Molecular Therapy
Nucleic Acids
Review

Genetically Encoded Reporter Genes for MicroRNA Imaging in Living Cells and Animals

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MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression by base paring with the complementary sequences of the target mRNAs, and then exert their function through degrading mRNA or inhibiting protein translation. They play a significant role as a regulatory factor in biological processes of organism development, cell proliferation, differentiation, and cell death. Some of the traditional methods for studying miRNAs, such as northern blot, real-time PCR, or microarray, have been extensively used to investigate the biological properties and expression patterns of miRNAs. However, these methods often require considerable time, cell samples, and the design of effective primers or specific probes. Therefore, in order to gain a deeper understanding of the role of miRNAs in biological processes and accelerate the clinical application of miRNAs in the field of disease treatment, non-invasive, sensitive, and efficient imaging methods are needed to visualize the dynamic expression of miRNAs in living cells and animals. In this study, we reviewed the recent progress in the genetically encoded reporter genes for miRNA imaging.

MicroRNAs (miRNAs) are endogenous non-coding small RNAs whose length is about 20–25 nt, encoded by approximately 3% of the human genomes.[1] The first miRNA was found in *Caenorhabditis elegans*, and thousands of miRNAs have been discovered in plants, animals, and viruses to date.[2] miRNAs completely or partially bind to the 3′ untranslated regions (UTRs) of target mRNAs to degrade mRNAs or repress protein translation.[3] A single miRNA can regulate the expression activity of multiple mRNAs, and its effector molecules play a role in different sites of cell signaling pathways and networks. At the same time, an mRNA is also regulated by multiple miRNAs; over 60% of all mRNA expression is regulated by miRNAs.[4] Therefore, miRNAs can switch quickly between cellular programs to form a dense molecular regulatory network.

miRNAs are negative gene regulators at the post-transcriptional level. Mature miRNAs are mainly formed in the nucleus and cytoplasm, and depend on RNA polymerase II, Drosha (double-stranded RNA-specific ribonuclease), and Dicer (RNase III endonuclease) in the formation process (Figure 1). First, under the action of RNA polymerase II, miRNA coding genes are transcribed into primary miRNAs (pri-miRNAs) in the nucleus. Subsequently, the precursor miRNAs (pre-miRNAs) are produced to form a hairpin structure with the specific enzyme action of Drosha. Next, the pre-miRNAs are transported from the nucleus to the cytoplasm under the action of Exportin 5 and then processed into mature double-strand miRNAs by Dicer. Finally, a strand of the duplex mature miRNA is brought into RNA-induced silencing complex (RISC) to base pair with the target mRNA and result in protein translation inhibition or mRNA degradation.[5–8]

In recent years, through analysis of a large amount of animal and human experimental data, it has been found that miRNAs play a key role as a regulatory factor in various biological or pathological processes, such as immune regulation, physiological metabolism, cell proliferation, differentiation, growth, and development by degrading or inhibiting the translation of target RNAs.[9] Overexpression or underexpression of miRNAs can cause various diseases. For instance, miR-122 plays an important role in the replication of hepatitis C virus (HCV).[10] miR-33 can lead to the significant increase of liver ABCA1 expression and plasma high density lipoprotein (HDL) level,[11] and the overexpression of miR-155 can result in chronic inflammation in humans.[12] In addition, miRNAs have been developed as a novel drug target, such as the emerging miRNA-based cancer therapy. Therefore, understanding more biological characteristics of miRNAs and their mechanisms of regulating biological processes through efficient and rapid detection methods is crucial for basic study and clinical application.

Previously, the expression and characteristics of miRNAs were mainly detected by northern blot, real-time PCR, microarray, etc. These methods require a long time of manual operation and are difficult to carry out due to a large-scale screening process. Moreover, miRNAs with small sequence differences cannot be accurately distinguished, and specific primers or probes are difficult to design. Therefore, in order to obtain biological information about miRNAs more efficiently, approaches with high sensitivity and high resolution, that are non-invasive, and that are easier to operate are needed to

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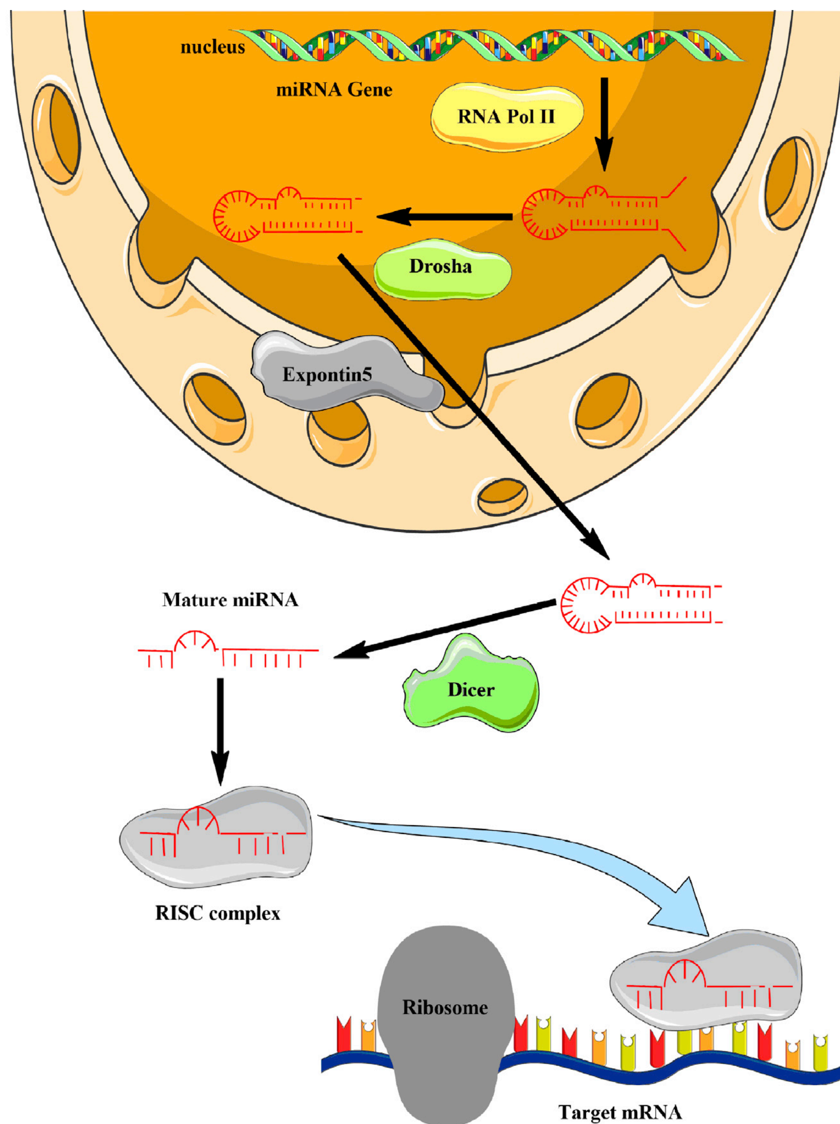

Figure 1. Schematic Overview of miRNA Biogenesis
miRNA genes are transcribed into primary miRNAs (pri-miRNAs) with the RNA polymerase II in the nucleus. Then the pri-miRNA is cleaved by Drosha, producing the precursor miRNAs with hairpin structure. Next, the pre-miRNAs are transported out of the nucleus under the action of Exportin 5 and then processed into mature miRNAs by Dicer. Mature miRNA enters into the RISC complex to bind the target mRNA and results in protein translation inhibition or mRNA degradation.

study the expression pattern of miRNAs. Recently, molecular imaging techniques involving positron emission tomography (PET), magnetic resonance imaging (MRI), and optical imaging have the advantages of higher sensitivity, quantitative analysis, and the ability to image in deep tissues, and they are widely used to detect and evaluate the biogenesis of miRNAs. In this paper, we first reviewed the traditional methods for detecting and evaluating miRNAs, then summarized the reporter gene probes-based imaging of miRNA biogenesis and regulation in living cells and animals.

Traditional Methods for Measuring miRNAs

Northern Blotting

Northern blotting is a process of quantitative visualization of specific RNA by hybridization of DNA with RNA to be detected.[13] First, electrophoretic separation (usually by denatured urea-polyacrylamide gels) is used to separate the RNA mixture by size and molecular weight of the RNA mixture. Then the separated RNA is transferred by semi-dry capillaries to nitrocellulose or positively charged nylon membranes, followed by hybridization with a radioactive ^{32}P-labeled DNA probe that allows for quantitative or deterministic analysis.[14] The intensity of the signal at the imprint represents the quantity of the target RNA, and the position of the signal represents the size of the target. Compared with other traditional methods, northern blotting requires a lot of manual operation, which is not conducive to large-scale screening and high-sensitivity and low-input RNA requirements. Moreover, radioactive isotopes pose some security risks for researchers.[15–17] In order to overcome a series of problems, the hybridizing probes for experiments need to be optimized.

The probe labeled with locked nucleic acid (LNA) can detect miRNA with high sensitivity and specificity, greatly reducing the detection time and increasing the sensitivity by 10 times.[18] However, the cost of hybridizing probes with this method is also greatly increased. The emergence of the 1-ethyl-3-(3-dimethyl-amino-propyl) carbodiimide (EDC)-mediated chemical cross-linking approach has replaced the conventional UV-cross-linking of RNA to nylon membranes.[19] This method is simple, relatively inexpensive, has a wide dynamic range, and is 50 times more sensitive than other traditional methods. However, with the increase of RNA size, the detection ability of EDC cross-linking also declined.

