FEBS Open Bio 5 (2015) 647–655

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

Bioimaging of microRNA124a-independent neuronal differentiation of human G2 neural stem cells

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ARTICLE INFO

Article history:
Received 27 July 2015
Revised 4 August 2015
Accepted 5 August 2015

Keywords:
G2 human neural stem cells
microRNAs
In vivo imaging
Neuronal differentiation
Reporter gene

ABSTRACT

Evaluation of the function of microRNAs (miRNAs or miRs) through miRNA expression profiles during neuronal differentiation plays a critical role not only in identifying unique miRNAs relevant to cellular development but also in understanding regulatory functions of the cell-specific miRNAs in living organisms. Here, we examined the microarray-based miRNA expression profiles of G2 cells (recently developed human neural stem cells) and monitored the expression pattern of known neuron-specific miR-9 and miR-124a during neuronal differentiation of G2 cells *in vitro* and *in vivo*. Of 500 miRNAs analyzed by microarray of G2 cells, the expression of 90 miRNAs was significantly increased during doxycycline-dependent neuronal differentiation of G2 cells and about 60 miRNAs showed a gradual enhancement of gene expression as neuronal differentiation progressed. Real-time PCR showed that expression of endogenous mature miR-9 was continuously and gradually increased in a pattern dependent on the period of neuronal differentiation of G2 cells while the increased expression of neuron-specific mature miR-124a was barely observed during neurogenesis. Our recently developed miRNA reporter imaging vectors (CMV/Gluc/3×PT_miR-9 and CMV/Gluc/3×PT_miR-124a) containing Gaussia luciferase, CMV promoter and three copies of complementary nucleotides of each corresponding miRNA showed that luciferase activity from CMV/Gluc/3×PT_miR-9 was gradually decreased both *in vitro* and *in vivo* in G2 cells induced to differentiate into neurons. However, *in vitro* and *in vivo* bioluminescence signals for CMV/Gluc/3×PT_miR-124a were not significantly different between undifferentiated and differentiated G2 cells. Our results demonstrate that biogenesis of neuron-specific miR-124a is not necessary for doxycycline-dependent neurogenesis of G2 cells.

1. Introduction

The investigation of key regulatory molecules during the process of neuronal development is important not only for identifying the crucial factors involved in determining neuronal fate specification at the molecular level, but also for elucidating the key activators needed to induce neuronal differentiation. Recently, microRNAs (miRNAs or miRs), which are non-coding RNA molecules of approximately 25 nucleotides, have emerged as critical gene regulators involved in the developmental process [1,2]. The study of regulatory functions and the expression profiles of microRNAs relevant to neuronal differentiation is necessary to understand the biological function of these microRNAs in neuronal development and to develop therapeutic agents for the repair of neurodegenerative disorders [3,4]. Previous

Abbreviations: miR, microRNA; Gluc, Gaussia luciferase; Fluc, Firefly Luciferase

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http://dx.doi.org/10.1016/j.fob.2015.08.003
2211-5463/

explorations into differential miRNA expression during neurogenesis have revealed the expression profiles of miRNAs not only in mouse and human embryonic carcinoma cells, which can be differentiated into neurons by retinoic acid (RA), but also in mouse and human embryonic stem cells, based on the use of a powerful tool, miRNA microarray analysis [5–7]. Recent evidence has indicated that the ability of neural stem cells to generate a neuronal lineage is regulated by a complex network between miRNAs and their target genes [8]. A variety of neuron-specific miRNAs including let-7, miR-124a, miR-9, and miR-132 have been shown to negatively regulate non-neuronal target genes such as PTBP-1 or the neuronal repressor, REST (RE-1 silencing transcription factor) [8–11]. Amid increasing interest in the development of treatments for neuron-related disorders, human neural stem cells offer therapeutic potential in terms of cell-based therapies for the repair of a wide range of neurological diseases in the field of human medicine. In this study, we used G2 cells, human neural stem cells that are easily differentiated into neurons, regulated through induction by tetracycline [12,13]. Genetically engineered G2 cells with v-myc overproduction under the control of tetracycline are able to maintain their self-renewal capacity. In contrast, withdrawal of tetracycline causes G2 cells to accelerate to a neuronal lineage, as proven in a previous study using measurement of the expression level of β-tubulin III, which is known as a neuronal marker.

