Biomaterials 34 (2013) 4803–4809

Contents lists available at SciVerse ScienceDirect

Biomaterials

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

Radioisotope imaging of microRNA-9-regulating neurogenesis using sodium iodide sympoter

Mi-hee Jo [a,1], Myoung Seok Jeong [a,1], Hae Young Ko [a], Chang Hyun Lee [a], Won Jun Kang [b], Soonhag Kim [a,*]

[a] Laboratory of Molecular Imaging, Department of Biomedical Science, CHA University, 605-21 Yoeksam 1-dong, Gangnam-gu, Seoul 135-081, South Korea
[b] Department of Radiology, Division of Nuclear Medicine, College of Medicine, Yonsei University, Seoul 120-752, South Korea

ARTICLE INFO

Article history:
Received 21 February 2013
Accepted 11 March 2013
Available online 29 March 2013

Keywords:
MicroRNA-9
Sodium iodide symporter
Radioisotope imaging
Neurogenesis

ABSTRACT

Since microRNAs (miRNA, miR) are known to be critical in various cellular processes and diseases, non-invasive molecular imaging system for miRNA is very important for imaging cellular therapy and disease diagnosis. In this study, we developed a radionuclide imaging system for miR-9 using sodium iodide symporter (NIS). During neuronal differentiation of P 19 cells induced by the treatment of retinoic acid (RA), *in vitro* and *in vivo* imaging demonstrated that the expression and activity of NIS from the miR-9 NIS reporter gene was clearly repressed by the increased expression and functional activity of miR-9 that bound with the target sequences in the NIS reporter gene and resulted in destabilized the transcriptional activity of NIS gene, compared with the undifferentiated P19 cells. The decreased activity of NIS from the differentiated P19 cells resulted in low uptake of radionuclide and decreased radioisotope signals. The NIS reporter gene-based miRNA imaging system showed a great specificity of imaging miRNA biogenesis during cellular developments. The miRNA NIS reporter gene will provide high sensitive imaging for visualizing miRNA-regulating cellular developments and diseases.

1. Introduction

Molecular imaging has been developed to visualize expression and functions of genes related to cellular developments or diseases *in vitro* and *in vivo*. One of the methods for molecular imaging is to use reporter genes transfected into the cells and track the localization and survival of grafted cells in live animal. Although the optical reporter genes including luciferases, green fluorescence protein and red fluorescence protein have been widely used to study gene function and cellular tracking *in vivo* [1–3], these reporter genes possess tissue attenuation which is a serious problem for depth imaging. To overcome the limitation of the optical reporter genes, radioisotope reporter genes including dopamine D_2 receptor (D_2R), sodium iodide symporter (NIS), and herpes simplex virus 1− thymidine kinase (HSV1-*tk*) are suitable for providing high sensitive imaging from small or large animals [4,5]. Especially, NIS gene function of transporting iodide anion into thyroid cells enables radioisotope imaging with single photon emission computed tomography (SPECT) or positron emission tomography using ^{99m}Tc-pertechnetate, ^{123}I and ^{125}I [6,7].

MicroRNAs (miRNA, miR) are a class of non-coding small RNAs of about 21 nucleotides that are negative regulators for gene expression of protein coding genes in many organs [8,9]. MiRNAs regulate diverse regulatory pathways including fat metabolism, apoptosis, differentiation and proliferation as well as diseases [10–14]. Among the several miRNAs, miR-9 is specially expressed in the brain and abundant in neurogenic regions in embryos and adults [15,16].

Most of currently conventional methods to analyze the miRNA expression are real-time PCR, northern blot and microarray which are time-consuming and laborious, and cannot repeated non-invasively on the same subjects [17,18]. Besides, considering short half-life of miRNAs in cells, these invasive methods that always require lysis of cells cannot provide the real-time information about miRNA expression in cells or living organisms. On the basis of the theory for miRNA hybridization with its target mRNA, our group has successfully visualized the biogenesis of miRNA function in intact cells and living organisms during neurogenesis, myogenesis and carcinogenesis using three different non-invasive miRNA imaging systems including 2 different reporter gene-based imaging methods using a bioluminescent reporter gene, *Gaussia* luciferase (Gluc) and magnetic resonance imaging (MRI) reporter gene,

* Corresponding author. Tel.: +82 2 555 5063; fax: +82 2 3668 7090.
E-mail address: kimsoonhag@empal.com (S. Kim).
[1] M. Jo and M.S. Jeong contributed equally to the work as a first author.

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http://dx.doi.org/10.1016/j.biomaterials.2013.03.032

transferring receptor (TfR), and a fluorescent miRNA molecular beacon [16,18–25]. Although both optical and MRI reporter genes of imaging miRNA expression provide a convenient method or a good spatial resolution, they can't provide imaging information about miRNA functions in depth imaging with high sensitivity. Therefore, development of radionuclide imaging using a radioisotope reporter gene may overcome such shortcomings of the existing miRNA reporter gene imaging systems by providing depth imaging with a great sensitivity, especially *in vivo*, about dynamic expression of miRNA.

