

## RESEARCH HIGHLIGHT

# Bioimaging of miRNA biogenesis using a color-tunable molecular beacon

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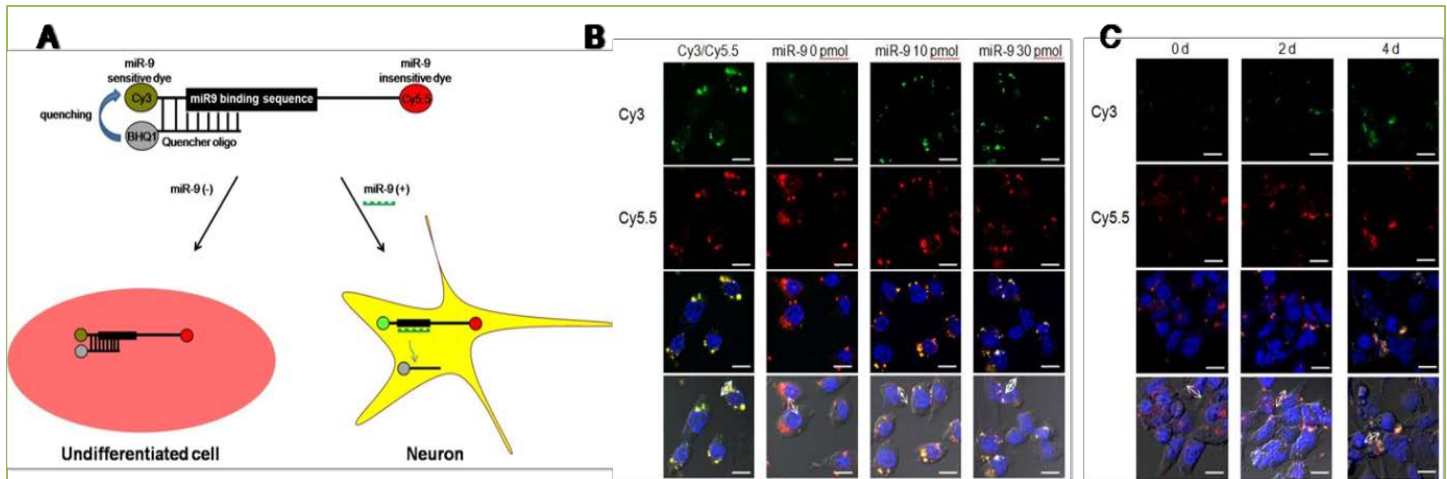
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Molecular imaging is a novel technology used to study cellular and molecular mechanisms. MicroRNAs (miRNAs or miRs) play important roles in clinical diseases. Bioimaging of miRNA is a powerful tool used to study various biological phenomena. Current bioimaging of miRNA biogenesis uses a reporter gene and a mono-fluorophore-based molecular beacon (MB) to monitor miRNA-regulating cellular developments. However, the miRNA reporter gene is unable to determine the miRNA function-mediating signal-off imaging activity of cell death. In addition, a miR MB has limited accuracy in detecting miRNA expression when fluorescence intensity in cells is weak. To overcome the disadvantages of both miRNA imaging systems, our group developed dual-fluorophore-based color-tunable miR MBs (ColoR MBs). These MBs consist of a partial duplex DNA oligonucleotide bearing a target miRNA binding site with a fluorophore (Cy3)/black hole quencher 1 (BHQ1) at one end as a reporter probe and another fluorophore (Cy5.5) without the quencher at the other end as a reference probe. The reference probe of ColoR MB is always turned on regardless of miRNA expression. In the absence of miRNA of interest, the fluorescence activity of the reporter probe from ColoR MB was quenched due to close proximity between the fluorophore and quencher. Therefore, cells expressing little or no miRNA of interest were only visualized by the reference probe, resulting in red color. When the target miRNA was expressed in cells, it bound to the miRNA binding site of the ColoR MB. The quencher then separated from the MB, resulting in the fluorescence brightness of the reporter probe. Cells were detected as a yellow color due to merging of green (Cy3) and red (Cy5.5). The reference probe of ColoR MB successfully differentiated the weak fluorescence signals of the reporter probe resulting from low expression of target miRNA or low delivery of the ColoR MB in cells. The color-tunable specificity of the ColoR MB for sensing color changes based on miRNA expression and miRNA-regulating cellular development overcame limitations of the miRNA reporter gene, which shows signal-off imaging activity in the presence of target miRNA.