Previous studies, which have involved a variety of in silico and *in vitro* experimental methods including bioinformatics as well as cellular and molecular approaches, have been hampered but the fact that the experimental environments did not accurately represent the biological phenomenon *in vivo* [14–16]. *In vivo* molecular imaging using an animal model is required to provide distinctive biological information for cells of interest. Here, we investigated a host of miRNA expression profiles using an underlying bioinformatics approach, and we evaluated the *in vivo* monitoring as well as *in vitro* analysis of miRNAs identified during neuronal differentiation of G2 cells. We examined the expression profiles of 500 human miRNAs expressed in G2 cells during neuronal differentiation using miRNA microarray analysis, and selected 68 miRNAs that showed a gradually increasing pattern of expression during neuronal differentiation of G2 cells. Of the miRNAs with significant increases in their expression levels during neuronal differentiation, *in vitro* and *in vivo* expression levels of two well-known neuron-specific miRNAs, miR-9 and miR-124a, were monitored during neuronal differentiation of G2 cells.

2. Materials and methods

2.1. Microarray analysis

The synthesis of target miRNA probes and hybridization reaction were conducted using an miRNA labeling reagent and hybridization kit (Agilent Technology, USA). Each 100 ng of total RNAs were dephosphorylated with ~15 units of calf intestine alkaline phosphatase (CIP), followed by RNA denaturation with ~40% DMSO and 10-min incubation at 100 °C. The dephosphorylated RNA was then ligated with pCp-Cy3 mononucleotide and purified using MicroBioSpin 6 columns (Bio-rad, USA). Labeled samples were then resuspended with Hi-RPM Hybridization buffer and Gene Expression Blocking Reagent, followed by boiling for 5 min at 100 °C and incubating on ice for 5 min. The denatured labeled probes were pipetted onto an assembled Agilent miRNA Microarray (15 K) and hybridized for 20 h at 55 °C with 20 rpm rotation in an Agilent Hybridization oven (Agilent Technology, USA). The hybridized microarrays were washed according to the manufacturer's washing protocol (Agilent Technology, USA).

2.2. Data acquisition and analysis

The hybridized images were scanned using Agilent's DNA microarray scanner and quantified using feature extraction software (Agilent Technology, Palo Alto, CA). All data normalization and identification of genes with significant changes in expression were performed using GeneSpringGX 7.3 (Agilent Technology, USA). The averages of the normalized ratios were obtained by dividing the average value of normalized signal channel intensity by the average value of normalized control channel intensity. Functional annotation of genes was performed according to the Gene Ontology™ Consortium (http://www.geneontology.org/index.shtml) by GeneSpringGX 7.3.

2.3. Cell culture and transient transfection studies

HB2.G2 cells were generated from HB1.F3, which is an immortalized human neural stem cell line derived from human fetal brain, and were cultured in DMEM medium containing 10% fetal bovine serum, 10 U/ml penicillin, 10 μg/ml streptomycin and 2 μg/ml of doxycycline [12]. To induce *in vitro* neuronal differentiation, G2 cells were cultured with DMEM medium without serum containing 5 ng/ml of epidermal growth factor (EGF). G2 differentiation medium was replaced with new EGF-containing differentiation medium every 2 days. The purified recombinant plasmids CMV/Gluc/3×PT_miR-124a, CMV/Gluc/3×PT_miR-9 and CMV/Fluc were transfected into each cell with lipofectamine reagent (Invitrogen, Grand Island, NY) diluted in OPTI-MEM medium (Gibco, Grand Island, NY). All transient transfections were carried out in triplicate.