In this study, we developed a radioisotope-based miRNA reporter gene using a NIS reporter gene. MiR-9 was selected for imaging neurogenesis of P19 cells. Using SPECT, NIS activity regulated by miR-9 was measured by the uptake of radioiodine using ^{125}I for *in vitro* imaging and ^{99m}Tc-pertechnetate for *in vivo* imaging.

2. Materials and methods

2.1. Construction of the miR-9 NIS reporter gene

DNA fragment encoding NIS gene (provided by Dr. Hwang, Seoul Medical University, Seoul) was inserted between *Xho*I and *Eco*RI of pDsRed2-N1 vector (Clontech, Palo Alto, CA) to construct the NIS reporter vector (CMV/NIS). The nucleotide sequences of mature miR-9 were acquired from the miRNAMap database (http://mirnamap.mbc.nctu.edu.tw) and we synthesized 3 copies of a perfect complementary target sequence of mature miR-9 (3XPT_miR-9) by annealing the following oligonucleotides: 5′-CGTTCATACAGCTAGATAACCAAAGATAGTATCATACAGCTAGATAACCAAAGATAGTATCATACAGCTAGATAACCAAAGAG-3′ and 5′-GATCCTCTTTGGTTATCTAGCTGTATGATACTATCTTTGGTTATCTAGCTGTATGATACTATCTTTGGTTATCTAGCTGTATGAACGGTAC-3′. The miR-9 NIS reporter vector was designed by inserting the 3XPT_miR-9 into the *Kpn*I and *Bam*HI site in the 3′ untranslated region (UTR) of the CMV/NIS (designed as CMV/NIS/3XPT_miR-9).

2.2. Cell culture and transfection

To study the specificity of the miR-9 NIS reporter gene, HeLa cells (human cervical cancer cell line) and P19 cells (mouse embryonic teratocarcinoma cell lines) were selected. HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Grand Island, NY) containing 10% fetal bovine serum (FBS, Grand Island, NY) and 1% antibiotics (Invitrogen, Grand Island, NY); this was passaged to 70–80% confluence. The P19 cells were maintained in minimal essential medium-alpha (a-MEM, Gibco, Grand Island, NY) containing 7.5% bovine calf serum (BS, Gibco, USA), 2.5% FBS and 1% antibiotics in a 5% CO_2-humidified chamber. For the induction of neuronal differentiation, DMEM/F12 (1:1) medium (Gibco, Grand Island, NY) containing 1% insulin–transferrin–selenium (ITS, Gibco, Grand Island, NY), 1% antibiotics and 0.5 μM all-trans-retinoic acid (RA Sigma, St Louis, MO) were used. The differentiation medium for P19 cells was replaced with new RA-containing differentiation medium every 2 days. To image miR-9-regulating neuronal differentiation of P19 cells induced by RA, the plate was coated with autoclaved 0.1% gelatin solution (Sigma, St Louis, MO). For the experiment of imaging miR-9 in HeLa cells, the miR-9 NIS reporter gene was transiently transfected with miR-9 precursor (pre-miR-9) or miR-124a precursor (Ambion, Austin, TX) using Lipofectamine and the Plus reagent (Invitrogen, Grand Island, NY). All of the transient transfections were carried out in triplicate.

2.3. Real-time PCR

Total RNAs were isolated from P19 cells the day after RA treatment using Trizol reagent (Invitrogen, Grand Island, NY). Small RNA was isolated from P19 cells using mirVana™ miRNA isolation kits (Ambion, USA), and then subjected to cDNA synthesis and qRT-PCR of mature miR-9 using the mirVana™ qRT-PCR primer Set and the mirVana™ qRT-PCR miRNA kit (both from Ambion, USA). The qRT-PCR was 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 64 °C for 30 s. The relative amounts of miR-9 were normalized versus the U6 snRNA primer set (Ambion, USA).

2.4. In vitro ^{125}I uptake assays of the miR-9 NIS reporter gene

NIS activity was analyzed 2 days after transfection of the CMV/NIS/3XPT_miR-9 into HeLa or P19 cells. All transfections were carried out in triplicate. Following incubation, the cells were washed with Hanks' balanced salt solution (HBSS) containing 0.5% bovine serum albumin with 3.7 kBq of carrier-free Na^{125}I and 10 μM NaI for 30 min. After incubation, the cells were washed twice, as quickly as possible, with 2 mL of iodide-free ice cold HBSS buffer, detached with 0.2% SDS, and radio activities were measured using a gamma counter (Cobra II, Canberra Packard, Meriden CT). A BCA™ Protein Assay Kit (Thermo scientific, USA) was used to quantify the protein for calibration. For the transfection efficiency-considered NIS activity of the CMV/NIS/3XPT_miR-9 from P19 cells, a firefly luciferase (Fluc) reporter gene regulated by the CMV promoter (CMV/Fluc, as an internal control) was co-transfected into undifferentiated and differentiated P19 cells. NIS activities 0, 2 and 4 days after neuronal differentiation of P19 cells transfected with the CMV/NIS/3XPT_miR-9 were normalized versus bioluminescence intensity of the CMV/Fluc.