Real-Time PCR

Real-time PCR is considered the gold standard for detecting miRNAs. Real-time PCR is an amplification technique that first utilized miRNA-specific or universal primers for reverse transcription (RT) to obtain cDNA; then SYBR Green or TaqMan probes are added into the PCR system to detect the whole PCR process in real time. Finally, quantitative analysis is carried out through the standard curve.[20] In the whole process, the preparation of samples and the

Table 1. Advantages and Disadvantages of Imaging Techniques Based on Reporter Genes

Imaging Technique	Advantages	Disadvantages	Reporter Gene
Nuclear medicine imaging	high sensitivity unrestricted detection depth accurate quantification simple extrapolation	high cost relatively low resolution	HSV1-TK NIS D2R
Magnetic resonance imaging	high spatial resolution unlimited detection depth possible for quantitative analysis	high cost poor sensitivity	β-galactosidase TYR TfR ferritin lysine-rich protein
Optical Imaging			
Fluorescence imaging	multiplex detection available low cost quick and simple method high sensitivity	depth limit high background	GFP YFP RFP
Bioluminescence imaging	highest sensitivity low cost low background quick and simple method	depth limit	Fluc Rluc Gluc

D2R, dopamine-D2 receptor somatostatin; GFP, green fluorescent protein; Fluc, Firefly luciferase; Gluc, Gaussia luciferase; HSV1-TK, herpes simplex virus type 1-thymidine kinase; NIS, sodium iodide symporter; RFP, red fluorescent protein; Rluc, Renilla luciferase; TfR, transferrin receptor; TYR, tyrosinase; YFP, yellow fluorescent protein.

extraction of RNA are of great importance. Due to the instability of miRNA, it is easy to degrade during the preparation and extraction process, while the analysis results of quantitative PCR (qPCR) largely depend on the purity and integrity of miRNA, so the fractionation of RNA extract can remove unnecessary pri-miRNA sequences and reduce the background in the qPCR process. qPCR requires less input RNA and is highly sensitive, providing a good balance between cost, accuracy, and sample size. But mature miRNAs are short, making it difficult to design effective specific primers or probes. It has been proposed to use pre-miRNA as a surrogate marker for mature miRNA, but the level of pre-miRNA cannot effectively indicate the level of mature miRNAs.

Some new cDNA synthesis methods, such as the use of miRNA-specific stem-loop primers, can distinguish between different forms of miRNAs without fractionation.[21] This method uses stem-loop structure primers, which can reduce the background of qPCR for more accurate quantitative detection of mature miRNAs, but this also hinders RT of isotopic heterogeneous sequences. The other method is to employ the extension of RT primers to complete the process of RT of circulating miRNA. miRNA produces tandem repeats of cDNA sequences through many times of intermolecular connections and RT.[22] In addition, Choi et al.[23] proposed a new qPCR technique based on hydrogel micro-columns to detect multiple miRNAs associated with Alzheimer's disease (AD). Jung et al.[24] designed a qPCR method, to analyze the expression of multiple miRNA targets using micro-particles of primer-immobilized networks as discrete reactors. Currently, real-time PCR technology plays a significant role in miRNA research and can be applied to accurate quantitative detection of miRNA expression.

Microarray

Based on the hybridization principle, microarray can simultaneously quantitatively detect the expression level of multiple miRNAs and analyze the regulation of miRNAs by analyzing the expression level of miRNAs. First, miRNAs were isolated and prepared, and multiple high-density fluorescent probes complementary to the target miRNA sequences were fixed in the solid phase to prepare the detection chip. Then the labeled miRNAs were hybridized with the detection chip to capture the signals. The most commonly used labeling method of miRNA is enzyme attachment labeling. One method is the fluorogenic labeled nucleotide or short oligonucleotide attached to the 3′-OH of RNA under the action of T4 ligase, and the other method is to add 3′-poly(A) tail to miRNA through the action of polyadenylate polymerase. Finally, relevant software is used to analyze the expression of miRNA. Because it was only a semi-flux test, qRT-PCR and other methods were needed to verify it.[25] Data analysis of miRNAs is usually divided into four steps: (1) control the quality of experimental data and normalization processing, (2) analyze the differential expression of miRNA, (3) predict the target genes of miRNA, and (4) functional exploration of the miRNA.[26] Microarray technology can simultaneously detect the expression of tens of thousands of miRNAs. High throughput is the biggest advantage of this method, and the cost is lower than other methods. However, this method has some inherent disadvantages, such as small dynamic range, low detection sensitivity, poor specificity, and poor repeatability. It is difficult to distinguish very small miRNAs.[27,28] Moreover, there are some challenges in data analysis, such as the diversity of microarray platforms, low repeatability, and lack of mature normalization methods, leading to errors in miRNA expression detection.[29]

Reporter Gene Strategies for miRNA Imaging

Currently, the common imaging techniques used for miRNA detection include nuclear medicine imaging, MRI, and optical imaging. All three imaging methods have their own advantages and disadvantages (Table 1). They have been widely employed to monitor miRNA expression pattern and functional regulation based on reporter gene systems.[30]

Nuclear Medicine Imaging

Clinical nuclear medicine imaging falls into two main categories: one is radionuclides, such as technetium-99m (^{99m}Tc) or iodine-131, which are called single-photon emission computed tomography (SPECT); and the other is radionuclide imaging that emits positrons, such as carbon-11, fluorine-18, and oxygen-15, which are called PET.[31] Radionuclide imaging is a non-invasive imaging, which has the advantages of high sensitivity, unrestricted detection depth, accurate quantification, and simple extrapolation.[32] PET and SPECT essentially use radioactive material with high enough contrast activity as a tracer to detect molecular signals and provide the image contrast required for the image.[33]

Using PET imaging technology, Mäkilä et al.[34] illustrated the general distribution and intravenous dynamics of miRNA target molecules in the body. However, due to the high cost of instruments for PET imaging, it is difficult to use nuclides for detection.

The herpes simplex virus type 1-thymidine kinase (HSV1-TK) is a therapeutic suicide gene and can also be used as a PET reporter gene. HSV1-TK interacts with the gene to cause cell death for radionuclide imaging. HSV1-TK encodes a catalytic phosphorylation of TK, an enzyme that phosphorylates modified thymidine analogs to thymidine phosphate, which cannot cross cell membranes. PET and SPECT detect HSV1-TK gene expression by observing phosphorylated thymidine.[35] The pyrimidine nucleoside derivatives and acycloguanosine derivatives have been reported as substrates for HSV1-TK. Radiologically labeled probes, such as ^{123}I-FIAU, ^{18}F-FHBG, or ^{124}I-FIAU, were delivered to cells, which were captured by phosphorylated thymidine analogs and then used for PET and SPECT imaging.[36] Besides the HSV1-TK reporter, the dopamine-D2 imaging system employed [^{18}F]fluoroethyl spiperone as radiation-labeled probe and dopamine-D2 receptor as reporter gene, but the expression and specificity of dopamine-D2 receptor on the cell membrane are low, so its application is limited.[37] To the best of our knowledge, no studies have reported the use of HSV1-TK or dopamine-D2 receptor as reporter genes for miRNA imaging. This may become a future research direction.

Sodium iodide symporter (NIS) gene is widely applied to *in vivo* nuclear medicine imaging on account of its low cytotoxicity, poor metabolic activity, few adverse reactions, and low acquired immunity as a human origin gene. In thyroid cells, two sodium ions and iodide ions enter the cell through the specific transporter NIS. Differing from the HSV1-TK and dopamine D2 receptor, the NIS gene can directly use radioactive iodine and ^{99m}Tc radionuclide probes.[38] Jo et al.[39] developed the radionuclide imaging system for miRNA-9 detection using NIS as reporter. They used cytomegalovirus (CMV) promoters (CMV/NIS) to drive the NIS reporter gene and inserted three reverse complementary target sequences (3×PT_miR-9) that could combine with miRNA-9 into the 3′ UTR of the NIS gene. This NIS reporter, designated as CMV/NIS/3×PT_miR-9, can serve as a reporter gene to detect miR-9. The systematic mechanism is that when the expression of miRNA-9 is low, the NIS activity of CMV/NIS/3×PT_miR-9 is relatively high, leading to the high uptake of radioactive iodine and hence enhanced radioisotope signals. On the contrary, when miRNA-9 is expressed, miRNA-9 would inhibit the expression of NIS gene, and thus the uptake of radioactive iodine decreases. SPECT was used to measure radio-iodine regulation of NIS by miRNA-9. In addition, our group also developed a human sodium iodide symporter (hNIS)/Firefly luciferase (Fluc) fusion gene and found that etoposide (VP-16) and 5-fluorouracil (5-FU) could regulate the dynamic expression of miRNA-16 in the drug resistance process of gastric cancer with ^{99m}Tc-pertechnetate gamma camera imaging and bioluminescence imaging.[40] Based on the similar design principle, Yang et al.[41] visualized let-7 expression in lung adenocarcinoma A549 cells using hNIS as the reporter gene. They employed both Cerenkov luminescence imaging (CLI) and gamma camera imaging to non-invasively visualize miRNA-7 expression, providing an alternative way to monitor the intracellular dynamic expression of miRNAs. However, the above-mentioned detection method using the NIS gene has some shortcomings, because the decrease of signals in SPECT imaging cannot confirm whether it is due to miRNA expression or due to cell death itself. To overcome this limitation, Simion et al.[42] designed a Cumate gene-inducible expression system RINES using the hNIS as the reporter gene (Figure 2). Only when endogenous or exogenous miRNA is present is the expression of NIS transgenic gene activated. Using this miRNA-ON reporter system, they elucidated the biological functioning of miRNA-23a in the apoptosis of skeletal muscle tissues by monitoring the changes of radioactive tracer uptake in the transfected cells or animals.[42] This is maybe the first study with positive radioisotope imaging of miRNA expression in living animals using the NIS reporter gene.

