MicroRNA-regulated transgene expression systems for gene therapy and virotherapy

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1. ABSTRACT

For safe and effective gene therapy, targeted tissue-restricted transgene expression is desirable. Various methods have been developed to achieve such expression, including the use of tissue-specific promoters. In addition to these approaches, a new system which can regulate transgene expression, including viral gene expression, by exploiting microRNAs (miRNAs) has recently been developed. miRNAs are approximately 22-nucleotide (nt)-long non-coding RNAs that translationally suppress or catalytically degrade target mRNA through binding to imperfectly complementary sequences in the 3’-untranslated region (UTR). In miRNA-regulated transgene expression systems, tandem copies of sequences perfectly complementary to the miRNAs are usually incorporated into the 3’-UTR of the transgene expression cassette, leading to the suppression of transgene expression in cells expressing the corresponding miRNAs. miRNA-mediated regulation of transgene expression was first demonstrated for lentivirus vectors, and subsequently this technology was applied to replication-incompetent adenovirus vectors, tumor-specific oncolytic adenovirus vectors, and recombinant live attenuated viruses for cancer therapy, and recombinant live attenuated viruses for vaccine therapy. The aim of this review is to highlight the applications of miRNA-regulated transgene expression systems for gene therapy and virotherapy.

2. INTRODUCTION

Transgenes should be delivered and expressed in a targeted tissue-specific manner to achieve safe and effective gene therapy. In order to specifically and actively deliver transgenes to the targeted tissue (active targeting), several approaches have been used. For example, ligation of monoclonal antibodies against the ligands specific for targeted cells to various gene delivery vehicles, including retrovirus vectors and adenovirus (Ad) vectors, has been used to successfully deliver transgenes to targeted tissues/organs (1, 2). Glycosylation of non-viral vectors, including cationic liposomes and polymers, has also been used to efficiently deliver transgenes to the liver, which expresses asialoglycoprotein receptors that recognize sugar moieties (3, 4). On the other hand, “passive targeting”, has also been achieved. In this technique, uptake of gene delivery vehicles by nontargeted cells is avoided, resulting in prolonged retention in the blood circulation and accumulation of gene delivery vehicles in targeted tissues with leaky vascular structures (e.g., tumors) via the enhanced permeability and retention (EPR) effects. For example, covalent conjugation of polyethylene-glycol (PEG) to Ad vectors results in a reduction of liver accumulation of Ad vectors (5) and an increase in tumor accumulation of Ad vectors (6).
miRNA-regulated transgene expression system

In addition to approaches which regulate the tissue distribution of gene delivery vehicles after in vivo administration, various methods have also been developed to regulate the transcription and translation of transgenes within the tissues. Among these approaches, a transcriptional targeting approach using tissue-specific promoters has been extensively studied (7, 8). Various types of tissue-specific promoters, including liver-specific human albumin promoter and tumor-specific telomerase reverse transcriptase promoter, have been shown to mediate targeted tissue-specific transgene expression in gene therapy studies (9-11).

Moreover, Naldini’s group recently demonstrated a new approach known as “transcriptional de-targeting”, which exploits the tissue-specific expression profiles of microRNAs (miRNAs) (12, 13). As described in detail below, miRNAs are approximately 22 nucleotide (nt)-long non-coding RNAs that are endogenously expressed in a tissue-specific pattern (14, 15). miRNAs bind to imperfectly complementary sequences in the 3’-untranslated region (UTR) of targeted miRNAs, and thereby suppress the expression of targeted genes by inhibiting the translation of the mRNA or degrading the mRNA (16, 17). Utilizing this miRNA-mediated post-transcriptional gene-silencing mechanism, transgene expression, including viral gene expression, can be efficiently suppressed in cells expressing the corresponding miRNAs, by insertion of the miRNA complementary sequences into the 3’-UTR of the transgene expression cassette (Figure 1). In transcriptional targeting using tissue-specific promoters, transgene expression is positively regulated in targeted tissues, whereas transgene expression is negatively controlled in a tissue-specific pattern in miRNA-regulated post-transcriptional de-targeting systems. Undesirable transgene expression in non-targeted tissues can be exclusively suppressed by miRNA-regulated transgene expression systems, leading to an enhanced safety profile. In addition, by successfully exploiting the tissue tropism of the gene delivery vehicles, targeted tissue-specific transgene expression can be achieved using miRNA-regulated transgene expression system. In this paper, we review the application of miRNA-regulated transgene expression systems for gene delivery vehicles, recombinant oncolytic viruses, and live attenuated vaccines.