2.4. qRT-PCR analysis

Total small RNA prepared from G2 cells at 0, 2, 4, and 6 days after neuronal differentiation was extracted by nuclease-free water using the *mir*Vana™ miRNA isolation kit (Ambion, Austin, TX). To verify the copy numbers of mature miR-124a and miR-9 during neuronal differentiation of G2 cells, qRT-PCR was carried out using the *mir*Vana™ qRT-PCR primer set (Ambion, Austin, TX) and *mir*Vana™ qRT-PCR miRNA detection kits (Ambion, Austin, TX). The levels of mature miR-124a and miR-9 were examined by PCR amplification conditions (95 °C for 3 min and 95 °C for 15 s, 60 °C for 30 s for up to 40 amplification cycles) with SYBR Premix Ex Taq™ (2×) (Takara, Japan). The U6 snRNA primer set (Ambion, Austin, TX) was used as a housekeeping RNA control for normalization of the data. All reactions were performed using the iCycler (Bio-Rad, Hercules, CA) system.

2.5. In vitro bioluminescence assay

Firefly luciferase assays and Gaussia luciferase assays were performed using luciferase assay kits (Applied Biosystems) or Gaussia luciferase assays kits (Targetingsystems, Cajon, CA). G2 cells were washed with phosphate-buffered saline (PBS) and lysed in lysis solution. G2 cell homogenates were collected and then transferred to a 96-well microplate. Bioluminescence intensity was calculated using a microplate luminometer (TR717; Applied Biosystems) with an integration time of 20 s.

2.6. Immunofluorescence analysis performed to verify the neuronal differentiation pattern using a neuronal marker in G2 cells

G2 cells were fixed with 4% formaldehyde for 20 min and washed 3 times for 10 min using PBS solution. Blocking and permeabilization procedures were performed simultaneously; the

reaction mixture of 20% normal goat serum and 0.2% TritonX-100 was added to the cells for 60 min. At each differentiation time point the prepared G2 cells were incubated with primary antibodies to Oct4 (Chemicon, Millipore, Watford, UK), Tuj1 (Covance, Princeton, NJ, USA), MAP2 (Sigma–Aldrich, St. Louis, MO, USA), GFAP (Cell Signaling Technology, Inc., Danvers. MA. USA), and O4 (R&D Systems, Inc., Minneapolis, MN, USA) overnight at 4 °C. After several washing steps for 10 min each, cells were incubated with Alexa Flour secondary antibody for 90 min. G2 cells were placed on a sterile cover slip with an aqueous mounting solution containing 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI) (Vector Laboratories, Inc, CA).

2.7. Preparation of PLLA scaffold

The fibrous poly (L-lactic acid) (PLLA) scaffold was kindly provided by S.J. Lee (Ewha Womans' University, College of Pharmacy). PLLA scaffolds were fabricated from poly L-lactic acid microfibers spun using a wet spinning method. For preparation of the G2 cell/PLLA scaffold complex, the sterilization step of PLLA scaffolds was carried out using 70% isopropyl alcohol overnight, and PLLA scaffolds were soaked with G2 culture medium for 12 h.