**Keywords:** Bioimaging; Molecular imaging; Molecular beacon; microRNA

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In the early 21<sup>st</sup> century, molecular imaging was developed for different cellular targets, including proteins



**Figure 1. ColoR9 MB structure and specificity of miR-9 expression.** (A) Schematic illustration of ColoR9 MB for sensing miR-9 expression-dependent color change. (B) Confocal microscopic analysis of ColoR9 MB in CHO cells. Fluorescence of Cy3 (excitation/emission: 546/563 nm, miR-9-sensitive dye) gradually increased with exogenous miR-9 concentration in CHO cells. (C) Confocal microscopic analysis of ColoR9 MB during P19 cell neuronal differentiation. Endogenous level of miR-9 using miRNA-MB generally increased during P19 (embryonic carcinoma) cell neurogenesis. Reporter probe (Cy3, green), reference probe (Cy5.5, excitation/emission: 683/705 nm, red). Scale bar, 10 mm. Reprinted with permission [8]. Copyright © 2014, Rights Managed by Nature Publishing Group.

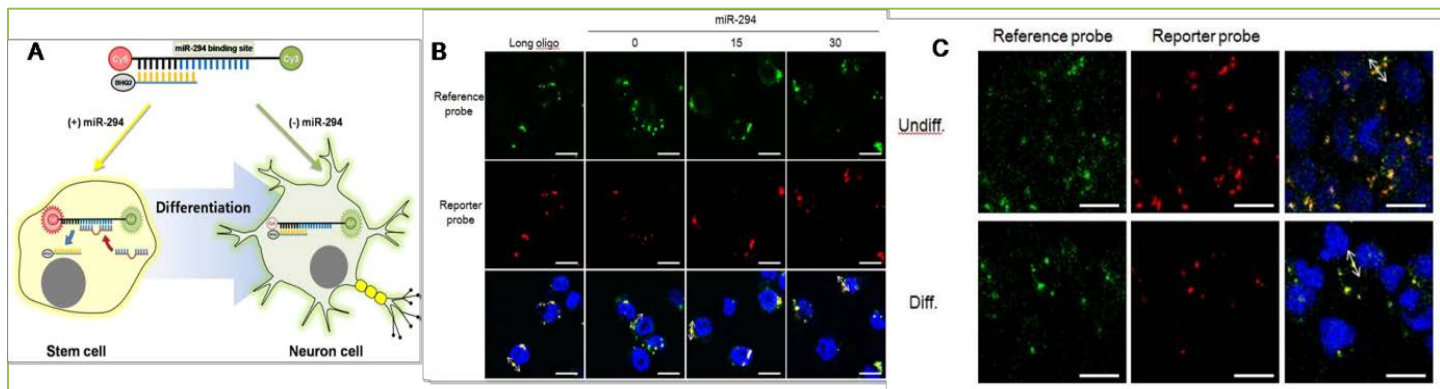
and peptide biomarkers involved in various cellular processes<sup>[1]</sup>. Imaging technology allows noninvasive study of the cellular and molecular mechanisms of fundamental molecular pathways inside organisms<sup>[2]</sup>. For this reason, interest in molecular imaging research has rapidly increased in recent years. Molecular imaging is expected to influence the diagnosis and therapy of cancer, neurological diseases, and cardiovascular diseases<sup>[3-9]</sup>.

MiRNAs are small non-coding RNAs 19-25 nucleotides in length that are transcribed into lengthy primary miRNAs (pri-miRNAa) by polymerase II in the nucleus and then processed into single-stranded mature miRNA. MiRNAs expressed in eukaryotic cells trigger sequence-specific gene silencing by complete or partial complementary base pairing with the 3' untranslated regions (UTRs) of target mRNAs<sup>[10-12]</sup>. Recently, investigators have discovered that miRNA functions to control cell proliferation, cell death, apoptosis, and differentiation. In addition, miRNAs play key roles in different biological processes and pathological states such as the development of cancers, neurogenesis, and myogenesis<sup>[4, 5, 7, 8]</sup>. Due to their individual and simultaneous regulation of hundreds of genes, such expression characteristics of miRNAs in cells could provide promising biomarkers for the diagnosis of diseases.

