Biomaterials 33 (2012) 6430–6437

Contents lists available at SciVerse ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Dual optical biosensors for imaging microRNA-1 during myogenesis

Won Jun Kang [a], Ye Lim Cho [a], Ju Ri Chae [a], Jong Doo Lee [a], Bahy A. Ali [b,d], Abdulaziz A. Al-Khedhairy [b], Chang Hyun Lee [c], Soonhag Kim [c,*]

[a] Division of Nuclear Medicine, Department of Radiology, Yonsei University College of Medicine, South Korea
[b] Department of Zoology, College of Science, King Saud University, Riyadh, Saudi Arabia
[c] Laboratory of Molecular Imaging, Department of Biomedical Science, CHA University, South Korea
[d] Department of Nucleic Acids Research, Genetic Engineering and Biotechnology Research Institute, City for Scientific Research and Technology Applications, Alexandria, Egypt

ARTICLE INFO

Article history:
Received 17 May 2012
Accepted 23 May 2012
Available online 12 June 2012

Keywords:
Biosensor
microRNA-1
Molecular beacon
Reporter gene
Myogenic differentiation
Molecular imaging

ABSTRACT

Dual optical microRNA (miRNA) imaging systems, bioluminescent reporter gene (a signal-off mechanism)- or fluorescent molecular beacon (MB) (a signal-on mechanism)-based miRNA imaging system, have individually allowed us to sense miRNA biogenesis in a noninvasive and iterative manner. Both of these imaging systems have shortcomings with respect to image quality. Therefore, we designed a dual optical imaging systems by simultaneous imaging of a miRNA-1 reporter gene (CMV/Gluc/3xPT_miR-1) and miRNA-1 MB in a single cell to overcome these limitations and used it to visualize miRNA-1, a highly expressed miRNA in cardiac and skeletal muscle. During myogenic differentiation of C2C12 cells, the bioluminescence intensity from CMV/Gluc/3xPT_miR-1 revealed a miRNA-1-dependent gradual decrease and the fluorescence intensity from miR-1 MB demonstrated a miRNA-1-dependent gradual increase both *in vitro* and *in vivo*. The dual miRNA-1 imaging systems, which provides the complementary imaging information about miRNA biogenesis, could be useful to sense miRNA expression during various biologic processes.

1. Introduction

MicroRNAs (miRNA, miR) are small non-coding RNAs that regulate gene expression post-transcriptionally [1,2]. MiRNAs are associated with multiple cellular processes including development, differentiation, proliferation, apoptosis, and disease progression [3–7]. MiRNAs are expressed in a tissue-specific manner, and several miRNAs including miRNA-1, miRNA-133, miRNA-181, miRNA-206, and miRNA-26a have been reported to be associated with cardiac and skeletal muscular proliferation and differentiation [8–14]. Of them, miRNA-1 is expressed specifically in skeletal and cardiac muscle, and increasing expression of miRNA-1 has been found in the developing heart [14–17].

The expression of specific miRNAs can be assessed using various methods including microarray, Northern blot and real-time polymerase chain reaction (PCR) [18–23]. However, dynamic changes in miRNA expression in the same subject during cellular processes can not be fully and repeatedly evaluated using these methods. Molecular imaging can be an appropriate method for monitoring miRNA expression during specific cellular processes in a noninvasive and iterative manner. Recently, we have developed two kinds of molecular imaging systems to visualize miRNA expression *in vitro* and *in vivo*. One consists of a bioluminescent miRNA reporter gene system that has binding sequences hybridized to a target miRNA in the 3′ untranslated regions (UTRs) of Gaussia luciferase (Gluc) [24–29]. Although this bioluminescence imaging system is good for *in vivo* imaging of miRNA due to the high signal to background ratio, it functions as a signal-off system because base pairing between binding sequences in the 3′ UTR of the reporter gene and the target miRNA inhibit Gluc expression. Such a signal-off system may be unable to differentiate between decreased Gluc signals resulting from increased miRNA expression and cellular death. The companion imaging system is a fluorescent signal-on imaging system using a MB-based miRNA imaging probe [30,31]. The miRNA MB is a stem-loop-structured DNA oligonucleotide with a fluorescent probe and a quenching molecule at each end with complementary binding sequences to a target miRNA located between them. The stem-loop structure in the miRNA MB is preserved in the absence of a target miRNA, resulting in a quenched fluorescence state by fluorescence resonance energy transfer (FRET) between a fluorescent probe and quenching molecule. In the presence of a target miRNA, binding to the miRNA MB allows

