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Molecular beacon-based bioimaging of multiple microRNAs during myogenesis

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ABSTRACT

MicroRNAs (miRNAs, miR) are associated with multiple cellular processes and diseases. Here, we designed fluorescent DNA probes composed of stem loop-structured DNA complementary to miRNAs and fluorophore–quencher pairs [molecular beacon (MB)] to simultaneously monitor the biogenesis of miR-206 and miR-26a, which are highly expressed during myogenic differentiation. C2C12 cells were transfected with an MB targeting miR-26a and containing a 6-FAM-BHQ1 pair (miRNA-26a MB) or an MB targeting miR-206 with a Texas Red-BHQ2 pair (miRNA-206 MB). *In vitro* and *in vivo* fluorescence analysis revealed that, only in differentiated single C2C12 cell, significantly increased fluorescence signals of miRNA-26a MB, miRNA-206 MB or simultaneous incubation of both beacons were detected due to the hybridization of miR-206 or miR-26a with their respective beacons, resulting in activation of fluorescence. Our MB-based miRNA imaging system may serve as a new imaging probe for monitoring multiple miRNAs during various cellular or disease processes associated with miRNAs.

1. Introduction

MicroRNAs (miRNA, miR) are composed of 20–22 non-coding nucleotides and regulate gene expression through translational repression and degradation of mRNA. These molecules are associated with diverse cellular activities (e.g., development, differentiation, proliferation, and apoptosis) as well as a number of clinically important diseases by virtue of their ability to induce degradation or translational repression of target mRNAs [1–6]. Several miRNAs have been found to be relevant to cardiac and skeletal muscular differentiation including miR-1, miR-133, miR-181, miR-195, miR-206, and miR-26a [7–9].

We have previously developed an *in vivo* system for imaging miRNA biogenesis to understand miRNA function in living organism. This imaging system, which is based on an optical reporter gene, is a signal-off system that works through destabilization of mRNA targets by miRNA [10–13]. A limitation of this signal-off system is its inability to differentiate between decreased signals resulting from reduced genetic expression or cellular loss. To overcome the limitations of the signal-off system, we have designed a signal-on miRNA imaging system using a fluorescent nanoparticle-based molecular beacon (MB) with a linear DNA structure. Using this approach, we have successfully imaged miRNA124a biogenesis during neurogenesis both *in vitro* and *in vivo* [14]. An MB is a synthetic stem loop-structured (hairpin) DNA oligonucleotide that binds to a target of interest and that has a reporter fluorophore at one end and a quencher at the other end [15]. In the absence of targets, emitted fluorescence is absorbed by quenching molecules as a result of fluorescence resonance energy transfer (FRET) between the fluorescence dye and the quencher. In contrast, the presence of targets induces the binding of target to the loop within the MB, causing the hairpin to open and recover fluorescence.

Tens of different miRNAs are simultaneously expressed in a single cell to determine or regulate cell fate [16]. However, none of the *in vivo* miRNA imaging systems has been used to simultaneously image multiple miRNAs in a living cell, an approach that would provide detailed imaging information about diverse miRNA expression patterns in a single cell. Here, we developed a simple hairpin MB-based *in vivo* imaging system to monitor multiple miRNAs at the same time. We focused on miR-26a and miR-206, which undergo biogenesis that is highly muscle specific and gradually increases during myogenesis. Two different MBs were designed for these molecules, one with 6-FAM fluorophore and black hole quencher 1 (BHQ1) and the other with Texas Red-X fluorophore and BHQ2. Using this approach, we could clearly and simultaneously visualize miR-26a and miR-206 during the myogenesis of C212 cells both *in vitro* and *in vivo*.

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2. Material and methods

2.1. Cell culture

C2C12 cells and HeLa cells were purchased from the American Type Culture Collection (ATCC). C2C12 cells and HeLa cells were cultured on growth media consisting of Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). The differentiation media for C2C12 cells contained DMEM and 1% FBS. Differentiation to myotubes was induced by switching the culture media to the differentiation media.

2.2. Quantification of expression in miRNA

The expression of miR-206 and miR-26a was quantified by real-time reverse polymerase chain reaction (RT-PCR) using the total RNA obtained from C2C12 cells on the differentiation day, D0, D2, D4, and D6. Each miRNA expression was represented relative to the expression of small RNA U6, which was used as an internal control of RT-PCR. The expression data were presented as means of relative expression values obtained from three samples with standard deviation. For the comparison of the mean, a *t*-test was performed with a *p*-value of 0.05 as significant.