2.8. In vivo bioluminescence images

All experimental animals were housed under specific pathogen-free conditions and handled in accordance with the guidelines issued by the Institutional Animal Care and Use Committee of Catholic Kwandong University International St. Mary's Hospital. G2 cells co-transfected with CMV/Fluc and CMV/Gluc3× PT_miRNA were incubated with PLLA scaffold pre-wetted with 70% ethanol in G2 cell medium for 2 h. For *in vivo* neuronal induction, 1×10^7 G2 cells carrying each plasmid vector were harvested in PBS and resuspended in G2 differentiation medium containing EGF. G2/scaffold coplex was subcutaneously implanted into both thighs of nude mice (male BALB/c, 8 weeks). To acquire bioluminescence images, the mice were anesthetized with 2% isoflurane flow in O_2 gas at a flow rate of 1 L/min through the nose cone by placing the mouse in the induction chamber. For acquisition of the *in vivo* Gaussia luciferase image, 0.01 mg of coelenterazine (10110-1, Biotium, Hayward, CA) was directly injected into the PLLA scaffold/G2 cell complex site. 3 mg/0.1 ml/mouse of luciferase substrate luciferin was intraperitoneally injected to acquire the firefly luciferase image. The bioluminescence imaging for the *in vivo* firefly luciferase signal was acquired approximately 6 h after acquisition of the Gaussia luciferase image by consideration of the decay time of a light signal with a half-life of Gaussia luciferase/coelenterazine reaction. Animals were placed in an IVIS-200 equipped with a CCD camera (Xenogen, California). Bioluminescence images were obtained by integrating and collecting light for 5 min.

2.9. Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM). The Student's *t*-test was used to calculate *P* values.

3. Results

3.1. Neuronal differentiation of G2 cells

G2 cells are human neural stem cells that have been modified by the addition of the v-myc oncogene through retroviral transduction for immortalization [12,13]. The schematic illustration summarizes the neuronal differentiation pattern of G2 cells (Fig. 1A). Briefly, v-myc oncogene expression is controlled by the presence of doxycycline (a tetracycline derivative). The G2 cell line is maintained on the condition of the presence of doxycycline, and can be induced to change to a neuronal lineage by the removal of doxycycline.

When G2 cells were induced into a neuronal lineage by the removal of serum and doxycycline and the addition of epidermal growth factor (EGF), an intensive neurite outgrowth pattern was observed to form over a number of days (Fig. 1B). The synaptic clefts of postsynaptic neuron-like cells were observed 4 days after neuronal differentiation. Immunofluorescence results verified that G2 cells were differentiated into a neuronal lineage since high expression of neuronal markers (TuJ1 and MAP2) was observed rather than glial markers (GFAP and O4) in confocal microscope images (Fig. 1C). Also, we verified that the expression of Oct4 (a marker for undifferentiated cells) in the cytoplasm of G2 cells was clearly decreased at 6 days after induction of neuronal differentiation by removal of doxycycline. These results indicate that the induction of G2 cells into a neuronal lineage by the removal of doxycycline was successful.

3.2. miRNA expression profile of G2 cells during neuronal differentiation

To investigate the miRNA expression profile during the differentiation of G2 cells into a neuronal lineage a 0.5-K miRNA microarray was hybridized with total RNA samples extracted from various time points of neuronal differentiation of G2 cells. The expression levels of 470 human miRNAs spotted onto each microarray grid was examined for samples collected at 0, 2, 4, and 6 days after induction of neuronal differentiation in G2 cells. On the basis of normalization and selection of fold-changed genes using GeneSpringGX 7.3 (Agilent's Human miRNA Microarray), time-series gene expression analysis revealed that 90 of 470 miRNAs showed up-regulation greater than 2-fold in at least one of the three time points examined after neuronal differentiation of G2 cells, compared with the undifferentiated state of G2 cells. In particular, let-7f, miR-29b, miR-374, miR-29c, miR-9 and miR-148 showed the most dramatically enhanced differences in gene expression after neuronal differentiation of G2 cells (Table 1). In total, 68 of these 90 miRNAs exhibited time-dependent alterations in their gene expression patterns that reached maximum levels on day 6 (Fig. 2). Several miRNAs have been reported to very specifically and gradually increase during neurogenesis including let-7a, let-7b, miR-9, miR-9*, miR-125a, miR-125b, miR-124a, miR-135a, miR-218, miR-20a, miR-128a and miR-128b [6,7,17,18]. Similar to the findings of other reports, the expression levels of let-7a, let-7b, miR-9, miR-9*, miR-125a and miR-125b in our experiments were gradually increased during the neuronal differentiation of G2 cells (Fig. 2 and Table 2). Interestingly, several miRNAs including miR-124a, miR-135a, miR-218, miR-20a, miR-128a and miR-128b, which were supposed to be gradually increased during normal neurogenesis, showed aberrant miRNA expression during neuronal differentiation of G2 cells (Table 1). None of these aberrantly expressed miRNAs exhibited a significant difference in the level of miRNA expression after neuronal differentiation of G2 cells.