MRI

MRI has been extensively applied in the clinical diagnosis of various diseases. MRI is non-invasive imaging modality with high spatial resolution and unlimited detection depth, especially for soft tissue. With the use of MRI reporter gene and probe, the sensitivity, specificity, and signal-to-noise ratio of MRI were effectively improved.[43] The reporter genes of MRI can be divided into three categories (Figure 3). The first category is reporter genes that encode enzymes (e.g., β-galactosidase and tyrosinase [TYR]). LacZ gene in *Escherichia coli* can be used to encode β-galactosidase. Using β-galactosidase as the reporter gene for MRI requires additional contrast agent. Currently, the most commonly used contrast agent is a paramagnetic $Gd3^{+}$ chelate -EgadMe.[44] LacZ gene enters the cell, encodes the production and expression of galactosidase, enhances inner sphere relaxation, and increases MR contrast of T1-weighted images (T1WIs).[45] The TYR gene can also enter the cell and encode TYR to synthesize melanin, providing T1 contrast for MRI.[46] The second category is reporter genes that encode the receptor on the cells (e.g., transferrin receptor [TfR]). When the TfR reporter gene is transferred to the target cell membrane, intracellular TfR is highly expressed, resulting in increased iron absorption and shortened T2 relaxation time.[47] The third category is endogenous reporter genes (e.g., ferritin reporter gene). Ferritin is ubiquitous in humans and binds specifically to iron, so ferritin can be used as a contrast agent for MR of T2-weighted images (T2WIs).[48] The ferritin reporter gene is not diluted because of cell division, which improves the stability of MRI signal. Moreover, iron exists in ferritin in the form of iron trivalent, which is non-toxic to the human body, making the ferritin reporter gene a perfect tracking probe for MRI.

To visualize miR-9 expression during neurogenesis of P19 cells, Jo et al.[49] developed a signal-on imaging system using TfR as a reporter gene. During the neurogenesis of P19 cells, high expression of miR-9 inhibited the expression of the TfR reporter gene and then led to high MR signal. Our group used TYR to design a reporter gene to monitor miR-9.[50] We inserted three target sequences into the 3′ UTR of the TYR vector that were fully complementary to miR-9 and obtained the CMV/TYR-3xTS reporter. We utilized the CMV/TYR-3xTS reporter gene to monitor miR-9 in living cells and animals. In the absence of miR-9, reporter genes are normally expressed and TYR

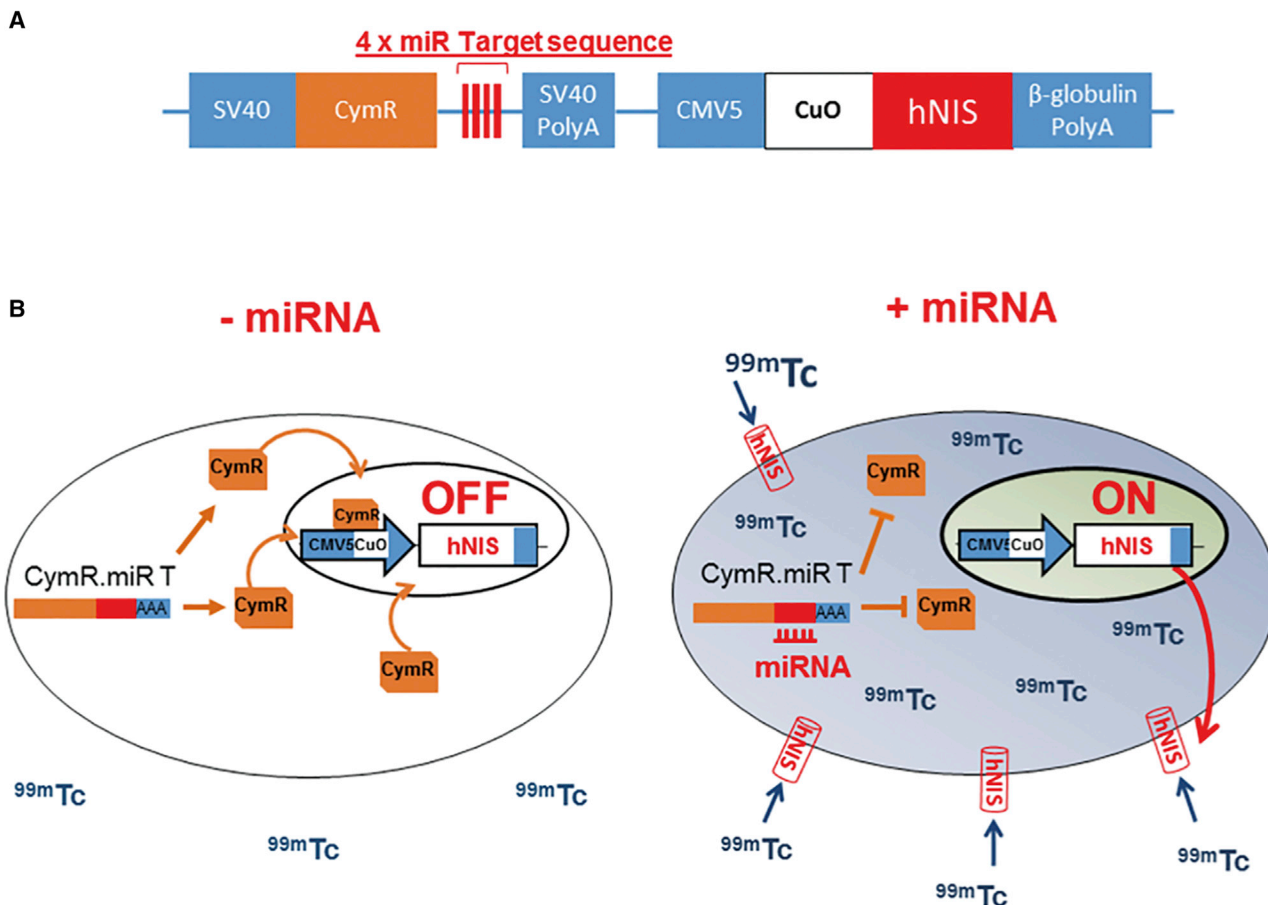

Figure 2. Schematic Diagram of the RINES System

(A) RINES system containing miRNA targeting sequence and hNIS reporter genes. The Simian vacuolating virus 40 (SV40) promoter drives the CymR repressor, and CymR induces the expression of CMV5/Cuo plasmid. When miRNA is expressed, it can bind to the CymR transcriptional repressor through base-pair binding and then induce its degradation through the RISC mechanism. (B) Left: the hNIS expression is switched off when the miRNA is not expressed, and CymR-mRNA translation produces CymR protein. CymR protein can bind to the Cuo operator sequence in the CMV5/Cuo inducible, then causes the hNIS system expression to be turned off. As a result, $^{99m}TcO_4^-$ is low or not present, which can be monitored with a SPECT camera. Right: miRNA is expressed and inhibited CymR protein production. Then hNIS is switched on, resulting in expression of hNIS at the cellular membrane, leading to accumulation of $^{99m}TcO_4^-$. (Adapted from Simion et al.[42] with permission. Copyright 2020 Public Library of Science.)

is produced to catalyze the synthesis of melanin. Following the treatment of 5-aza-2′-deoxycytidine in A549 cells to promote DNA methylation, miR-9 is activated, leading to the inhibition of TYR reporter and melanin synthesis. In addition to being a MRI reporter, melanin can also act as the functional target for photoacoustic imaging (PAI). We demonstrated that miR-9 activity could be visualized by the TYR reporter through the detection of melanin content, TYR enzyme activity, and PAI signal. However, because of experimental conditions, we did not perform MRI of miR-9 in this study. We would explore the use of TYR as a dual reporter gene for both MRI and PAI of miRNA expression in the biological process in future studies.

Optical Imaging

Different optical imaging strategies can be selected according to the imaging purpose. Currently, there are two commonly used strategies to design miRNA reporters, namely, the photoreporter system containing miRNA transcription products and the photoreporter system containing miRNA target sequences.[5] Photoreporter systems generally consist of regulatory promoters and photogene carriers (encoding fluorescent proteins or bioluminescent proteins). When reporter genes are transfected into cells, they bind to endogenous miRNAs, which can regulate specific reporter mRNAs or their translation, eventually leading to a change of propensity and activity in reporter gene. Cells or small animals are transfected with fluorescent protein or luciferase gene, and then can be imaged through the *in vivo* imaging system. Therefore, the effects of endogenous miRNAs can be visualized by optical imaging of fluorescent proteins or luciferase.[51]

Fluorescence Imaging

Fluorescence imaging uses fluorophores (fluorescent proteins), which are excited by external sources with shorter wavelengths than emitted