2.3. Confirmation of myogenic differentiation with RT-PCR and western blot analysis

Myogenic differentiation was confirmed by reverse polymerase chain reaction (RT-PCR) using the total RNA obtained from C2C12 cells on the differentiation day, D0, D1, D2, D3, and D4. Total RNA from each C2C12 cell was isolated using Trizol reagent (Invitrogen). First strand cDNA was synthesized using a random hexamer and SuperScrip II reverse transcriptase (Invitrogen) based on manufacturer's instruction. Myogenin primer (Forward: 5′ TACGTCCATCGTGGACAG CAT 3′, reverse: 5′ TCAGCTAAATTCCCTCGCTGG 3′) and β-actin primer (forward: 5′ TGACGGGGT CACCCAACTGTGCCCATCTA 3′, reverse: 5′ CTAGAAGCATTTGCGGTGGACGATGGAGGG 3′) were manufactured by Bionics (Korea). After PCR amplication for myogenon and β-actin with i-Taq polymerase (iNtRON, Korea), gel eledctrophoresis was performed with 2% agarose gel.

Quantification of myogenin of C2C12 cells at differentiation day, D0, D2, D4, and D6 was measured by western blot analysis. C2C12 cells at each differentiation day were lysed in a buffer containing 10 mM Tris–HCl (pH 7.5), 1 mM DTT, 20% glycerol, 1 mM EDTA, and protease inhibitor mixture. Protein concentrations were determined using BCA protein kits (Pierce, US), and the resulting lysates were cleared by centrifugation. Membranes were blocked in TBS-T (20 mM Tris, 137 mM NaCl, 0.1% Tween 20) containing 3% nonfat dried milk and incubated with myogenin antibody (1:5000 dilution, Abcam, UK) or α-tubulin antibody (1:5000 dilution, Sigma, Saint Louis, MO) for 120 min. The membranes were then washed 3 times with TBS-T, and anti-mouse IgG (H + L) was added. Immunochemical detection was performed, using WEST-zol (plus) (intron) by means of Las 3000.

2.4. Design of miRNA-linked molecular beacon

Of several miRNA highly expressed in myogenic differentiation of C2C12 cells, miR-206 and miR-26a were tested in the study, which are known to be highly expressed during myogenic differentiation. miRNA-133a was selected as a non-specific control. Mouse mature miRNA-26a, miRNA-206, and miRNA-133a were purchased from Bionics (BIONICS, Seoul, Korea). The sequences of miRNAs are as follows: miRNA-26a (5′UUCAAGUAAUCCAGGAUAGGCU3′), miRNA-206 (5′UGGAAUGUAAGGAAGUGUGUGG3′), miRNA-133a (5′UUUGGUCCCCUUCAACCAGCUG 3′). MBs with reciprocal sequences of miR-206 and miR-26a were synthesized (Fig. 1). The miRNA-206-linked MB was designed to have Texas Red-X (absorbance/emission wavelength: 583/603 nm) as fluorophore and BHQ2 as a quencher. The miRNA-26a-linked MB was designed to have 6-FAM (absorbance/emission wavelength: 494/525 nm) as fluorophore and BHQ1 as a quencher.

2.5. Fluorescence intensity

HeLa cells (human cervix cancer cell, American Type Culture Collection) were seeded onto a 24 well plate to the amount of 2×10^4 cells in each well. After 24 h, 50 pmole of miRNA-26a MB or 50 pmole of miRNA-206 MB was transfected to HeLa cells. Transfection was performed with LipofectAMINE Plus reagent (Invitrogen). For each well, 1 μl of Plus reagent and 1 μl of LipofectAMINE were used.

Target miRNA-26a and miRNA-206 were applied to each well plate containing each MB and transfected HeLa cells with a concentration of 0, 0.01, 0.025, 0.05, 0.1, 0.25, 0.75, and 1 nM, respectively. Mature miRNA-133 (1 nM) was used as a negative control. After incubation for 3 h at 37 °C, fluorescence intensity was measured (Varioskan Flash, Thermo Scientific Co.).

C2C12 cells were seeded onto a 24 well plates to the amount of 2×10^4 cells on each well. After 24 h, 50 pmole of miRNA-26a MB and/or 50 pmole of miRNA-206 MB were transfected to C2C12 cells. The transfection procedure in C2C12 cells was the same as that in HeLa cells. However, target miRNA was not added to C2C12 cells. After incubation for 3 h at 37 °C, the growth media (DMEM, 10% FBS) were replaced by differentiation media (DMEM, 1% FBS) to induce myogenic differentiation. For cells after differentiation at 0 day, 2 days, 4 days, and 6 days, fluorescence intensity was measured (Varioskan Flash, Thermo Scientific Co.).