3.3. Aberrant gene expression pattern of neuron-specific miR-124a during neuronal differentiation of G2 cells

Among the miRNAs aberrantly expressed during neurogenesis of G2 cells, miR-124a was selected to use for verification of its expression using real-time PCR. In normal neurogenesis, miR-124a gradually increases in expression during neurogenesis and is highly neuron-specific [6,19–21]. Similarly to the results of the microRNA microarray, real-time PCR measurement of miR-124a

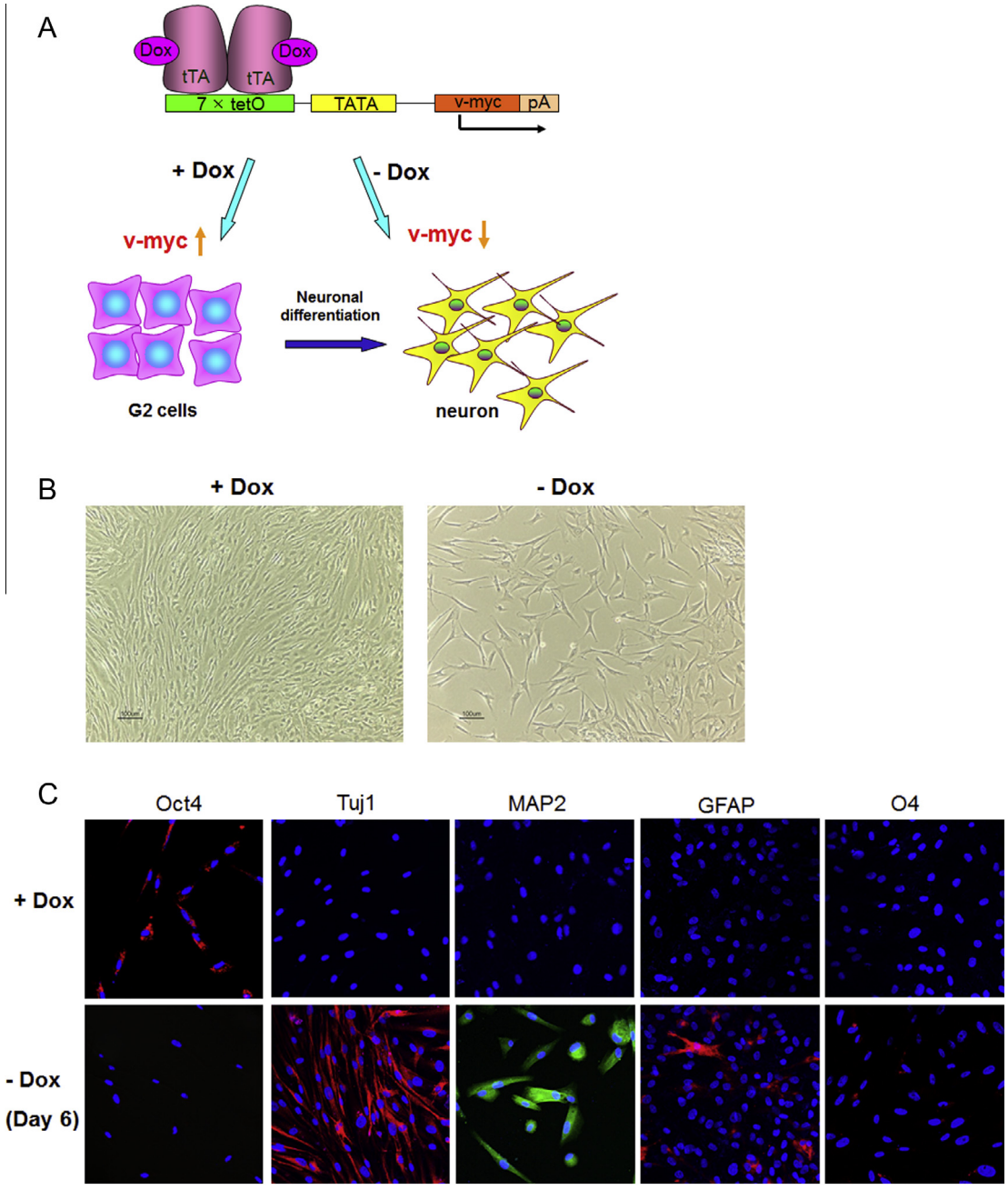

Fig. 1. Characterization of neuronal differentiation in G2 human neural stem cells. (A) Schematic illustration of the tetracycline-dependent neuronal differentiation of the G2 cell line. The HB2.G2 cell line was infected with two different viral vector systems, CMV-regulated tetracycline regulatory gene and tetracycline responsive element-dependent v-myc expression vector. In the presence of doxycycline, tTA and doxycycline complex binds to 7× tetO sequences within the tetracycline responsive element, causing the v-myc oncogene to be expressed and thereby maintaining the self-renewal of the immortalized human neural stem cells, HB2.G2. In contrast, the neuronal differentiation of the G2 cell line can be induced by the removal of doxycycline. (B) G2 cells maintained in DMEM containing 10% FBS with doxycycline (2 μg/ml) were transferred into DMEM containing 5 ng/ml of epidermal growth factor (EGF) in serum-free conditions to induce neuronal differentiation. Neurite-like processes were observed at 4 days after induction of neuronal differentiation in G2 cells. (C) G2 cells at 6 days after induction of the neuronal lineage showed an immunoreactive pattern to Tuj1, MAP2, GFAP, and O4. While a high fluorescence signal using the Oct4 antibody was detected in undifferentiated G2 cells, only very weak non-specific signals of Oct4 were found in differentiated G2 cells due to reduced expression of this protein. Scale bar, 20 μm.