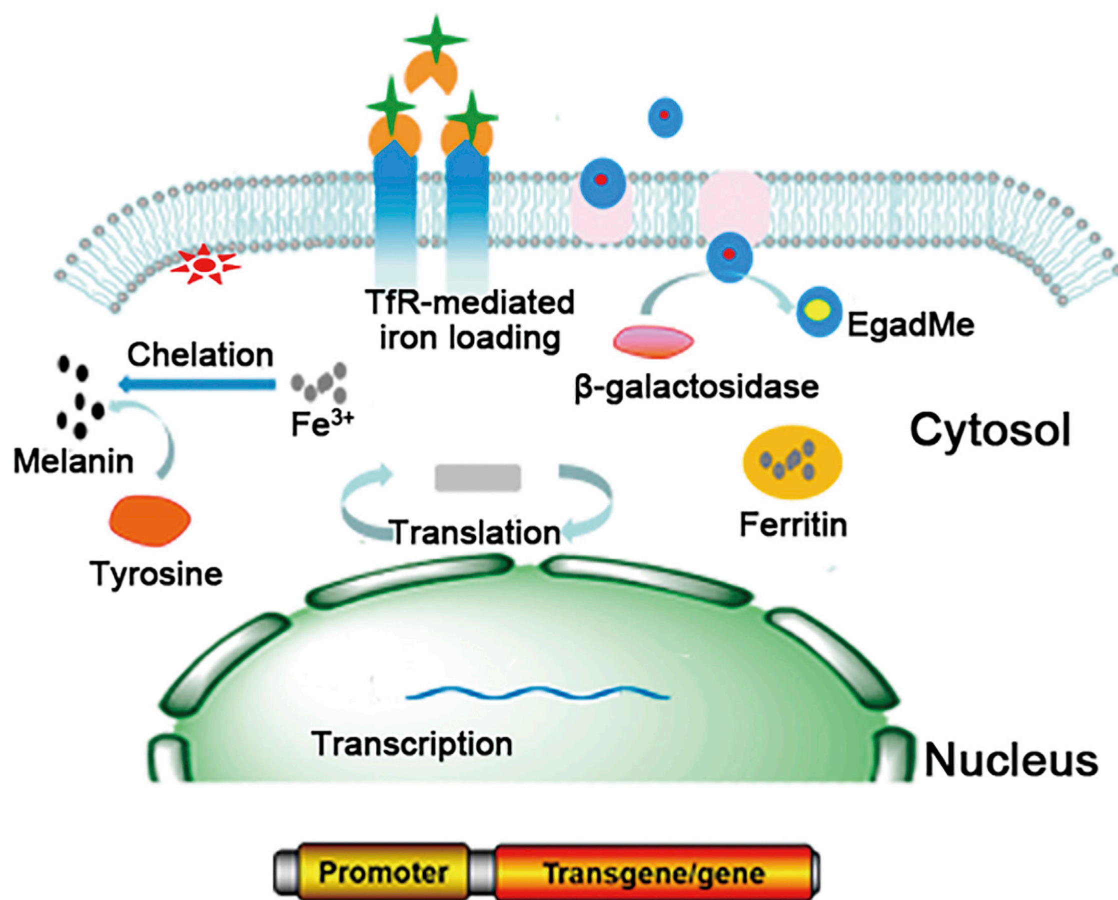

Figure 3. Commonly Used MRI Reporter Genes

β-Galactosidase as the reporter gene requires additional contrast agent, such as EgadMe. β-Galactosidase can enhance MR contrast on T1-weighted images; TYR gene can also enter the cell and encode tyrosinase to synthesize melanin, providing T1 contrast for MRI; when TfR binds with iron-loaded transferrin molecule, it is internalized through endocytosis, resulting in increased iron absorption and shortened T2 relaxation time; ferritin binds specifically to iron, which can act as a MR contrast agent. (Adapted from Yang et al.[43] with permission.Copyright 2020 MDPI)

light. Fluorescent proteins include green fluorescent protein (GFP), yellow fluorescent protein (YFP), red fluorescent protein (RFP), etc. Because the GFP was isolated from jellyfish in 1962, it has been used extensively as a reporter gene for optical imaging to study the various biological process and events.[52] To monitor the role of miRNAs in zebrafish brain formation, Giraldez et al.[53] designed a GFP sensor that contains three perfect target sites for miRNAs. They identified a new miR-430 family, which exerts an enormous function on the embryonic development of zebrafish. They found that the absence of the miR-430 family led to the brain morphogenesis defects of the maternal-zygotic dicer mutant.

There are many kinds of fluorescent proteins with unique emission wavelengths, and there is not much overlap in the emission spectrum. Based on this, fluorescent proteins can be used to construct a multicolor imaging system, and the dynamic expression of multiple miRNA activities can be monitored at the same time. Because enhanced GFP (EGFP) and DsRed have a maximum emission wavelength of 509 and 583 nm, respectively, they were very suitable for non-cross, two-color imaging with simultaneous excitation. Kato et al.[54] developed a dual-fluorescent imaging system by inserting three segment repeats that were completely complementary to miR-133 into the 3′ UTR of GFP of a retrovirus vector. In this system, retroviral vectors encode both GFP and RFP fluorescent proteins, and the expression level of GFP is affected by miR-133, whereas the expression level of RFP is not. They used the reporter system to dynamically monitor the expression level of miR-133 in C2C12 myoblast cells. They also developed a human immunodeficiency virus-1 (HIV-1)-based lentiviral vector system, which encodes two fluorescent proteins.[55] The lentiviral vectors were transfected into adenovirus-infected cells and under the control of two different bidirectional promoters. The fluorescence changes of RFP and GFP can be used to quantitatively monitor adenovirus infection and miRNA expression. In this system, the coordinated expression of bidirectional encoded fluorescent proteins can reduce the risk caused by the random integration of lentiviral vectors into the cellular genome.

The chromosome-integrated virus vector-based system enables steady expression of reporter genes in cells, which makes long-term monitoring of miRNA possible. Nevertheless, the activity of viral promoters varies widely depending on genomic background, cell type, and epigenetic control. These characteristics may reduce the sensitivity and stability of miRNA monitoring during cell reprogramming or differentiation. To overcome these limitations, Sano et al.[56] developed a fluorescence-based system for monitoring of miRNA expression in living cells using a replication-defective and persistent vector Sendai virus (SeVdp). SeVdp vector can stabilize the expression of transgenic gene in various types of mammalian cells without chromosomal integration. The system harbors fluorescent EGFP as reporter and keima-red (KR) as internal reference genes. They found that miR-124 significantly increased during neuronal transformation via examining EGFP signal by fluorescence microscopy. In addition, to continuously monitor miRNA activity, Nakanishi et al[73]. developed a miRNA-responsive non-viral reporter vector driven by a single promoter to transcribe two reporter mRNA genes simultaneously. One is the reference mRNA containing the gene of enhanced cyan fluorescent protein (ECFP), and the other is miRNA-responsive mRNA containing the gene of RFP. When the corresponding miRNA activity is enhanced, the expression level of miRNA-responsive mRNA is inhibited, and the expression level of reference mRNA is not affected. Therefore, miRNA activity can be quantified by measuring the fluorescence ratio.

Due to its high stability, however, GFP has a slow turnover rate. So the monitoring of the rapid dynamic expression of miRNA is not suitable with GFP. To solve this problem, Ando et al.[57] developed a new time-lapse imaging method for fast dynamic imaging of miRNA activity by using destabilized fluorescent proteins (dsFPs) with protein degradation sequence (PEST sequence), which has a fast turnover rate. Moreover, they also developed a double imaging system by using destabilized cyan fluorescent protein (dsCFP) and destabilized Venus protein (dsVenus). A PEST sequence was also fused to the C terminus of CFP or both the C terminus and N terminus of Venus to make it unstable. miR-9-5p and miR-9-3p were simultaneously expressed in single cells and had similar dynamics detected by this system.

Most of the miRNA reporter systems mentioned above are generated by introducing three or four copies of target sequences for the miRNA of interest in the 3′ UTR of the reporter gene. In this case, the reporter system works as a signal-off system because the endogenous miRNAs act as small interfering RNAs (siRNAs) and downregulate or turn off the reporter expression. To exclude the false negative signals that are not caused by miRNA expression, Amendola et al.[58] designed a dual-switch miR-on system to detect endogenous miRNA expression using fluorescent imaging. The system consists of two parts: one is the transcriptional repressor regulated by miRNA expression, and the other is the reporter cassette regulated through the repressor. GFP reporter genes begin to be expressed when miRNAs are activated and inhibit transcription factor translation. Besides this signal-on reporter system, Ying et al.[59] designed a fluorophore sulforhodamine (SR)-dinitroaniline (DN)-conjugated luminescent RNA aptamer for fluorescent imaging of miRNAs in cells (Figure 4). The sensor uses two independent aptamers for miRNA hybridization, and the combination of the appropriately folded aptamers and SR-DN can be used to detect fluorescence signals. The plasmid of the sensor is composed of an RNA sensor expressing tRNA support and two independent promoters regulating GFP. The miRNA expression can regulate the RNA sensor to produce orange fluorescence, whereas the GFP is driven by the promoters, and the green fluorescence signal generated can be used as an internal reference. They showed that this genetically encoded RNA sensor enabled selective and sensitive detection of miRNAs in different tumor cells. Although fluorescence imaging has the advantages of high sensitivity and multiplexing for *in vitro* detection, it has encountered some inherent difficulties for *in vivo* detection, such as high background, spontaneous fluorescence of the organism itself, low signal noise, and limited tissue penetration. Therefore, alternative optical reporters are needed to monitor miRNA activity in living animals.

Bioluminescence Imaging

Fluc, Renilla luciferase (Rluc), and Gaussia luciferase (Gluc) are currently commonly employed optical genes encoding bioluminescent proteins. Different optical genes can catalyze the corresponding substrate to produce optical signals.[38] Fluc catalyzes luciferin to produce carbon dioxide, phosphate, and light under the combined action of adenosine triphosphate (ATP), magnesium, and oxygen. Rluc and Gluc act on the coelenteron and catalyze the production of coelenteron amide, carbon dioxide, and light.[47] miRNA imaging based on luciferase reporter genes can be used not only to observe the biogenesis of miRNAs, including both pre-miRNAs and mature forms of miRNAs, but also to observe the functions of miRNAs. Compared with fluorescence protein-based imaging, bioluminescence imaging detects there action between luciferase and substrate, so its specificity is stronger. Moreover, bioluminescence imaging does not require *in vitro* laser irradiation, and its high signal-to-background ratio is better than fluorescence imaging. Although bioluminescence imaging is superior to fluorescence imaging in depth, it has significant limitations compared with PET, SPECT, and MRI.