* Corresponding author. Laboratory of Molecular Imaging, Department of Applied Bioscience, CHA University, 605-21, Yeoksam1-dong, Gangnam-gu, Seoul 130-081, South Korea. Tel.: +82 2 555 5063; fax: +82 2 3668 7090.
E-mail address: kimsoonhag@empal.com (S. Kim).

0142-9612/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2012.05.056

substantial distance between the fluorescent probe and quenching molecule, causing recovery of fluorescence. Therefore, the fluorescence signals of the miRNA MB imaging system are increased in proportion to increased target miRNA. Although the miRNA MB is a convenient signal-on imaging system, it is still difficult to obtain high quality *in vivo* images due to a high autofluorescence background caused by external light sources.

A molecular imaging system consisting of both a reporter gene and a MB to monitor miRNA *in vitro* and *in vivo* has advantages and disadvantages. To overcome the shortcomings of each individual miRNA imaging system by providing complementary information, a dual optical miRNA imaging systems consisting of both reporter gene-based signal-off and MB-based signal-on imaging probes was used to monitor the muscle-specific miRNA-1. Using the dual optical miRNA-1 imaging systems, bioluminescence and fluorescence imaging modalities could successfully and simultaneously visualize miRNA-1 biogenesis in a single C2C12 cell (a mouse myoblast cell line) during myogenesis by the miRNA-1 reporter gene and the miR-1 MB *in vitro* and *in vivo*.

2. Materials and methods

2.1. Cell culture

C2C12 and HeLa cells were purchased from the American Type Culture Collection (ATCC). The culture medium for C2C12 and HeLa cells consisted of Dulbecco's modified Eagle's medium (DMEM) and 10% fetal bovine serum (FBS). Medium was changed to DMEM with 1% FBS for induction of differentiation.

2.2. Quantification of miRNA-1 expression

Expression of miRNA-1 from C2C12, HT-ori3, ARO, H9C2 (ATCC; CRL-1446), C6 (ATCC; CCL-107), and HeLa cells were quantified by real-time reverse transcription (RT)-PCR using total RNA. HT-ori3 and ARO cells were kindly provided by Dr. Dongsoo Lee in Seoul National University. Real-time PCR of mature miR-1 was conducted using the mirVanaTM qRT-PCR primer Set and the mirVanaTM qRT-PCR miRNA kit (both from Ambion). PCRs were performed in triplicate using an iCycer (Bio-Rad, USA) and SYBR Premix Ex Taq™ (2×; Takara, Japan) at 95 °C for 3 min and 40 cycles of 95 °C for 15 s and 60 °C for 30 s. The relative amounts of each mature miRNA were normalized versus the U6 snRNA primer set (Ambion) using the equation $2-\Delta\Delta CT$, where $\Delta CT = (CTmiRNA-CTU6RNA)$, $\Delta\Delta CT = (\Delta CT-\Delta CTmiRNA$ of HT-ori3). Data were expressed as mean and standard deviation of realtive values obtained from three different samples. *T*-test was performed with significant *p*-value of 0.05 for comparison.