3. MECHANISM OF MI RNA-MEDIATED POST-TRANSCRIPTIONAL GENE SILENCING

miRNAs are approximately 22 nt-long single-stranded non-coding RNAs that were first identified in C.elegans in 1993 by Lee et al. (18). miRNAs are ubiquitously expressed in eukaryotes, and form base pairs with imperfectly complementary sequences in the 3’-UTR of target mRNAs, resulting in post-transcriptional gene silencing via suppression of mRNA translation and/or catalytic degradation of target mRNA. More than 30% of genes are expected to be regulated by miRNAs, and one miRNA would regulate several hundreds genes (18, 19). Many studies have demonstrated that miRNAs play a major role in various diseases, including cancer, inflammatory responses, and chronic diseases (20-22). Stepwise processing and cellular localization of miRNAs are shown in Figure 2. miRNAs are mainly transcribed by RNA polymerase II as part of one arm of an approximately 80-nt stem-loop that forms part of a several hundred nt-long miRNA precursor termed a pri-miRNA (23). Pri-miRNAs are often generated from the intron or UTR of an mRNA (24). This stem-loop structure is cleaved in the nucleus by the nuclear RNase III Drosha coupled with its cofactor DiGeorge syndrome critical region 8 (DGCR8), producing approximately 60-nt hairpin loop termed a pre-miRNA (25, 26). Pre-miRNAs are then exported from the nucleus into the cytoplasm by exportin-5 (27), and subsequently cleaved by the cellular RNase III Dicer (28). Dicer processing produces an miRNA duplex. After cleavage by Dicer, one strand (the guide strand) of the miRNA duplex is incorporated into the RNA-induced silencing complex (RISC), which contains argonaute 2 (Ago2). The mature miRNA functions to guide the recognition of target mRNAs, while the passenger (sense) strand is degraded. Which strand will be incorporated into RISC depends on the thermal instability at the 5’ and 3’ ends of the miRNA duplex (29). The strand with higher 5’ thermal instability remains in the RISC and works as a guide strand. Although various mechanisms of miRNA-mediated post-transcriptional gene silencing have been proposed (30-34), further examination is required to elucidate the exact mechanism of miRNA-mediated post-transcriptional gene silencing; however, a perfect complementarity between seven consecutive nucleotides in the 3'-UTR of the target mRNA and the 2-8 nt at the 5’ end of the miRNA, termed the “seed sequence”, is known to be required for the efficient suppression of target gene expression (35).

More than 900 miRNAs have currently been identified in humans (http://www.mirbase.org/), and most of these miRNAs are differentially expressed in different tissues in order to regulate tissue-specific gene expression. Table 1 shows the miRNAs expressed in a tissue-specific pattern. The tissue-specific expression pattern of miRNAs is the most important property for the miRNA-regulated transgene expression system. The expression levels of transgenes encoding miRNA complementary sequences in the 3’-UTR are reduced in cells abundantly expressing the corresponding miRNA. Therefore, we should pay attention to which miRNAs are highly expressed in cells in which transgene expression should be avoided.

In addition to the tissue-specific expression profile of miRNAs, several miRNAs are specifically down-regulated or up-regulated in tumors (Table 2). Recent studies have revealed that human miRNAs are frequently encoded at fragile sites of the genome, which are associated with cancer (36, 37). For example, miR-15a and miR-16a, which are often down-regulated in chronic lymphocytic leukemia B cells, are located at chromosome 13q14 (38). Deletion of this region is often found in cancer patients (38). Furthermore, expression levels of miRNA-processing factors, including Dicer and Ago2, are also down- or up-regulated in several types of cancer (39-41). Alteration of the expression profiles of miRNAs and miRNA-processing factors would contribute to tumorigenesis and metastasis.
miRNA-regulated transgene expression system

Figure 1. Schematic diagram of an miRNA-regulated transgene expression cassette. Multiple copies of the sequences perfectly complementary to the miRNAs were inserted into the 3'-UTR of the transgene expression cassette. pA, poly A signal; RISC, RNA-induced silencing complex.