2.6. Confocal microscopy

HeLa cells were seeded onto a 6 well plate containing cover glass to the amount of 2×10^4 cells per well. After 24 h, 50 pmole of miRNA-26a MB and/or 50 pmole of miRNA-206 MB were transfected with LipofectAMINE Plus reagent (Invitrogen). After the application of target miRNA-26a and miRNA-206 with a concentration of 0, 0.25, 0.5, 0.75, and 1 nM, respectively, for 3 h at 37 °C, cells were washed twice for 15 min with D-PBS (Gibco, US). Then, cells were fixed with 500 μl of 3.7% formaldehyde (Sigma, US), and washed twice with D-PBS for 15 min again. After DAPI staining (Vector Laboratories, CA), confocal specimens were prepared. Cells treated with mature miRNA-133a (1 nM) were also prepared after the same procedure as the control.

C2C12 cells were seeded onto a 6 well plate containing cover glass to the amount of 0.1×10^4 cells per well. After 24 h, 50 pmole of miRNA-26a MB or 50 pmole of miRNA-206 MB was transfected with LipofectAMINE Plus reagent (Invitrogen). After incubation for 3 h at 37 °C, the growth media were replaced by differentiation media (DMEM, 1% FBS). Cells were collected at 0 day, 2 days, 4 days, and 6 days after induction of differentiation, and washed with D-PBS twice for 15 min. After fixation with 500 μl of 3.7% formaldehyde, cells were washed again with D-PBS. After DAPI staining, confocal specimens were prepared.

For dual fluorescence imaging of miR-206 and miR-26a, C2C12 cells were prepared with the same protocol of single imaging, as was previously described. 50 pmole of miRNA-26a MB and 50 pmole of miRNA-206 MB were co-transfected with LipofectAMINE Plus reagent (Invitrogen) in the same C2C12 cells. Subsequent procedures for confocal imaging were the same as single fluorescence imaging.

2.7. In vivo fluorescence imaging

All *in vivo* experiments were approved by institutional animal care and use committee. Differentiation was induced in C2C12 cells by differentiation media (DMEM, 1% FBS), which was transfected with the miRNA-26a MB and miRNA-206 MB. 1×10^7 cells were subcutaneously injected into the thigh of three nude mice. *In vivo* fluorescence and bioluminescence image were obtained by using IVIS spectrum (Xenogen, California) for 3 days of differentiation ($n = 3$, mice).

2.8. Statistical analysis

Data are displayed as mean ± standard errors of means (SEM) and were calculated with the Student's *t*-test. Statistical significance was accepted at *P*-values under 0.05.

3. Results

3.1. Specificity of the MBs for detecting miRNA-26a and miRNA-206

Two different MBs with sequences that were complementary to mature miRNA-26a or miRNA-206 were designed and synthesized (Fig. 1). For simultaneous imaging of both miRNAs, one end of the hairpin DNA oligonucleotide containing the miR-26a- or miR-206a-binding sequence was labeled with 6-FAM (absorbance/emission wavelength: 494/525 nm) or Texas Red-X (absorbance/emission wavelength: 583/603 nm), respectively. The other end of the miR-26a-binding sequence was labeled with BHQ1 (miR-26a MB) and that of the miR-206a-binding sequence with BHQ2 (miR-206 MB). When mature miRNA-26a or miRNA-206 is expressed and binds to the loop of each MB, a conformational change would be expected occur in each MB. This change should result in the separation of the fluorophore and quencher, producing a fluorescence signal.

The specificity of both MBs was tested by suspending each in solution with synthesized 22-nucleotide (mature) miR-26a or miR-206. Both the miR-26a MB and miR-206 MB showed a dose-dependent and gradual increase in fluorescence in the presence of miR-26a or miR-206, while both remained in a quenched state in the presence of mature miR-133 (Fig. S1a, b). The stability and quenching efficiency of each MB was also verified in a tube by assessing fluorescence intensity in PBS and opti-MEM media for up to 6 days (Fig. S1c, d). Fluorescence intensity of both MBs was quenched for up to 6 days in the absence of miR-26a or miR-206 but increased after exposure to 1 nM of relevant miRNA.