Most current methods for analyzing the levels of endogenous miRNA expression include northern blotting, microarray and real-time polymerase chain reaction (PCR). However, these methods do not reflect real conditions of living cells or organisms. Many methods are also invasive, such as tissue biopsy<sup>[5, 13]</sup>. To overcome these limitations, we

succeeded in developing a few of novel miRNA optical imaging systems using a bioluminescent luciferase reporter gene and a fluorophore-based molecular beacon (MB) to visualize miRNA-regulating cellular developments<sup>[4, 5, 7-9]</sup>. The bioluminescent miRNA reporter gene was designed to detect target miRNAs expressions by cloning perfectly complementary sequences of mature miRNA in the 3'UTR of luciferase<sup>[5]</sup>. There have been successive reports on miRNA reporter gene imaging systems for monitoring miR-1, miR-9, miR-23a, miR-221, and miR124a biogenesis, including myogenesis, neurogenesis, and carcinogenesis<sup>[13-17]</sup>. In the presence of target miRNAs, the imaging system is a signal-off system that operates through destabilization of the luciferase reporter gene by miRNA of interest<sup>[4, 5, 13, 16, 18]</sup>. Signal-off systems are limited in differentiating low bioluminescent signals from miRNA expression or cellular loss and are difficult to construct the miRNA reporter gene. Besides, these signal-off systems remain distant goals for clinical application due to their attenuation of optical signals in a living animal.

To overcome limitations of inability to figure out the miRNA function-mediating signal-off imaging activity from low miRNA expression or cellular death, a signal-on miRNA imaging system was designed using a mono-fluorophore-based MB with a linear DNA structure (miR MB)<sup>[4]</sup>. The miR MB is a short stem-loop-structured or linear-structured DNA oligonucleotide hybridization probe containing miRNA binding sequence with a quenching molecule and fluorescent dye at the end of each oligonucleotide<sup>[8]</sup>. When target miRNA is absent, the fluorescence signals of miR MBs are quenched due to a



**Figure 2. ColoR294 MB structure and specificity of miR-294 expression.** (A) Schematic diagram of ColoR294 MB for sensing miR-294 expression-dependent color change. (B) Confocal microscope images of ColoR294 MB in CHO cells. ColoR294 MBs were transfected into CHO cells. Mature miR-294 was applied to each plate well in a dose-dependent manner (0, 15, and 30 pmol of miR-294). Green fluorescence (Cy3) gradually changed to yellow fluorescence (mix of green and red) with exogenous miR-294 concentration in CHO cells. (C) Confocal microscope images of ColoR294 MB during neuronal differentiation of P19 cells. After neuronal differentiation of P19 cells, yellow color changed to green (Cy3). Reporter probe (Cy5, red), reference probe (Cy3, green). Scale bar 10 mm. Reprinted with permission [7] from The Royal Society of Chemistry.

result of fluorescence resonance energy transfer (FRET). When the miR MBs bind to their correspondent miRNAs or the quencher separates from the miR MB, resulting in a signal-on imaging signal [7, 8]. We successfully visualized expressions of miR-1, miR-26a, miR124a, miR-126, miR-206, and miR-221 by miR MBs *in vitro* and *in vivo*. These miRNAs are highly expressed during myogenesis, neurogenesis, and carcinogenesis [4, 6, 14, 19]. However, it is still difficult to obtain accurate information from the mono-fluorophore-based miR MB when fluorescence intensity in cells is relatively weak.

Our group recently developed a new miRNA bioimaging system to detect miRNA expression-dependent color changes using dual-fluorophore-based color-tunable miR MBs (ColoR MBs) [7, 8]. The ColoR MB imaging system overcame disadvantages of both miRNA reporter genes and miR MBs. A color-tunable miR-9 MB (ColoR9 MB) was designed to visualize color changes for miR-9 expression and miR-9 regulating neurogenesis [8]. The ColoR9 MB consists of a partial duplex DNA oligonucleotide bearing a miR-9 binding site with Cy3 (green, miR-9 sensitive dye)/black hole quencher 1 (BHQ1) at one end as a reporter probe and another fluorophore (Cy5.5, red, miR-9 insensitive dye) without the quencher at the other end as a reference probe (Figure 1-A). When miR-9 is absent, only Cy5.5 signals of the reporter probe from the ColoR9 MB were detected as red because the fluorescence activity of the reporter probe is efficiently quenched due to the FRET between Cy3 and BHQ1. When it is present, miR-9 binds to the ColoR9 MB miR-9 binding site, BHQ1 is separated from the reporter probe, and Cy3 green fluorescence is visualized. Therefore, the ColoR9 MB was detected as yellow due to the merging of green and red. To confirm the feasibility of sensing color