Figure 2. Intracellular maturation steps of miRNAs and the mechanism of miRNA-mediated post-transcriptional gene silencing.
Table 1. Tissue/organ-specific microRNAs.

<table>
<thead>
<tr>
<th>Tissue/Organ</th>
<th>miRNA</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>miR-9, miR-124, miR-125</td>
<td>80, 101</td>
</tr>
<tr>
<td>Heart</td>
<td>miR-1, miR-133a, miR-208</td>
<td>100, 102, 103</td>
</tr>
<tr>
<td>Blood cells</td>
<td>miR-16, miR-142-5p, miR-142-3p, miR-223</td>
<td>104</td>
</tr>
<tr>
<td>Liver</td>
<td>miR-122a</td>
<td>59</td>
</tr>
<tr>
<td>Pancreas</td>
<td>miR-375</td>
<td>105</td>
</tr>
<tr>
<td>Spleen</td>
<td>miR-142-5p, miR-142-3p, miR-150</td>
<td>104, 106</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>miR-1, miR-133a, miR-206</td>
<td>102, 107</td>
</tr>
<tr>
<td>Kidney</td>
<td>miR-192, miR-215, miR-216</td>
<td>108</td>
</tr>
<tr>
<td>Ovary</td>
<td>miR-542-5p</td>
<td>109</td>
</tr>
<tr>
<td>Testis</td>
<td>miR-10b, miR-23a, miR-204</td>
<td>110</td>
</tr>
</tbody>
</table>

Table 2. MicroRNAs selectively up- or down-regulated in tumor cells.

<table>
<thead>
<tr>
<th>Organ</th>
<th>miRNA</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast Cancer</td>
<td>miR-21 (U), miR-125b (D), miR-145 (D)</td>
<td>111, 112</td>
</tr>
<tr>
<td>Glioblastoma</td>
<td>miR-7 (D), miR-21 (U), miR-221 (U)</td>
<td>113, 114</td>
</tr>
<tr>
<td>Lung Cancer</td>
<td>Let-7 (D), miR-155 (U), miR-17-92 (U)</td>
<td>75, 82, 115</td>
</tr>
<tr>
<td>Liver Cancer</td>
<td>miR-18 (U), miR-122a (D), miR-199a (D)</td>
<td>116, 117</td>
</tr>
<tr>
<td>Bladder Cancer</td>
<td>miR-133a (D), miR-133b (D), miR-143 (D)</td>
<td>118, 119</td>
</tr>
<tr>
<td>Prostate Cancer</td>
<td>miR-125b (D), miR-145 (D), Let-7c (D)</td>
<td>120</td>
</tr>
<tr>
<td>Ovary Cancer</td>
<td>miR-143 (D), miR-199a (D), miR-200c (U)</td>
<td>121</td>
</tr>
<tr>
<td>Colorectal Cancer</td>
<td>miR-31 (U), miR-143 (D), miR-145 (D)</td>
<td>73, 74, 122</td>
</tr>
<tr>
<td>Chronic Lymphatic Leukemia</td>
<td>miR-15 (D), miR-16 (D)</td>
<td>123</td>
</tr>
<tr>
<td>Burkitt Lymphoma</td>
<td>miR-155 (D)</td>
<td>124</td>
</tr>
</tbody>
</table>

D; down-regulated, U; up-regulated

As described in Sections 5-2 and 5-3, the differences in miRNA-expression levels between tumor and normal cells can be exploited for miRNA-mediated regulation of transgene expression, especially for oncolytic viruses.