2.5. Immunofluorescence analysis of neuronal differentiation of P19 cells

P19 cells or matrigel-incorporated P19 cells were fixed with 4% formaldehyde for 15 min, and then washed two times for 5 min with PBS, and gentle shaking was provided during incubation. The blocking and permeabilization procedures were performed simultaneously with 20% normal goat serum reaction mixture and 0.1% Triton X-100 for 60 min. The Oct-4 or Tuj1 protein was detected by a 1:1000 dilution of anti-Oct-4 antibody (Chemicon, Millipore, Watford, UK) or anti-Tuj1 antibody (Chemicon, Millipore, Watford, UK) and incubated overnight at 4 °C. After three washes for 5 min each, Alexa-488 and -594 Fluor secondary antibody conjugates were added and the mixture was incubated for 90 min. The P19 cells were placed on a cover slip and mounted with an aqueous mounting solution containing DAPI (Vector Laboratories, Inc., CA). The fluorescence signal was detected by confocal laser scanning microscopy (LSM 510; Carl Zeiss, Weimer, Germany).

2.6. In vivo SPECT imaging

All animals used in the *in vivo* experiments were housed under specific pathogen-free conditions and the experiments were approved by the institutional animal care and use committee in CHA University. The CMV/NIS/3XPT_miR-9 was co-transfected into P19 cells with the CMV/Fluc. 2×10^6 cells were harvested in 150 μL of PBS and resuspended within 150 μL of matrigel. These cells were implanted into both thighs of a male ICR mice (7 weeks old; $n = 3$); the left thigh, without the RA treatment, was used as a control, and the right thigh was treated with RA for induction of neuronal differentiation. All mice received intraperitoneal injections of 70 μL of a Zoletil (Virbac, Carros, France) and Rompun (Bayer, Seoul, Korea) solution (2:1) for anesthesia18.5 MBq (500 μCi) of ^{99m}Tc-pertechnetate was intraperitoenally injected. For image acquisition, mice were anesthetized by injecting 50 μL of a ketamine and xylazine (2:1) solution intraperitoenally, and placed in a spread-prone position. At 30 min and 60 min after injection, scintigraphic image was acquired with a gamma-camera (ADAC ARGUS single detector, Oak Ridge, NC) equipped with a 6-mm-sized pinhole collimator. Sequential studies were performed using same protocol on Day 0, Day 1 and Day 2 after cell implantation. The *in vivo* fluorescence images of the CMV/Fluc were acquired using an IVIS spectrum (Xenogen, CA) and a red filter (605/30 nm, excitation filter; 660/20 nm, emission filter). A camera was used to acquire images at a constant exposure time (1 s). ROIs were drawn around the area of uptake in the right and left thighs on the SPECT images. The average counts per pixel were recorded for both thighs of the mouse. For *in vivo* bioluminescence imaging, 4 mg of D-luciferin for Fluc imaging was intraperitoneally injected and acquired the bioluminescence imaging by the IVIS spectrum (Xenogen, CA).

2.7. Statistical analysis

All data are presented as the means ± SD calculated from quadruple wells and significant differences between samples were assessed using a Student's *t*-test ($^*P < 0.05$, $^{**}P < 0.005$).

3. Results

3.1. Design and function of a NIS reporter gene-based miRNA imaging system

To non-invasively monitor miR-9 expression during neurogenesis by a radioisotope reporter gene-based miRNA imaging system, NIS reporter gene regulated by cytomegalovirus (CMV) promoter (CMV/NIS) was first selected and further designed to have perfectly targeted binding sequences against mature miR-9 by inserting three copies of reverse complimentary sequences (3XPT_miR-9), which is highly expressed in the neuron, into the 3′ UTR of the NIS reporter gene (CMV/NIS/3XPT_miR-9) as a miR-9 NIS reporter gene (Fig. 1). The imaging mechanism of the miR-9 NIS reporter gene is described as follows; in the absence of miR-9, NIS activity of the CMV/NIS/3XPT_miR-9 in cells will be increased due to no function of miR-9, result in higher uptake of radioiodine and produce strong signals of radioisotope imaging. In contrast, when miR-9s are expressed in cells, miR-9 binds to the 3XPT_miR-9 of the CMV/NIS/3XPT_miR-9. This hybridization of miR-9 with the target