change for miR-9 expression, ColoR9 MBs with various concentrations (0, 10, and 30 pmol) exogenous miR-9 were co-transfected into Chinese hamster ovary (CHO) cells that express no endogenous miR-9 [8]. The green fluorescence (Cy3) from the reporter probe was quenched in CHO cells in the absence of exogenous miR-9. Therefore, CHO cells transfected with ColoR9 MBs were visualized as a red color from the reference probe. When exogenous miR-9 was transfected into CHO cells, the Cy3 green fluorescence brightness of the reporter probe increased in a dose-dependent manner. CHO cells transfected with ColoR9 MBs were visualized as yellow due to merging of green and red (Figure 1-B). ColoR9 MB was also used to demonstrate miR-9 expression during neurogenesis of P19 cell (embryonic carcinoma). In confocal microscopic imaging of P19 cells during neurogenesis, fluorescence from the reference probe was constantly detected regardless of miR-9 expression and neurogenesis. However, the fluorescence from the reporter probe was quenched before neuronal differentiation. The fluorescence signals of the reporter probe were gradually recovered after neurogenesis of P19 cells (Figure 1-C). Therefore, undifferentiated P19 cells with weak or no miR-9 expression were visualized as a red color, while neuronal differentiated P19 cells with high miR-9 expression were displayed as a yellow color due to mixing of green from the reporter probe and red from the reference probe.

Another color-tunable miR-294 MB (ColoR294 MB) was designed to monitor the miR-294 expression-dependent color change in cells [7]. MiR-294 is highly expressed in undifferentiated embryo stem cells, and the structure of ColoR294 MB resembles that of the ColoR9 MB [7]. The ColoR294 MB consists of a partial double-stranded DNA oligonucleotide (oligo) with a long oligo containing a miR-9

binding site labeled with Cy5 at the 5' end as a reporter probe and Cy3 at the 3' end as a reference probe (Figure 2-A). A short oligo containing BHQ2 partially hybridized with the 5' end of the long oligo. Similar to the imaging mechanism of the ColoR9 MB, Cy3 green fluorescence activity of the reference probe was constantly visualized irrespective of miR-294 expression. When miR-294 is expressed in stem cells, complementary binding of miR-294 to the miR-294 binding site induced the separation of the quencher from the ColoR294 MB and activated the Cy5 red fluorescence of the reporter probe. Therefore, cells were visualized as yellow due to merging of green and red. When miR-294 is not expressed after neuronal differentiation, the red fluorescence activity of the reporter probe was quenched to result in green cells. To study the feasibility of detection of miR-294 expression-dependent color change, ColoR294 MBs were transfected into CHO cells with exogenous miR-294. Green fluorescence (Cy3) from the reference probe was continually detected, while red fluorescence (Cy5) from the reporter probe was increased in CHO cells with increasing concentration of exogenous miR-294 (Figure 2-B). The results showed that CHO cells transfected with the ColoR294 MB without and with the treatment of exogenous miR-294 were visualized as green and yellow due to merging of green and red, respectively. The miR-294 expression gradually and significantly decreased during neuronal differentiation in P19 cells. In undifferentiated P19 cells, the fluorescence activity of the reporter probe from the ColoR294 MB was detected as red, while the reference probe from Cy3 was constantly visualized as green. During neuronal differentiation, the red color from the reporter probe was efficiently quenched due to close proximity of Cy3 and BHQ2 caused by decreased endogenous miR-294 expression. However, a green color was constantly detected from the reference probe. Consequently, ColoR294 MB demonstrated that undifferentiated P19 cells were yellow due to merging of green from the reporter probe and red from the reference probe. Neuronal differentiated P19 cells with significantly decreased miR-294 expression were displayed as a green color from the reference probe (Figure 2-C).

Many investigators are interested in miRNAs that play important roles in cellular development and clinical diseases. An understanding of miRNA functions will affect the development of miRNA-based diagnosis and therapies. MiRNA imaging systems using a bioluminescent luciferase reporter gene or fluorophore-based MB to visualize miRNA-regulating cellular developments have been developed. These methods are important in the early study and precise diagnosis of miRNA involving-cellular developments and diseases. We successfully developed and demonstrated the ColoR MB, which provides target miRNA expression-dependent color change and overcomes the

limitations of prior miRNA imaging systems<sup>[4, 7, 8, 13, 14, 17]</sup>. The ColoR MB clearly increased fluorescence signals of the reporter probe in the presence of target miRNAs and consistently visualized fluorescence brightness of the reference probe irrespective of miRNA expression<sup>[7, 8]</sup>. The advantages of the ColoR MB include its relatively low cost, easy synthesis, sensing specificity and easy analysis. Molecular imaging by the ColoR MB is a feasible mechanism for understanding the interactions of complex miRNA regulatory networks in disease for use in diagnosis and treatment.

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