4. FACTORS TO BE CONSIDERED FOR THE CONSTRUCTION OF MIRNA-REGULATED TRANSGENE EXPRESSION SYSTEMS

The simplest, but most important factor for construction of an miRNA-regulated transgene expression system is the copy number of miRNA-complementary sequences in the 3’-UTR. In studies using miRNA-regulated transgene expression systems, four copies of miRNA target sequences are often incorporated into the transgene expression cassette (12, 13, 42-46), because insertion of four copies of miRNA target sequences has been shown to more efficiently suppress the reporter gene expression than insertion of two copies, while the suppression levels are comparable between four and six copies (47). In addition to optimization of the copy number of miRNA target sequences, the use of a combination of more than one miRNA target sequence seems a promising approach to mediate a further reduction in gene expression (48), especially when the expression levels of the miRNAs are not high enough to efficiently suppress the gene expression. In such cases, the different miRNAs would cooperatively suppress gene expression.

The expression levels of miRNAs are a crucial determinant for the miRNA-mediated regulation of gene expression. Brown et al. demonstrated that there is a threshold miRNA expression level (100 copies/pg small RNA) for the reliable suppression of gene expression (12). We also observed that highly expressed miRNAs tend to mediate a larger reduction in the transgene expression; however, the miRNA expression levels are not the only factor determining the levels of gene suppression in the miRNA-mediated gene expression system. Secondary structures around the region incorporating miRNA complementary sequences would also be involved in the miRNA-mediated suppression levels, as described below.

Sequences perfectly complementary to miRNAs are incorporated into the gene expression cassette in miRNA-mediated gene expression systems. Although miRNAs usually bind to endogenous target mRNA with sequences that are imperfectly complementary to the miRNAs, and induce post-transcriptional gene silencing, insertion of perfectly complementary sequences leads to larger reduction in the gene expression than insertion of imperfectly complementary sequences (47, 49). In addition, Ebert et al. reported that the association between imperfectly complementary sequences and RISC would be more stable than that between perfectly complementary sequences and RISC (50), suggesting that recycling of RISC for post-transcriptional silencing might be faster for perfectly complementary sequences than imperfectly complementary sequences and that the faster recycling of RISC might lead to the more efficient suppression of transgene expression.

In general, the miRNA-regulated gene expression cassette remains to be fully optimized for specific and efficient miRNA-mediated regulation. Secondary structures of mRNA-encoding miRNA target sequences would be a crucial determinant for efficient suppression of transgene expression via miRNAs; however, such secondary structures have not been fully examined. Several factors which determine the secondary structures, including the sequences between tandem copies of miRNA target sequences, should be evaluated for optimization of the miRNA-regulated gene expression cassette.

5. APPLICATION OF MIRNA-REGULATED TRANSGENE EXPRESSION SYSTEMS

5.1. Regulation of suicide gene expression

Among the various approaches to cancer gene therapy, suicide cancer gene therapy is considered to be one
of the most promising, and has already been studied in human clinical trials (51, 52). In suicide gene therapy, a suicide gene, which encodes an enzyme converting nontoxic prodrugs into toxic compounds, is expressed in target cancer cells, and nontoxic prodrugs are separately administered to patients, resulting in induction of cancer cell death. Herpes simplex virus thymidine kinase (HSVtk) and ganciclovir (GCV) are often used as a suicide gene and prodrug, respectively. Nontoxic GCV is phosphorylated by HSVtk, resulting in inhibition of DNA replication. In addition, the cytotoxic effects of phosphorylated GCV are enhanced by a so-called bystander effect, namely, the transfer of phosphorylated GCV from HSVtk-expressing cells to neighboring cells through cellular gap junctions (53, 54). As described above, the phosphorylated GCV inhibits DNA synthesis, and thus the phosphorylated GCV is more toxic to actively dividing tumor cells than nontargeting cells. However, the phosphorylated GCV is also toxic to normal cells which do not actively divide (55, 56), indicating that HSVtk expression in non-target cells should be avoided.

