possibly disturbing another important RNAi machinery factor, Drosha, which is required for the miRNA shuttle strategy. In rAAV mediated miRNA replacement or enforcement, the impact of ectopic miRNAs on RNAi machinery and homeostasis of endogenous miRNAs as well as the off-target effects in targeted tissue and unwanted tissues have not been addressed. On the other hand, AAV delivered TuD RNA has demonstrated efficacy, specificity and safety of

miRNA inhibition [34], but extensive studies particularly in large animal models are still required.

Overall miRNA therapeutics is an emerging field filled with lots of hope. Because of their roles as master regulators in many diseases, miRNAs can achieve previously unreachable medical benefit when compared with conventional mono-target therapeutics. Targeting of multiple genes is the strength of using miRNAs and miRNA inhibitors as therapeutics reagents, but the unique property is also its weakness. Without thoroughly understanding the miRNA functions, miRNA-based therapeutics will be double-edged swords in many cases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AAV	adeno-associated virus
miRNA	microRNA
pri-miRNA	primary miRNA
pre-miRNA	precursor miRNA
NSCLC	non-small-cell lung cancer
HCC	hepatocellular carcinoma
TAC	Transverse aortic constriction
HCV	hepatitis C virus
AMOs	anti-miRNA oligonucleotides
TuD RNAs	tough decoy RNAs
ITR	inverted terminal repeat
scAAV	self-complimentary AAV
HDL	high-density lipoprotein
LDL	low-density lipoprotein
SBMA	spinal and bulbar muscular atrophy
RISC	RNA-induced silencing complexes

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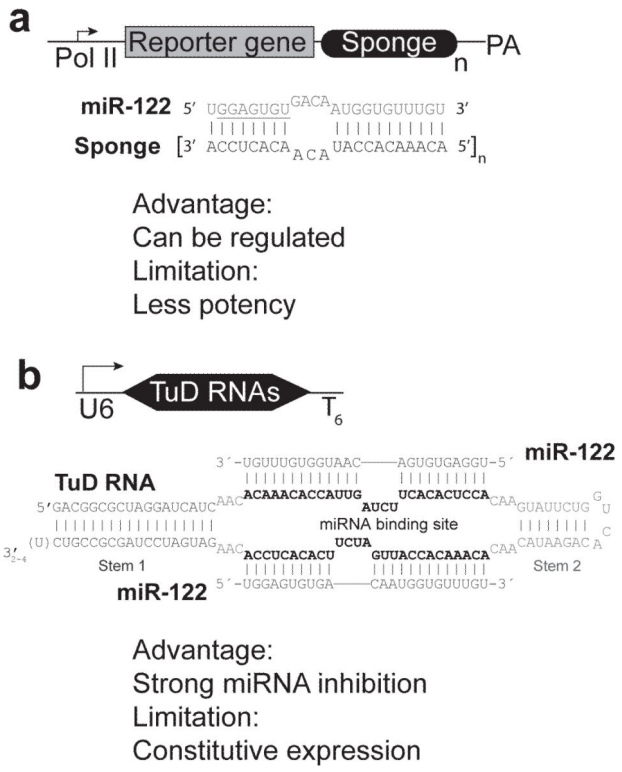

Figure 1.

(A) Sponge is tandem repeats of miRNA binding site after reporter gene driven by Pol II promoters. The imperfect paring between microRNA and sponge is diagrammed for miR-122. **(B)** Tough decoy RNAs (TuDs) contain two single-stranded miRNA binding sites, flanked by double-stranded stems that enhance stability and promote nuclear export. U6 promoter is used for high level expression of the miRNA inhibitors.

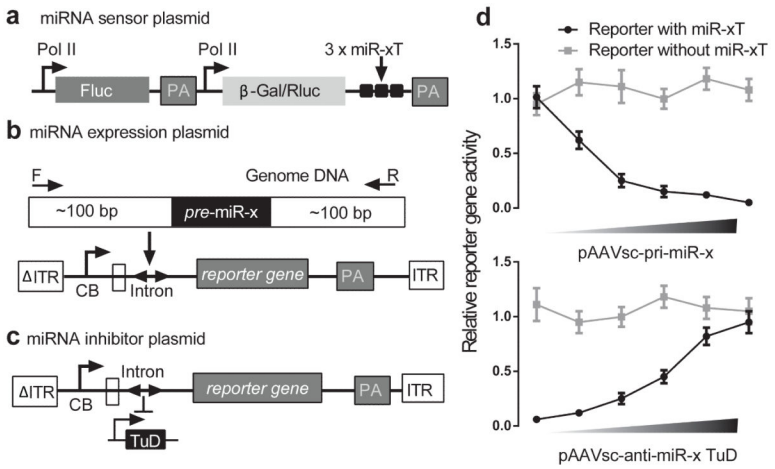

Figure 2.

Validation of miRNA toolkit in HEK293 cells. **(A)** miRNA sensor plasmid to monitor the miRNA activity. The strategy for construction of AAV plasmids expressing functional pri-miRNA fragments **(B)** and miRNA inhibitors, TuD RNAs **(C)**. Cross validation of miRNA expression and inhibition plasmids **(D)**. Top at **(D)** shows increasing amounts of a pri-miRNA producing vector inhibits expression of a miRNA sensor plasmid in HEK293 cells. The bottom shows the de-repression from anti-miR TuDs in a dose response manner when we fix the amount of pri-miR plasmids. ITR, inverted terminal repeat; ΔITR, mutated ITR; CB, chicken β-actin promoter with CMV enhancer; U6, U6 promoter; PA, poly (A); pre-miR, precursor miRNA; Fluc, Firefly luciferase; Rluc, Renilla luciferase; β-Gal, β-galactosidase; 3 × miR-xT, 3 miRNA perfect target sites.

Table 1

Summary of delivering miRNA or inhibitors to targeted tissues by the combinations of AAV serotypes and route of vector administration

		Route of Vector Administration	
Target Tissue	**AAV Serotype**	**Neonates**	**Adults**
Liver	AAV8	Not suitable because of hepatocyte division	Intravenous
Pancreas	AAV7, AAV8, or AAV9	Not efficient	Intravenous or retrograde bile duct injection
Heart	AAV9	Intravenous or intrapericardial	Intravenous
Lung	AAV5, AAV9, or rh.10	Intravenous	Intratracheal or intranasal
Skeletal Muscle	AAV1, AAV7, or AAV9	Intravenous of AAV9 only	Intramuscular or isolated limb perfusion (AAV9)
Brain	AAV9, rh.8, or rh.10	Intravenous or intracerebral ventricular	Intravenous or intracranial