Ko et al.[60–62] developed a series of luciferase-based reporter systems for monitoring the miRNA biogenesis and function. For instance, they designed a luciferase imaging system, which contained three target sequences against miRNA in the 3′ UTR of luciferase gene, for monitoring the role of miR-124a or miR-9 in neurogenesis of P19 cells.[60–62] They also employed the Gluc reporter system to conduct long-term and non-invasive monitoring of miR-221 biogenesis and regulation in papillary thyroid carcinoma.[63,64] When Gluc reporter was being transfected into cells, Gluc activity was decreased when miR-221 was present in the cells. The decreased Gluc activity indirectly reflected the expression level of miR-221. Like bicolor fluorescence imaging, a dual-luciferase reporter imaging system for detecting miRNAs can also be constructed based on the different substrates of luciferase genes. Lee et al.'s[65] team designed a dual-luciferase imaging system to detect expression and post-transcriptional regulation of miR-23a using Fluc and Gluc. The substrates of these two luciferases are different, and there

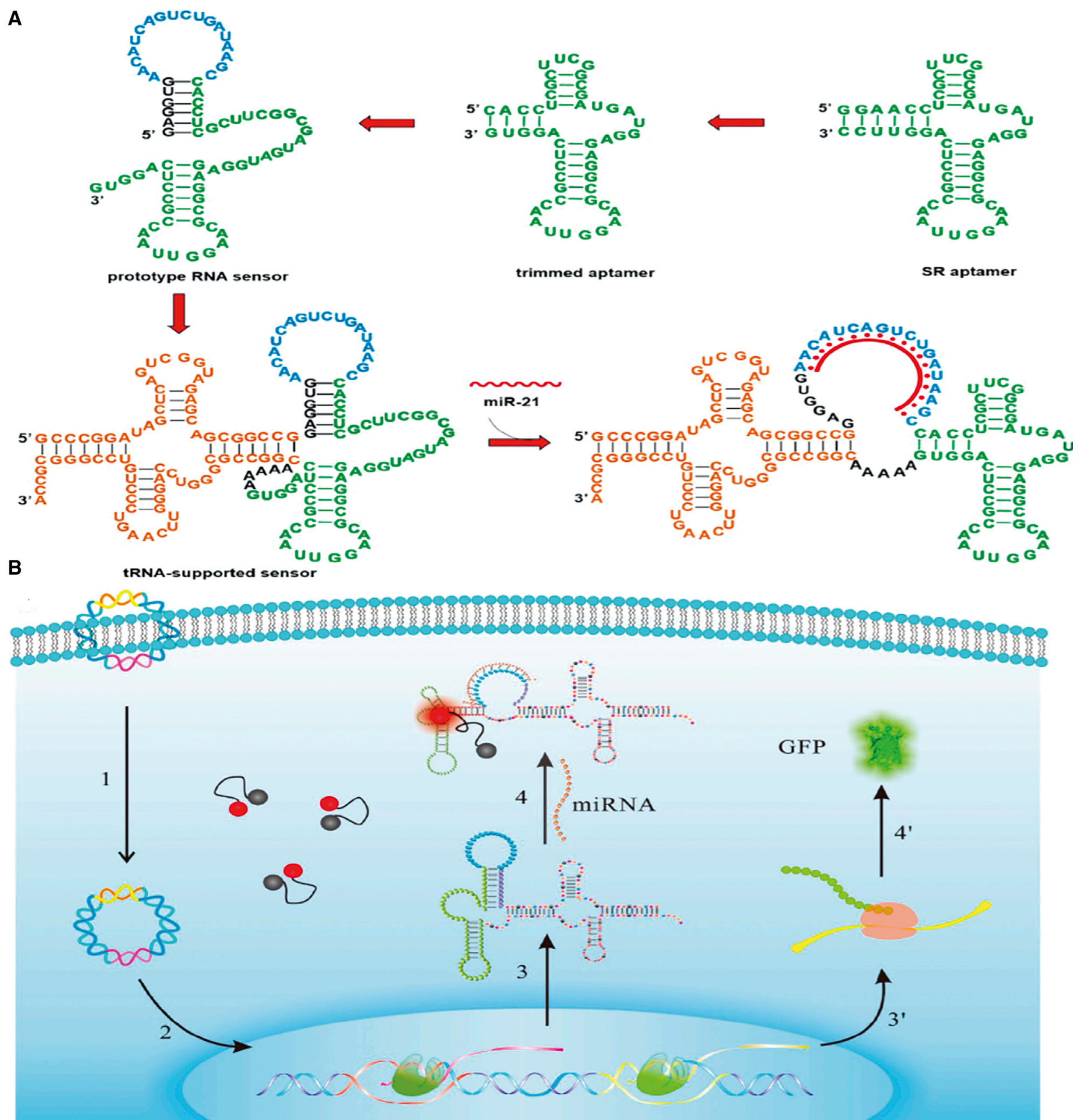

Figure 4. Luminescent RNA Aptamer for Fluorescent Imaging of miRNAs

(A) Designing light-up RNA sensor. The sensor uses two independent aptamers for miRNA hybridization, and the combination of the appropriately folded aptamers and SR-DN can be used to detect fluorescence signals. Designing a prototype sensor by pruning the SR-binding aptamer in the terminal stem and then binding to a miR-responsive stem-loop motif. Aptamer sensor design in the recombinant tRNA can realize the efficient expression of the sensor. The tRNA-supported RNA sensor can combine with quenched fluorescence to the SR-DN conjugate with the presence of the target miR. (B) Genetically encoded RNA sensor detecting miRNAs in tumor cells. The plasmid of the sensor is composed of an RNA sensor expressing tRNA support and two independent promoters regulating GFP. The miRNA expression can regulate the RNA sensor to produce orange fluorescence, while the GFP is driven by the promoters, and the green fluorescence signal generated can be used as an internal reference. (Adapted from Ying et al.[59] with permission. Copyright 2020 American Chemical Society)

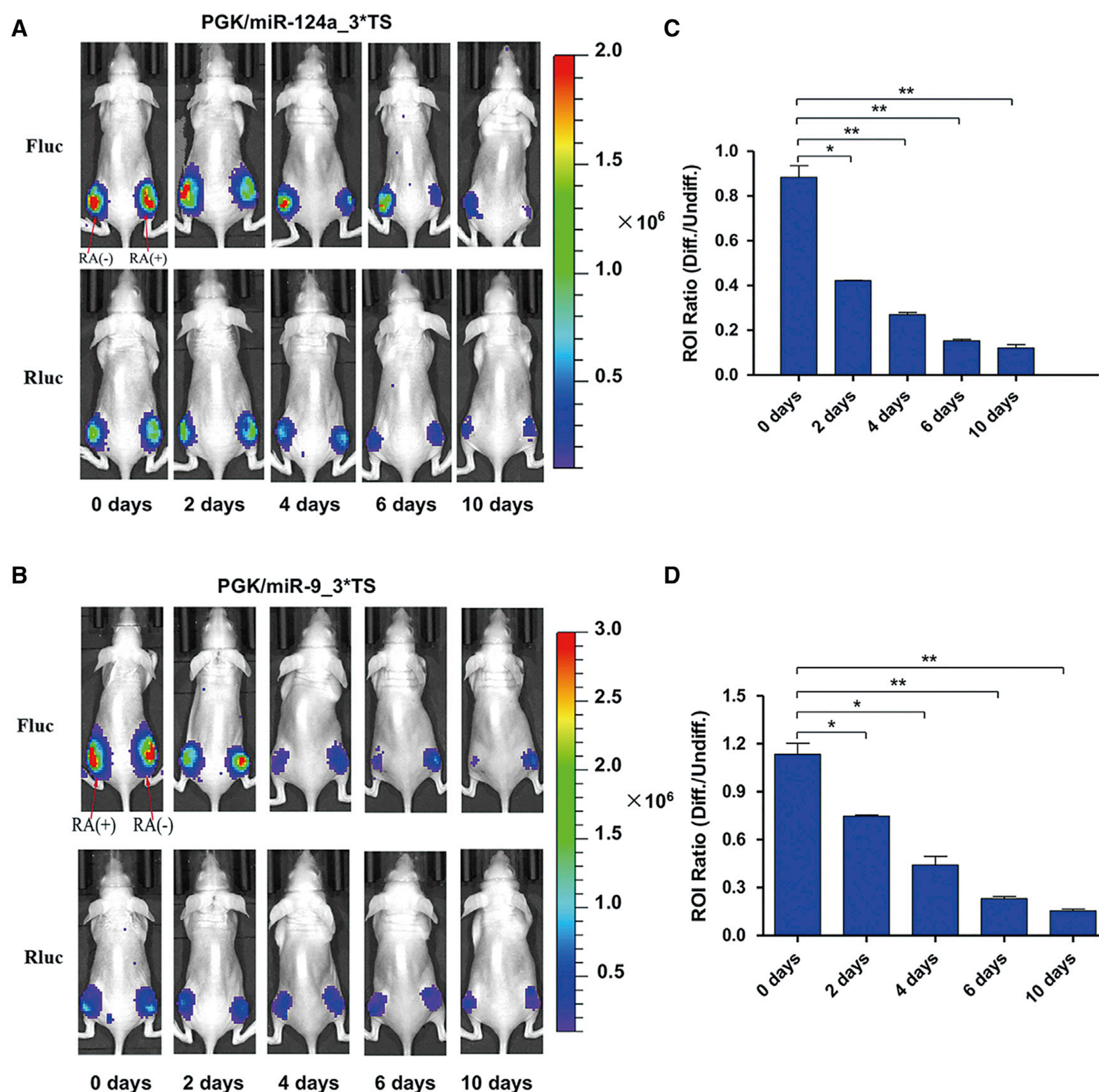

Figure 5. Bioluminescence Imaging of the Neurogenic Processes of miR-124a and miR-9 in Nude Mice

(A and B) Transfecting with reporter genes (A) PGK/mir-124a_3*TS and (B) PGK/mir-9_3*TS plasmids into $1*10^7$ P19 cells. Then $1*10^7$ P19 cells were implanted into the subcutaneous tissue of the thighs of nude mice; one thigh was treated with RA. Fluc (upper) or Rluc (lower) optical signals were imaged at different time periods. Fluc signal strength gradually decreased, and Rluc signal strength basically did not change. (C) Quantitative detection of bioluminescence signals in the region of interest (ROI). In the cells transfected with PGK/mir-124a_3*TS, the relative Fluc/Rluc ratio between the differentiated and undifferentiated groups decreased gradually over time. (D) In the PGK/miR9_3*TS cells implantation mice, similar patterns of reduction were also found between the two groups. Data was presented as means ± SD. *$p < 0.05$, **$p < 0.01$. (Adapted from Zheng et al.[67] with permission. Copyright 2020 American Chemical Society)

is no cross-activity between enzymes, so the activity of endogenous pri-miRNA and mature miRNA can be monitored simultaneously. They employed this dual system to evaluate the expression patterns of miR-23a in nerve cells at different stages of differentiation. They found that pri-miR-23a gradually decreased and mature miR-23a gradually decreased during the neurogenesis of P19 by measuring the activity changes of Fluc and Gluc.