In adenovirus (Ad) vector-mediated suicide gene therapy, severe hepatotoxicity is induced because of the strong hepatotropism of Ad vectors, although efficient antitumor effects are also observed (57, 58). Even after local administration of Ad vectors expressing HSVtk into the tumors, the Ad vectors are disseminated from the tumor into the systemic circulation and efficiently transduce the liver, leading to severe hepatotoxicity. In order to suppress the Ad vector-mediated transduction in the liver, four copies of sequences complementary to liver-specific miR-122a have been included in the 3'-UTR of the HSVtk expression cassette (42). miR-122a is highly expressed in the liver hepatocytes (135,000 copies/cell in human primary hepatocytes) (59). Insertion of the miR-122a complementary sequences mediates a dramatic, approximately 100-fold reduction in the hepatic transduction with the Ad vector following intratumoral administration, resulting in suppression of the hepatotoxicity induced by the HSVtk-expressing Ad vector (body weight loss and elevation in serum alanine aminotransferase levels). On the other hand, the antitumor effects of the HSVtk-expressing Ad vectors are not altered by inclusion of the miR-122a complementary sequences. Reduction in HSVtk expression in nontarget cells through the insertion of the miRNA target sequences into baculovirus vectors has also been demonstrated (48). In a study by Wu et al., in addition to a glioma-specific promoter (a glial fibrillary acidic protein (GFAP) promoter), three copies of each of miR-31, -127, and -143 (total 9 copies) were incorporated into the HSVtk expression cassette in the baculovirus vectors to suppress the HSVtk expression in normal neural cells and to realize glioma cell-specific HSVtk expression (48). miR-31, -127, and -143 showed significantly higher expression levels in normal neural cells than glioma cells. Reduction in HSVtk gene expression in nontarget cells leads to suppression of HSVtk/GCV-induced side effects, which makes it possible to increase the injected doses of HSVtk-expressing vectors and GCV, resulting in enhanced antitumor effects.

5.2. Regulation of DNA virus replication

Recently, much attention has been focused on oncolytic viruses, which show antitumor effects via specific replication of viruses in tumor cells. Oncolytic viruses are classified into two groups. The first, nonrecombinant viruses, takes advantage of tumor-specific mutations or virus receptors overexpressed in tumor cells, leading to tumor-specific infection. The viruses of the second group, recombinant viruses, are genetically engineered to show tumor-specific replication. In the case of oncolytic DNA viruses, most of the viruses that have been developed are based on Ad and herpesvirus, and clinical trials using these oncolytic DNA viruses have been performed (60-62).

Various types of oncolytic Ads have been developed; however, the most popular types of oncolytic Ads are those containing the E1A gene expression cassette driven by a tumor-specific promoter. The E1A gene is indispensable for self-replication of Ads. Various types of tumor-specific promoters, including the alpha-fetoprotein promoter (63), prostate-specific antigen promoter (64), osteocalcin promoter (65), and cyclooxygenase-2 promoter (66), are used for expression of the E1A gene. The tumor-specific E1A expression renders the oncolytic Ads tumor-specifically replicative; however, to a lesser extent, oncolytic viruses also replicate in normal cells, leading to unexpected toxicity. In order to suppress the replication of oncolytic Ads in normal cells, an miRNA-regulated gene expression system was included in the virus genome.

Among the various types of normal cells, liver hepatocytes are one of the major targets via which oncolytic Ads mediate side effects, because Ads originally have strong hepatotropism, as described above. Oncolytic Ads accumulate in the liver after dissemination from the tumor into the blood stream, leading to the concern that oncolytic Ads might replicate in the hepatocytes. In order to suppress the replication of oncolytic Ads in the hepatocytes, tandem copies of sequences complementary to liver-specific miR-122a were inserted into the 3'-UTR of the E1 gene (67-69). Recombinant Ads containing miR-122a complementary sequences in the wild-type E1A promoter-driven E1 gene expression cassette exhibited reduced replication in Huh-7 cells, which is a hepatoma cell line expressing a high level of miR-122a, and reduced hepatotoxicity in mice (68). However, oncolytic Ads replicate and are disseminated from the injected tumors, and infect un.injected, distal tumors after intratumoral injection (70, 71), indicating that Ads would infect not only hepatocytes but also normal cells over the body after dissemination from the tumor. We have developed oncolytic Ads containing multiple copies of sequences complementary to normal cell-specific miRNAs in order to suppress the replication of oncolytic Ads in not only hepatocytes but also other types of normal cells (72). miRNA array analysis of tumors and the adjacent normal tissues revealed that several miRNAs, including miR-143, -145, and -199a, are exclusively down-regulated in tumors (73-75). Oncolytic Ads containing target sequences for miR-143, -145, and -199a exhibited dramatically reduced replication in normal human primary lung fibroblasts and
prostate stromal cells, without altering the antitumor effects.