2.3. Design of miRNA-1 molecular beacon

Mouse miRNA-1 was purchased from Bionics (Bionics, Seoul, Korea). The sequence of miRNA-1 was as follows: 5′TGGAATGTAAAGAAGTATGTAT3′. MB having reverse complimentary sequences to miRNA-1 were synthesized. MiR-1 MB was designed to have Texas Red-X (absorbance/emission wavelength: 590/615 nm) as a fluorophore and BHQ2 as a quencher. The sequences of miRNA-1 molecular beacon was as follows: Texas Red-5′ TGGAAATACATACTTCTTTACATTCCA 3′-BHQ2. As a control, miRNA-9 was purchased from Bionics. The sequence of miRNA-9 was as follows: 5′ UAAAGCUAGAUAACCGAAAGU 3′.

2.4. Fluorescence intensity of molecular beacon

HeLa cells (ATCC) were seeded into 24-well plates at 2×10^4 cells per well. After 24 h, cells were transfected with 50 pmole of miR-1 MB using LipofectAMINE Plus reagent (Invitrogen). Fluorescence intensity was measured from day 0 to day 6 to confirm stability of the MB in HeLa cells.

To validate the correlation of miRNA-1 and the miR-1 MB, target miRNA-1 was applied to each well containing miR-1 MB (50 pmol)-transfected HeLa cells at concentrations of 0, 0.01, 0.025, 0.05, 0.1, 0.5, 0.75, and 1 nM. One nanomole of target miRNA-9 was used as a negative control. After incubation for 3 h at 37 °C, fluorescence intensity was measured at absorbance/emission wavelength with 590/615 nm (Varioskan Flash, Thermo Scientific Co.). C2C12 cells were seeded into 24-well plates at 2×10^4 cells per well. After 24 h, 50 pmole of miR-1 MB was transfected into the C2C12 cells. The transfection procedure was the same as for the HeLa cells. After incubation for 3 h at 37 °C, growth medium was replaced by differentiation medium (DMEM, 1% FBS) to induce myogenic differentiation. On differentiation days 0, 2, 4, and 6, fluorescence intensity was measured to visualize MB activity responding to intrinsic miRNA-1 expression (Varioskan Flash, Thermo Scientific Co.).

2.5. Confocal microscopy of miRNA-1 molecular beacon

C2C12 cells (0.1×10^4) were seeded into 6-well plates containing cover glass. After 24 h, 50 pmole of miRNA-1 was transfected using LipofectAMINE (Invitrogen). After incubation for 3 h at 37 °C, growth medium was replaced by differentiation medium (DMEM, 1% FBS). Cells were collected at days 0, 2, 4, and 6 after induction of differentiation. After washing with Dulbecco's phosphate-buffered saline (D-PBS) twice for 15 min, cells were fixed with 500 μl of 3.7% formaldehyde for 15 min at 4 °C. After washing with D-PBS again, DAPI (4′,6-diamidino-2-phenylindole) staining (Vector Laboratories, CA) was performed, and confocal images were obtained (Carl Zeiss Inc., Germany).

2.6. Construction of CMV/Fluc and CMV/Gluc/3xPT_miRNA-1 vectors

A vector containing the firefly luciferase (Fluc) gene driven by a CMV promoter (designated as CMV/Fluc) was constructed. The Fluc reporter system was used to monitor the cellular homing and quantification of viable cells. We constructed the Gluc reporter system (CMV/Gluc/3xPT_miR-1) containing three copies of a perfect complementary miRNA-1 target sequence and fused it after the Gluc stop codon. The sequence of 3xPT_ miR-1 is: 5′ tcgagaatctagtATACATACTTCTTTACATTCCAtagta ATACATACTTCTTTACATTCCAtagtaATACATACTTCTTTACATTCCAt3′, 5′ ctagaTGGAA TGTAAAGAAGTATGTATtactaTGGAATGTAAAGAAGTATGTATtactaTGGAATGTAAAGAA GTATGTATAcTagattc 3′. The double-stranded oligonuleotide was subcloned into the Xho1 and Xba1 sites of CMV/Gluc to create CMV/Gluc/3xPT_miR-1. Three copies of perfect miRNA-1 targets before the poly (A) tail enhanced the response of CMV/Gluc to the repressive activity of miRNA-1.