Unlike the above dual-luciferase reporters, Tu et al.[66] built a dual-luciferase reporter system, in which three copies of the miR-22 target sequence were inserted into the 3′ UTR of Gluc, whereas CMV/Fluc with no target sequences was used as a positive control. They used the system to successfully repeatedly and non-invasively monitor the dynamic expression of miR-22 in isoproterenol-induced cardiac hypertrophy. Furthermore, our group also designed a dual-luciferase reporter system to continuously detect the dynamic expression of miR-124a and miR-9 in neural models *in vitro* and *in vivo*[67] (Figure 5). In this system, the Fluc gene and Rluc gene on the vector are controlled by two different promoters. The 3′ UTR of the Fluc gene contains three complementary sequences of miRNA; thus, Fluc

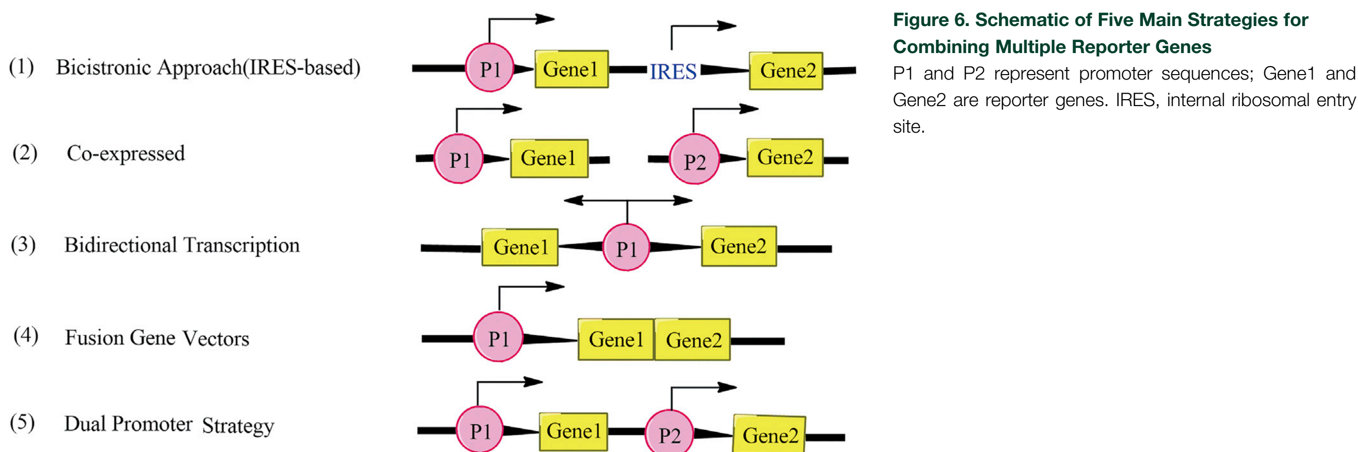

Figure 6. Schematic of Five Main Strategies for Combining Multiple Reporter Genes
P1 and P2 represent promoter sequences; Gene1 and Gene2 are reporter genes. IRES, internal ribosomal entry site.

expression is regulated by the miRNA of interest. In contrast, Rluc does not contain miRNA complementary sequences, so it is not affected by miRNA activity and can be used as an internal reference to correct transfection efficiency. After retinoic acid (RA) treatment, miR-9 or miR-124a is expressed and then binds to the 3′ UTR of Fluc, thus inhibiting the expression of Fluc and weakening the Fluc signal. As a consequence, the differential expression profiles of miR-9 or miR-124a in a neurogenesis model induced by RA can be tracked by bioluminescence imaging analysis using this dual-luciferase reporter gene imaging system.

Besides the above-mentioned luciferase-based signal-off miRNA reporter systems, several signal-on luciferase reporters have been developed to monitor positively the endogenous expression pattern of miRNAs in cells and animal models. These signal-on systems are usually based on the genetic switches, for instance, the Tet-Krab,[58] Tet R,[68] and Cumate[69] regulatable expression systems. Ezzine et al.[70] developed a Cumate-based RNAi-Inducible Luciferase Expression System (RILES) to monitor the dynamic expression of miRNA in real time. After transfecting RILES into target cells, the expression of miRNA inhibits the transcriptional repressor, thereby activating the expression of luciferase reporter genes and the emission of optical signals. So the endogenous miRNA could be quantitatively and qualitatively detected through bioluminescence imaging. Using this RILES miRNA reporter, they successfully monitored the regulatory patterns of myomiR-1, -133, and -206 in mouse skeletal muscle and the expression of miRNA-122 in mouse liver.

Multimodal Imaging

Because any single imaging technique has its advantages and disadvantages, it does not provide all the necessary conditions for research. Radionuclide imaging, for example, has the advantages of high sensitivity, and the quantitative detection and detecting depth are not limited, but the resolution is relatively low and the cost is high. MRI features high spatial distribution rate, unlimited detection depth, and extraction of physiological and anatomical information. However, the low sensitivity and high cost make MRI, although studied for many years, still in the preliminary stage and rarely used for genetic testing. Optical imaging has the characteristics of simple operation, short collection time, low cost, and high sensitivity, but it has the problems of optical signal attenuation, the limited detection depth, and two-dimensional image. Therefore, the application of multimode non-invasive imaging for the study of miRNAs based on reporter genes is of great significance.

Multimodal imaging techniques often combine two or more models to obtain information using fused reporter genes. There are two approaches currently used for reporter imaging. One is to combine labeling strategy with reporter genes, involving physical labeling with contrast agents (such as micro-sized particles of iron oxide [MPIOs]) and reporter genes (such as luciferase or EGFP). The other is to combine two or more reporter genes, such as multimodal reporter genes. There are five main strategies for combining multiple reporter genes (Figure 6).[37,47,71] The first strategy is the bicistronic reporter using an internal ribosomal entry site (IRES) to connect the two genes. Second, two labeled foreign genes are transfected into the same cell as two vectors. The third strategy is bidirectional transcription, in which the promoter located at the center is used to express a reporter gene in each direction. The fourth strategy is that the two genes are within the same reading frame of a fusion vector and controlled by the same promoter or enhancer. The fifth is multiple reporter gene constructs and their own vectors are controlled by their own promoters.

Based on the combined labeling strategy, Jo et al.[49] developed a multimodal imaging system for detecting miRNAs by using the TfR gene as the reporter gene. The system combined a TfR reporter gene and a magnetic fluorescence (MF) nanoparticle-conjugated peptide targeting TfR (MF targeting TfR). When the miR-9 was highly expressed, the expression of the TfR reporter gene was repressed, leading to the reduced fluorescence intensity and enhanced MR signal. In this system, the fluorescence activity is shown as signal-off system. On the contrary, the MR signal is shown as signal-on system. This probe for reverse complementary multimode imaging provides a new way to detect miRNA expression in cell development and disease. Based on

the IRES reporter strategy, Cho et al.[72] designed a luciferase-EGFP transgenetic reporter where enhanced Fluc (effluc) and EGFP were linked with an IRES sequence. This fused reporter contained three copies of perfect target sequences of miR-430, miR-1, or miR-133, allowing monitoring of the temporal changes of miRNA expression patterns in transgenic mice by bioluminescence imaging.[72] In addition, our group also developed a dual reporter gene by fusing the hNIS and Fluc gene into a single open reading frame (ORF) to form the hNIS/Fluc reporter, which was used for bioluminescence imaging and ^{99m}Tc-pertechnetate gamma camera imaging of the dynamic expression of miRNA-16 in the chemoresistance of gastric cancer.[40]

Conclusion and Future Perspectives

Emerging studies have indicated that miRNAs play a critical role in various biological processes, neurological diseases, tumors, and other diseases by regulating the expression of target mRNAs. It is of great clinical significance to reveal the biological characteristics and dynamic expression patterns of miRNAs. Traditional approaches, such as northern blotting and RT-PCR, are often time consuming and laborious, unable to achieve non-invasive detection. Therefore, alternative *in vivo* imaging techniques are needed for quantitative detection of miRNA to evaluate the biological characteristics and expression of miRNAs in a biological context.

The application of reporter gene-based molecular imaging can efficiently and non-invasively provide the dynamic expression profiles of miRNAs and obtain much more meaningful biological information. The development of a mature reporter system is the key to study the regulation mechanism of miRNAs. A reporter gene system usually consists of reporter genes and substrates that interact with the products of reporter genes. The expression of miRNAs can be non-invasively detected by imaging technology based on a reporter system. With reporter genes, we can visually detect the expression and regulation of miRNAs in cells, which can improve more effective information for the diagnosis and treatment of patients. Reporter gene imaging can provide clinical and practical three-dimensional structure information. In particular, the rapid development of multimodal imaging, by combining multiple reporter genes to identify multiple targets, over time can continuously monitor gene expression and accurately define its location. Reporter genes can make imaging strategies faster at relatively low cost. In the case that multiple consecutive biopsies cannot be performed, reporter gene imaging can be used to obtain spatial information of the whole body, as well as target organs and tumors. Also, imaging does not cause any interference to the tissues during the biopsy. This advantage has considerable potential to address toxicity problems in biopsies. The development of genetically encoded reporter genes will lead to the diagnosis and treatment of diseases, such as tumors, and most importantly, it holds the promise of direct transfer to human clinical trials through animal model experiments and promotes the research progress of gene therapy. However, reporter genes are still at the primary stage of clinical application, and there is still room for improvement in many aspects of practice. For example, whether the transfection of the reporter gene is in the optimal position, the expression time of the reporter gene in the target cells and the optimal time for its detection are not clear. Moreover, the reporter gene has great uncertainty in immunogenicity, probe targeting, and gene mutation. These uncertainties bring great challenges to the application of miRNA imaging technology in the study of small-animal models and human clinical practice. Intracellular miRNA imaging is more challenging than imaging by targeting molecules on the cell surface.