An oncolytic herpesvirus carrying a miRNA-regulated replication system has also been developed (76). The ICP4 gene is indispensable for self-replication of herpes simplex virus-1 (HSV-1). Four or five tandem copies of sequences complementary to miR-143 or -145 were inserted into the 3'-UTR of the ICP-4 gene. The HSV-1 amplicons containing the sequences complementary to miR-143 exhibited a significant reduction in virus spread in normal tissue after intratumoral injection, without altering the antitumor effect of the HSV-1 amplicons.

5.3. Regulation of RNA virus replication

In addition to the oncolytic DNA viruses described above, an miRNA-regulated virus replication system has been included in oncolytic RNA viruses. Vesicular stomatitis virus (VSV), which is a negative-stranded RNA virus, was genetically modified to contain the miRNA complementary sequences in the 3’-UTR of the M gene or L gene (43). The M gene encodes a matrix protein indispensable for viral replication. The L gene is expressed at the lowest levels of all viral genes, suggesting that this gene would be vulnerable to miRNA-mediated suppression. VSV has been considered a promising vaccine platform and anticancer agent; however, previous studies have demonstrated that VSV causes severe neuropathogenesis (77-79). In order to suppress the replication of VSV in neural cells, sequences complementary to miR-125, which is highly expressed in all cells in the brain (80), were inserted into the 3'-UTR of the M or L gene. Recombinant VSV encoding miR-125 target sequences exhibited significantly reduced neurotoxicity after intracranial administration, although the in vivo antitumor effects of the recombinant VSV were not disturbed. Edge et al. incorporated three copies of Let-7a target sequences into the 3'-UTR of the M gene to suppress the replication of VSV in normal cells (81). The expression levels of Let-7a were demonstrated to be exclusively down-regulated in the tumor cells (82). VSV containing the Let-7a complementary sequences showed reduced replication in the primary human fibroblast cell line MG38, while efficient replication of the recombinant VSV was achieved in A549 cells (a human lung adenocarcinoma epithelial cell line).

Tropism of coxsackievirus A21 (CVA21), which is a positive single-stranded RNA virus, was also regulated by incorporation of the miRNA complementary sequences (45). CVA21 has been demonstrated to mediate efficient oncolytic activity (83), although it has also been shown to cause respiratory tract infection and myositis (84, 85). Two copies each of sequences complementary to muscle-specific miR-133 and -206 were inserted into the 3'-UTR of the virus genome to suppress the replication of CVA21 in muscle cells and myositis. Insertion of the sequences complementary to miR-133 and -206 dramatically reduced the symptoms of myositis following intratumoral administration.

MiRNA-mediated post-transcriptional gene silencing is available for the development of live attenuated vaccines. Live attenuated vaccines are the most effective against infectious diseases; however, there are no rational approaches to attenuate virus pathogenicity. Classically, live attenuated vaccines are often developed by altering the cell tropism of a virus after repeated passage in cell culture, yet a limited number of safe and effective vaccines have been developed. In order to rationally design safe and effective live attenuated vaccines, miRNA complementary sequences were inserted into the virus genome, resulting in alteration of virus tropism. Barnes et al. developed a recombinant poliovirus encoding sequences complementary to Let-7a, which is ubiquitously expressed, or neuron-specific miR-124 (86). Polioviruses infect motor neurons in the spinal cord and brainstem, leading to poliomyelitis. Incorporation of the miRNA target sequences resulted in efficient suppression of the replication of the recombinant polioviruses, leading to enhanced safety profiles after inoculation. The protective immunity induced by inoculation with the recombinant polioviruses was not altered by insertion of the miRNA complementary sequences. Recombinant influenza viruses encoding miRNA complementary sequences have also been developed (87). In order to develop attenuated influenza viruses which exhibit protective effects in humans without reducing the vaccine yield in eggs, sequences complementary to miR-93, which is ubiquitously expressed in mammals but not expressed in birds, were inserted into the virus genome. The miRNA complementary sequences were incorporated into the open reading frame of segment five, which encodes a nucleoprotein, in the recombinant influenza virus because there were no appropriate sites for insertion of miRNA target sequences in the genome; however, influenza viruses H1N1 and H5N1 were significantly attenuated by insertion of the miR-93 target sequences without altering virus yield in eggs.