As a negative control, CMV/Gluc/3xPT_miR-9 containing three copies of a perfect complementary miR-9 target sequence was constructed (25). The sequence of 3xPT_miR-9 is: 5′ tcgagaaTCTAGTTCATACAGCTAGATAACCAAAGAtagtaTCATA CAGCTAGATAACCAAAGA tagtaTCATACAGCTAGATAACCAAAGAt3′, 5′ctagaTCTTTG GTTATCTAGCTGTATGAtactaTCTTTGGTTATCTA GCTGTATGAtactaTCTTTGGTTATCTAG CTGTATGAactagattc 3′.

2.7. Luciferase assay for Fluc and Gluc activities

HeLa cells were seeded into 24-well plates at 5×10^4 cells per well. After 24 h incubation, transfection of the CMV/Fluc and CMV/Gluc/3xPT_miR-1 vectors was performed using LipofectAMINE Plus reagent (Invitrogen). CMV/Gluc/3xPT_miR-9 was transfected as a negative control. Target miRNA-1 was administered at concentrations of 0, 0.5, 1, 2.5, and 5 nmole. 4–8 h after transfection, cells were washed with phosphate-buffered saline (PBS) and treated with 100 μl of lysis solution. Supernatants were plated in microplates and Gluc and Fluc activities were measured using a Synergy MX (Bio-Tek, Ltd.) using a luciferase assay kit (TargetingSystems, USA). All results are displayed as means ± standard deviation (SD; $n = 4$).

Bioluminescence imaging obtained from an *ex vivo* specimen of a C2C12 cell-injected mouse was acquired by IVIS spectrum (Xenogen). Tissue samples from both thighs were sectioned 2 days after implantation and treated with 100 μl of lysis solution. Supernatants were plated in microplates and Gluc and Fluc activities were measured using a luciferase assay kit (TargetingSystems, USA).

Myogenic differentiation was induced in C2C12 cells as previously described (31). On days 0, 2, 4, and 6, C2C12 cells were seeded into 24-well plates at 5×10^4 cells per well. After 24 h incubation, cells were transfected with the CMV/Fluc and CMV/Gluc/3xPT_miR-1 vectors using LipofectAMINE Plus reagent (Invitrogen). Luciferase activity was measured in the C2C12 cells on days 0, 2, 4, and 6.

2.8. In vivo imaging of miRNA-1 by the dual optical miRNA-1 imaging systems

All *in vivo* experiments were housed under specific pathogen-free conditions and approved by institutional animal care and use committee in Yonsei University.

For *in vivo* imaging of miRNA-1 during myogenesis, CMV/Gluc/3xPT_miR-1 and miR-1 MB with an internal control, CMV/Fluc, were co-transfected into C2C12 cells. Myogenic differentiation was induced by changing the C2C12 medium (DMEM, 1% FBS). 1×10^7 of differentiated and undifferentiated C2C12 cells were injected subcutaneously into the right thigh and left thigh of nude mice (male BALB/c, 7 weeks old), respectively. The mice was scanned using IVIS spectrum (Xenogen, CA) ($n = 4$, mice). For the bioluminescence imaging of CMV/Gluc/3xPT_miR-1 and CMV/Fluc and fluorescence imaging of miR-1 MB, mice were anesthetized in an IVIS imaging chamber equipped with a small holder connected to an isoflurane and oxygen tank. Anesthetic gas was administered with 2% isoflurane in O_2 gas at a flow rate of 1 L min^{-1} through a nose cone. For bioluminescence imaging, 4 mg of D-luciferin for Fluc imaging and 100 μg of coelenterazine for Gluc imaging were intraperitoneally injected. The *in vivo* fluorescence imaging was conducted using a red filter (570–620 nm, band-pass filter; 600 nm, long-pass filter). A camera was used to acquire captured images at constant exposure times (1 s).

2.9. Histologic exam

Three days after injection of C2C12 cells, mice were sacrificed and implanted C2C12 tissue was isolated. C2C12 tissue was fixed in 4% paraformaldehyde and