Nuclear medicine imaging technology is widely used because of its high sensitivity and unrestricted detection depth. NIS is often used as the reporter gene in nuclear medicine imaging and can also be combined with other imaging technologies for multimodal imaging. The HSV1-TK reporter gene is not currently used for non-invasive imaging of miRNAs, providing a direction for utilizing HSV1-TK as a miRNA reporter. However, a limitation of non-human reporter genes, such as HSV1-TK and its mutants, lies in their potential immunogenicity, thus preventing their use in clinical application. One way to reduce this risk is to employ a mammalian source reporter construct, such as hNIS, which may be more feasible in the clinical settings. MRI technology has been widely used in clinical disease diagnosis. Most of the MRI technology employs TfR or TYR as the reporter genes and can be combined with other imaging technology to detect miRNAs. Optical imaging is also used widely for visualizing miRNAs using fluorescent proteins or luciferase. Currently, multiple miRNAs can be detected simultaneously with one or more imaging techniques. However, the information obtained by any single imaging technology is limited, and the use of multimodality imaging can obtain more biological information of miRNAs. At present, the single imaging method has some limitations, such as low sensitivity, high *in vivo* background signal, low spatial resolution, and poor tissue penetration. The multimode imaging method can combine the advantages of each imaging method to overcome the limitation of a single imaging method to the greatest extent. Therefore, it would monitor more complex biological information and has a good prospect of disease diagnosis. Future research on the development of more efficient and clinically relevant multimodality reporter genes would promote the development of miRNA-based diagnosis and therapy in various diseases, and advance the field of miRNA imaging into clinical application.

AUTHOR CONTRIBUTIONS

Y.S. and Z.X. wrote the draft; F.W. revised the manuscript and approved the publication of the content.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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REFERENCES

1. Sekar, T.V., Mohanram, R.K., Foygel, K., and Paulmurugan, R. (2013). Therapeutic evaluation of microRNAs by molecular imaging. Theranostics *3*, 964–985.
2. Di Leva, G., Garofalo, M., and Croce, C.M. (2014). MicroRNAs in cancer. Annu. Rev. Pathol. *9*, 287–314.
3. He, D., Wong, K.-W., Dong, Z., and Li, H.-W. (2018). Recent progress in live cell mRNA/microRNA imaging probes based on smart and versatile nanomaterials. J. Mater. Chem. B Mater. Biol. Med. *6*, 7773–7793.
4. Bartel, D.P., and Chen, C.Z. (2004). Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. Nat. Rev. Genet. *5*, 396–400.
5. Oh, S.W., Hwang, D.W., and Lee, D.S. (2013). In vivo monitoring of microRNA biogenesis using reporter gene imaging. Theranostics *3*, 1004–1011.
6. Bhaskaran, M., and Mohan, M. (2014). MicroRNAs: history, biogenesis, and their evolving role in animal development and disease. Vet. Pathol. *51*, 759–774.
7. Xie, M., and Steitz, J.A. (2014). Versatile microRNA biogenesis in animals and their viruses. RNA Biol. *11*, 673–681.
8. Treiber, T., Treiber, N., Plessmann, U., Harlander, S., Daiβ, J.-L., Eichner, N., Lehmann, G., Schall, K., Urlaub, H., and Meister, G. (2017). A Compendium of RNA-Binding Proteins that Regulate MicroRNA Biogenesis. Mol. Cell *66*, 270–284.e13.
9. Ambros, V. (2003). MicroRNA pathways in flies and worms: growth, death, fat, stress, and timing. Cell *113*, 673–676.
10. Sarnow, P., and Sagan, S.M. (2016). Unraveling the Mysterious Interactions Between Hepatitis C Virus RNA and Liver-Specific MicroRNA-122. Annu. Rev. Virol. *3*, 309–332.
11. Rayner, K.J., Sheedy, F.J., Esau, C.C., Hussain, F.N., Temel, R.E., Parathath, S., van Gils, J.M., Rayner, A.J., Chang, A.N., Suarez, Y., et al. (2011). Antagonism of miR-33 in mice promotes reverse cholesterol transport and regression of atherosclerosis. J. Clin. Invest. *121*, 2921–2931.
12. Christopher, A.F., Kaur, R.P., Kaur, G., Kaur, A., Gupta, V., and Bansal, P. (2016). MicroRNA therapeutics: Discovering novel targets and developing specific therapy. Perspect. Clin. Res. *7*, 68–74.
13. McClure, L.V., Lin, Y.-T., and Sullivan, C.S. (2011). Detection of viral microRNAs by northern blot analysis. Methods Mol. Biol. *721*, 153–171.
14. Schwarzkopf, M., and Pierce, N.A. (2016). Multiplexed miRNA northern blots via hybridization chain reaction. Nucleic Acids Res. *44*, e129.
15. Válóczi, A., Hornyik, C., Varga, N., Burgyán, J., Kauppinen, S., and Havelda, Z. (2004). Sensitive and specific detection of microRNAs by northern blot analysis using LNA-modified oligonucleotide probes. Nucleic Acids Res. *32*, e175.
16. Kim, S.W., Li, Z., Moore, P.S., Monaghan, A.P., Chang, Y., Nichols, M., and John, B. (2010). A sensitive non-radioactive northern blot method to detect small RNAs. Nucleic Acids Res. *38*, e98.
17. Johnson, B.N., and Mutharasan, R. (2014). Biosensor-based microRNA detection: techniques, design, performance, and challenges. Analyst (Lond.) *139*, 1576–1588.
18. Várallyay, E., Burgyán, J., and Havelda, Z. (2008). MicroRNA detection by northern blotting using locked nucleic acid probes. Nat. Protoc. *3*, 190–196.
19. Pall, G.S., and Hamilton, A.J. (2008). Improved northern blot method for enhanced detection of small RNA. Nat. Protoc. *3*, 1077–1084.
20. Tan, G.W., and Tan, L.P. (2017). High-Throughput RT-qPCR for the Analysis of Circulating MicroRNAs. Methods Mol. Biol. *1580*, 7–19.
21. Chen, C., Ridzon, D.A., Broomer, A.J., Zhou, Z., Lee, D.H., Nguyen, J.T., Barbisin, M., Xu, N.L., Mahuvakar, V.R., Andersen, M.R., et al. (2005). Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res. *33*, e179.
22. Kumar, P., Johnston, B.H., and Kazakov, S.A. (2011). miR-ID: a novel, circularization-based platform for detection of microRNAs. RNA *17*, 365–380.
23. Choi, W., Yeom, S.Y., Kim, J., Jung, S., Jung, S., Shim, T.S., Kim, S.K., Kang, J.Y., Lee, S.H., Cho, I.J., et al. (2018). Hydrogel micropost-based qPCR for multiplex detection of miRNAs associated with Alzheimer's disease. Biosens. Bioelectron. *101*, 235–244.
24. Jung, S., Kim, J., Lee, D.J., Oh, E.H., Lim, H., Kim, K.P., Choi, N., Kim, T.S., and Kim, S.K. (2016). Extensible Multiplex Real-time PCR of MicroRNA Using Microparticles. Sci. Rep. *6*, 22975.
25. Sand, M., Skrygan, M., Georgas, D., Sand, D., Hahn, S.A., Gambichler, T., Altmeyer, P., and Bechara, F.G. (2012). Microarray analysis of microRNA expression in cutaneous squamous cell carcinoma. J. Dermatol. Sci. *68*, 119–126.
26. Mastriani, E., Zhai, R., and Zhu, S. (2018). Microarray-Based MicroRNA Expression Data Analysis with Bioconductor. Methods Mol. Biol. *1751*, 127–138.
27. Pritchard, C.C., Cheng, H.H., and Tewari, M. (2012). MicroRNA profiling: approaches and considerations. Nat. Rev. Genet. *13*, 358–369.
28. Tam, S., de Borja, R., Tsao, M.S., and McPherson, J.D. (2014). Robust global microRNA expression profiling using next-generation sequencing technologies. Lab. Invest. *94*, 350–358.
29. Wang, B., and Xi, Y. (2013). Challenges for MicroRNA Microarray Data Analysis. Microarrays (Basel) *2*, 34–50.
30. Wang, F., Niu, G., Chen, X., and Cao, F. (2011). Molecular imaging of microRNAs. Eur. J. Nucl. Med. Mol. Imaging *38*, 1572–1579.
31. Gambhir, S.S., Herschman, H.R., Cherry, S.R., Barrio, J.R., Satyamurthy, N., Toyokuni, T., Phelps, M.E., Larson, S.M., Balatoni, J., Finn, R., et al. (2000). Imaging transgene expression with radionuclide imaging technologies. Neoplasia *2*, 118–138.
32. Liang, Q., Gotts, J., Satyamurthy, N., Barrio, J., Phelps, M.E., Gambhir, S.S., and Herschman, H.R. (2002). Noninvasive, repetitive, quantitative measurement of gene expression from a bicistronic message by positron emission tomography, following gene transfer with adenovirus. Mol. Ther. *6*, 73–82.
33. Sharma, V., Luker, G.D., and Piwnica-Worms, D. (2002). Molecular imaging of gene expression and protein function in vivo with PET and SPECT. J. Magn. Reson. Imaging *16*, 336–351.
34. Mäkilä, J., Kiviniemi, A., Saanijoki, T., Liljenbäck, H., Käkelä, M., Jadhav, S., Poijärvi-Virta, P., Lönnberg, H., Laitala-Leinonen, T., Virta, P., and Roivainen, A. (2019). Noninvasive and Quantitative Monitoring of the Distributions and Kinetics of MicroRNA-Targeting Molecules in Vivo by Positron Emission Tomography. Mol. Pharm. *16*, 1507–1515.
35. Lan, X., Liu, Y., He, Y., Wu, T., Zhang, B., Gao, Z., An, R., and Zhang, Y. (2010). Autoradiography study and SPECT imaging of reporter gene HSV1-tk expression in heart. Nucl. Med. Biol. *37*, 371–380.
36. Niu, G., and Chen, X. (2012). Molecular imaging with activatable reporter systems. Theranostics *2*, 413–423.
37. Youn, H., and Hong, K.J. (2012). In vivo Noninvasive Small Animal Molecular Imaging. Osong Public Health Res. Perspect. *3*, 48–59.
38. Youn, H., and Chung, J.K. (2013). Reporter gene imaging. AJR Am. J. Roentgenol. *201*, W206–W214.
39. Jo, M.H., Jeong, M.S., Ko, H.Y., Lee, C.H., Kang, W.J., and Kim, S. (2013). Radioisotope imaging of microRNA-9-regulating neurogenesis using sodium iodide sympoter. Biomaterials *34*, 4803–4809.
40. Wang, F., Song, X., Li, X., Xin, J., Wang, S., Yang, W., Wang, J., Wu, K., Chen, X., Liang, J., et al. (2013). Noninvasive visualization of microRNA-16 in the chemoresistance of gastric cancer using a dual reporter gene imaging system. PLoS ONE *8*, e61792.
41. Yang, W., Qin, W., Hu, Z., Suo, Y., Zhao, R., Ma, X., Wang, T., Liang, J., Tian, J., and Wang, J. (2012). Comparison of Cerenkov luminescence imaging (CLI) and gamma camera imaging for visualization of let-7 expression in lung adenocarcinoma A549 cells. Nucl. Med. Biol. *39*, 948–953.
42. Simion, V., Sobilo, J., Clemoncon, R., Natkunarajah, S., Ezzine, S., Abdallah, F., Lerondel, S., Pichon, C., and Baril, P. (2017). Positive radionuclide imaging of miRNA expression using RILES and the human sodium iodide symporter as reporter gene is feasible and supports a protective role of miRNA-23a in response to muscular atrophy. PLoS ONE *12*, e0177492.
43. Yang, C., Tian, R., Liu, T., and Liu, G. (2016). MRI Reporter Genes for Noninvasive Molecular Imaging. Molecules *21*, 580.