One major concern with RNA viruses containing an miRNA-regulated replication system is the potential for mutation in the miRNA complementary sequences, which could result in escape from the miRNA-mediated regulation of virus tropism. Viral RNA polymerase has high error rates (1x10⁻³ to 1x10⁻⁵ errors per nucleotide site per round of infection) (88). Mutations in the miRNA target sequences have been found in the recombinant CVA21 isolated from a few of the mice treated with the recombinant CVA21 containing the miRNA complementary sequences (45), although no mutation was found in the miRNA target sequences of the VSV genome (43) or the influenza virus genome (87). The best way to avoid the escape of an RNA virus from the miRNA-regulated virus tropism by mutation in the miRNA target sequences would be to increase the copy numbers and insertion sites of the miRNA target sequences. Alternatively, insertion of miRNA complementary sequences into the open reading frame of genes in which mutation is not readily allowed for replication is a promising approach, if the miRNA complementary sequences encoded in the open reading frame can efficiently suppress the target gene expression. miR-93 target sequences inserted into the open reading frame of the influenza virus nucleoprotein gene, which is highly conserved, were not mutated after either both in vitro or in vivo infection (87).
miRNA-regulated transgene expression system

The genome type of single-stranded RNA viruses, i.e., positive-strand or negative-strand, would also affect the miRNA-mediated suppression efficiency of target genes. miRNAs directly target the positive-stranded RNA genome, but miRNAs bind to the mRNA which is transcribed from the negative-stranded RNA genome. VSV, which is a negative-stranded virus, was more resistant to the miRNA-regulated virus replication system than positive-stranded CVA21 (43). The difference in the resistance to the miRNA-mediated suppression of virus replication might be partly due to the difference in the genome type between CVA21 and VSV.

5.4. Regulation of immune responses against transgene products

Undesirable immune responses against transgene products should be avoided in order to obtain sustained gene expression in gene therapy, especially for inherited genetic diseases, such as hemophilia. The generation of neutralizing antibodies to transgene products leads to rapid clearance of transgene products from the blood circulation, and induction of transgene product-specific cytotoxic T cells results in elimination of cells expressing the transgene products (89-91). Various strategies have been developed for the immune suppression against transgene products (92, 93), including use of immune suppressive agents (94, 95); however, a strategy of specifically blocking only the immune responses against transgene products is preferable. For this purpose, the miRNA-regulated transgene expression system offers a highly promising strategy. Incorporation of four copies of sequences complementary to miR-142-3p, which is specifically expressed in hematopoietic cells, including antigen-presenting cells (APCs), prevented the induction of immune responses against clotting factor IX (FIX) (96), green fluorescence protein (GFP) (97), and bilirubin-uridine 5’-diphosphate-glucuronosyltransferase (UGT1A1) (98), via the suppression of transgene expression in APCs. Suppression of transgene expression in APCs by insertion of the miR-142-3p complementary sequences induced a population of transgene product-specific cytotoxic T cells, leading to immunologic tolerance to transgene products. Both humoral and cellular immunity can be suppressed by this system. Sustained transgene expression via the miR-142-3p-regulated transgene expression cassette significantly enhanced the therapeutic effects. Brown et al. demonstrated that introduction of FIX into hemophilia B mice via a lentivirus vector encoding the miR-142-3p target sequences into the 3’-UTR of the FIX expression cassette successfully resulted in sustained FIX expression without anti-FIX immune responses, and phenotypic correction of the bleeding diathesis in hemophilia B mice (96). It remains to be clarified how the regulatory T cells are induced by miR-142-3p-mediated suppression of transgene expression in APCs. In a study by Annoni et al., the simultaneous suppression of transgene expression in both hepatocytes and APCs by incorporation of target sequences to miR-142-3p and liver-specific miR-122a reduced a population of regulatory T cells, indicating that not only suppression of transgene expression in APCs but also transgene expression in hepatocytes is crucial for induction of transgene product-specific regulatory T cells by suppression of transgene expression in APCs (97). In the miR-142-3p-regulated transgene expression system for induction of immune tolerance, choice of promoters is important. Immune tolerance against the transgene products was successfully induced by insertion of the miR-142-3p target sequences into the expression cassette containing the liver-specific promoter or phosphoglycerate kinase (PGK) promoter; however, the induction of immune tolerance against the transgene products was not found when a cytomegavirus promoter that, ubiquitously and strongly drives the transcription, was used (46). Combinatorial regulation of transgene expression via tissue-specific promoters and miRNAs would be required for the induction of immune tolerance.

5.5. Other applications

The miRNA-regulated transgene expression system has also been used to track the differentiation of progenitor/stem cells. Sachdeva et al. demonstrated that the differentiation status of ES cells could be checked and visualized by using an ES cell-specific miRNA-regulated GFP-expression system (44). Mouse and human ES cells were transduced with lentivirus vectors containing the miR-292 target sequences in the 3'-UTR of the GFP expression cassette. miR-292 is abundantly expressed in undifferentiated ES cells; however, miR-292 expression is significantly downregulated after differentiation. GFP expression in mouse and human ES cells was hardly detected in undifferentiated ES cells; however, GFP expression was significantly increased after differentiation into neural progenitor cells due to the down-regulation of miR-292. These authors further demonstrated that the pluripotent marker gene Oct4 was not expressed in the GFP-positive neural progenitor cells and that purification of GFP-positive cells by fluorescence activated cell sorting (FACS) improved the survival of the transplanted grafts.

miRNAs can be knocked-down in vivo by an miRNA-regulated transgene expression system (50, 99). Ebert et al. demonstrated that over-expression of transcripts containing tandem binding sites to an miRNA of interest (a method referred to as an “miRNA sponge”) resulted in derepression of endogenous genes targeted by the miRNA (50). Transcripts containing miRNA target sites serve as a competitive inhibitor (decoy) of the miRNA. The derepression levels of the miRNA target gene expression by the miRNA sponge were comparable to those by chemically modified antisense oligonucleotides against the miRNA (50). The role of miR-133 in cardiac hypertrophy was demonstrated by using Ad vectors expressing the GFP gene containing the miR-133 target sequences in the 3’-UTR (100). The advantage of the miRNA sponge over chemically modified antisense oligonucleotides is that the miRNA sponge can stably derepress the miRNA target genes by integrating the miRNA sponge expression cassette into the genome. In addition, various types of viral vectors, which mediate efficient in vivo transduction, are currently available for the delivery of miRNA sponge expression cassettes.

6. CONCLUDING REMARKS

In this review, we have described the features of the miRNA-regulated transgene expression system. The
miRNA-regulated transgene expression system

miRNA-regulated transgene expression system has several advantages. For example, regulation of transgene expression can be achieved simply by the insertion of approximately 100 bp sequences encoding several copies of miRNA complementary sequences, using conventional genetic engineering techniques. Because the miRNA complementary sequences are small in size, their inclusion does not cause a problem in terms of the packaging capacity for foreign genes, which is often a big problem for viral vectors. In addition, the miRNA-regulated transgene expression system can be easily applied to both non-viral and viral vectors. The miRNA-regulated gene expression system could become a major technique not only for the regulation of transgene expression in gene therapies, but also for regulation of the expression of viral genes in cancer therapies using tumor-specific oncolytic viruses and vaccine therapies using recombinant live attenuated viruses.

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**Key Words:** MicroRNA, Gene therapy, Virotherapy, Transgene expression cassette, 3'-untranslated region.

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