44. Louie, A.Y., Hüber, M.M., Ahrens, E.T., Rothbächer, U., Moats, R., Jacobs, R.E., Fraser, S.E., and Meade, T.J. (2000). In vivo visualization of gene expression using magnetic resonance imaging. Nat. Biotechnol. *18*, 321–325.

45. Bengtsson, N.E., Brown, G., Scott, E.W., and Walter, G.A. (2010). lacZ as a genetic reporter for real-time MRI. Magn. Reson. Med. *63*, 745–753.

46. Feng, H., Xia, X., Li, C., Song, Y., Qin, C., Zhang, Y., and Lan, X. (2015). TYR as a multifunctional reporter gene regulated by the Tet-on system for multimodality imaging: an in vitro study. Sci. Rep. *5*, 15502.

47. Kang, J.H., and Chung, J.K. (2008). Molecular-genetic imaging based on reporter gene expression. J. Nucl. Med. *49* (*Suppl 2*), 164S–179S.

48. He, X., Cai, J., Liu, B., Zhong, Y., and Qin, Y. (2015). Cellular magnetic resonance imaging contrast generated by the ferritin heavy chain genetic reporter under the control of a Tet-On switch. Stem Cell Res. Ther. *6*, 207.

49. Jo, M.H., Ali, B.A., Al-Khedhairy, A.A., Lee, C.H., Kim, B., Haam, S., Huh, Y.M., Ko, H.Y., and Kim, S. (2012). A reverse complementary multimodal imaging system to visualize microRNA9-involved neurogenesis using peptide targeting transferrin receptor-conjugated magnetic fluorescence nanoparticles. Biomaterials *33*, 6456–6467.

50. Zheng, H., Zhou, L., Shi, Y., Tian, J., and Wang, F. (2018). Tyrosinase-Based Reporter Gene for Photoacoustic Imaging of MicroRNA-9 Regulated by DNA Methylation in Living Subjects. Mol. Ther. Nucleic Acids *11*, 34–40.

51. Baril, P., Ezzine, S., and Pichon, C. (2015). Monitoring the spatiotemporal activities of miRNAs in small animal models using molecular imaging modalities. Int. J. Mol. Sci. *16*, 4947–4972.

52. Tsien, R.Y. (1998). The green fluorescent protein. Annu. Rev. Biochem. *67*, 509–544.

53. Giraldez, A.J., Cinalli, R.M., Glasner, M.E., Enright, A.J., Thomson, J.M., Baskerville, S., Hammond, S.M., Bartel, D.P., and Schier, A.F. (2005). MicroRNAs regulate brain morphogenesis in zebrafish. Science *308*, 833–838.

54. Kato, Y., Miyaki, S., Yokoyama, S., Omori, S., Inoue, A., Horiuchi, M., and Asahara, H. (2009). Real-time functional imaging for monitoring miR-133 during myogenic differentiation. Int. J. Biochem. Cell Biol. *41*, 2225–2231.

55. Kato, Y., Sawata, S.Y., and Inoue, A. (2010). A lentiviral vector encoding two fluorescent proteins enables imaging of adenoviral infection via adenovirus-encoded miRNAs in single living cells. J. Biochem. *147*, 63–71.

56. Sano, M., Ohtaka, M., Iijima, M., Nakasu, A., Kato, Y., and Nakanishi, M. (2017). Sensitive and long-term monitoring of intracellular microRNAs using a non-integrating cytoplasmic RNA vector. Sci. Rep. *7*, 12673.

57. Ando, H., Hirose, M., Kurosawa, G., Impey, S., and Mikoshiba, K. (2017). Time-lapse imaging of microRNA activity reveals the kinetics of microRNA activation in single living cells. Sci. Rep. *7*, 12642.

58. Amendola, M., Giustacchini, A., Gentner, B., and Naldini, L. (2013). A double-switch vector system positively regulates transgene expression by endogenous microRNA expression (miR-ON vector). Mol. Ther. *21*, 934–946.

59. Ying, Z.M., Wu, Z., Tu, B., Tan, W., and Jiang, J.H. (2017). Genetically Encoded Fluorescent RNA Sensor for Ratiometric Imaging of MicroRNA in Living Tumor Cells. J. Am. Chem. Soc. *139*, 9779–9782.

60. Ko, M.H., Kim, S., Hwang, D.W., Ko, H.Y., Kim, Y.H., and Lee, D.S. (2008). Bioimaging of the unbalanced expression of microRNA9 and microRNA9* during the neuronal differentiation of P19 cells. FEBS J. *275*, 2605–2616.

61. Ko, H.Y., Hwang, D.W., Lee, D.S., and Kim, S. (2009). A reporter gene imaging system for monitoring microRNA biogenesis. Nat. Protoc. *4*, 1663–1669.

62. Ko, H.Y., Lee, D.S., and Kim, S. (2009). Noninvasive imaging of microRNA124a-mediated repression of the chromosome 14 ORF 24 gene during neurogenesis. FEBS J. *276*, 4854–4865.

63. Kim, H.J., Kim, Y.H., Lee, D.S., Chung, J.K., and Kim, S. (2008). In vivo imaging of functional targeting of miR-221 in papillary thyroid carcinoma. J. Nucl. Med. *49*, 1686–1693.

64. Kim, H.J., Chung, J.K., Hwang, D.W., Lee, D.S., and Kim, S. (2009). In vivo imaging of miR-221 biogenesis in papillary thyroid carcinoma. Mol. Imaging Biol. *11*, 71–78.

65. Lee, J.Y., Kim, S., Hwang, D.W., Jeong, J.M., Chung, J.K., Lee, M.C., and Lee, D.S. (2008). Development of a dual-luciferase reporter system for in vivo visualization of MicroRNA biogenesis and posttranscriptional regulation. J. Nucl. Med. *49*, 285–294.

66. Tu, Y., Wan, L., Zhao, D., Bu, L., Dong, D., Yin, Z., Cheng, Z., and Shen, B. (2014). In vitro and in vivo direct monitoring of miRNA-22 expression in isoproterenol-induced cardiac hypertrophy by bioluminescence imaging. Eur. J. Nucl. Med. Mol. Imaging *41*, 972–984.

67. Zheng, H., Wang, X., Chen, S., Shi, X., Xie, J., Mao, W., Tian, J., and Wang, F. (2019). Real-Time Functional Bioimaging of Neuron-Specific MicroRNA Dynamics during Neuronal Differentiation Using a Dual Luciferase Reporter. ACS Chem. Neurosci. *10*, 1696–1705.

68. Stieger, K., Belbellaa, B., Le Guiner, C., Moullier, P., and Rolling, F. (2009). In vivo gene regulation using tetracycline-regulatable systems. Adv. Drug Deliv. Rev. *61*, 527–541.

69. Mullick, A., Xu, Y., Warren, R., Koutroumanis, M., Guilbault, C., Broussau, S., Malenfant, F., Bourget, L., Lamoureux, L., Lo, R., et al. (2006). The cumate gene-switch: a system for regulated expression in mammalian cells. BMC Biotechnol. *6*, 43.

70. Ezzine, S., Vassaux, G., Pitard, B., Barteau, B., Malinge, J.M., Midoux, P., Pichon, C., and Baril, P. (2013). RILES, a novel method for temporal analysis of the in vivo regulation of miRNA expression. Nucleic Acids Res. *41*, e192.

71. Li, M., Wang, Y., Liu, M., and Lan, X. (2018). Multimodality reporter gene imaging: Construction strategies and application. Theranostics *8*, 2954–2973.

72. Choi, Y., Hwang, D.W., Kim, M.Y., Kim, J.Y., Sun, W., and Lee, D.S. (2016). Transgenic Mouse Expressing Optical MicroRNA Reporter for Monitoring MicroRNA-124 Action during Development. Front. Mol. Neurosci. *9*, 52.

73. Nakanishi, H., Miki, K., Komatsu, K.R., Umeda, M., Mochizuki, M., Inagaki, A., et al. (2017). Monitoring and visualizing microRNA dynamics during live cell differentiation using microRNA-responsive non-viral reporter vectors. Biomaterials *128*, 121–135.