showed no significant gradual increase in miRNA expression after differentiation of G2 cells while expression of miR-9, used as a positive control, was gradually increased over time during neuronal differentiation of G2 cells (Fig. 3A). The miR-124a expression pattern was also studied in P19 cells, which are embryonal teratocarcinoma cells that have been differentiated into a neuronal lineage controlled by treatment with RA. Similar to results shown in other reports for the miR-124a expression pattern during neurogenesis, the miR-124a expression level was gradually increased during the progression of neuronal differentiation in RA-treated P19 cells [7,19] (Supplementary Fig. 1).

VAMP3 (vesicle-associated membrane protein 3) is necessary for the efficient fusion between a synaptic vesicle and the synaptic membrane, and it is targeted and transcriptionally repressed by miR-124a during neurogenesis [22]. To see the functional activity of miR-124a during neurogenesis of G2 cells the expression of VAMP3 was measured by RT-PCR. The mRNA expression level obtained by RT-PCR demonstrated that VAMP3 was not

Table 1
MicroiRNAs expression profile during neurogenesis of G2 cells.

miRNAs	UD	2 D	4 D	6D
Up-regulated miRNAs				
hsa-miR-7	1	522.2198	523.2151	292.2855
hsa-miR-29b	1	42.74225	57.29627	76.20358
hsa-miR-374	1	24.42929	31.31131	36.88374
hsa-miR-758	1	16.15773	10.68004	14.79463
hsa-miR-29c	1	13.68596	19.7659	27.33652
hsa-miR-9	1	10.60056	15.83843	26.63348
hsa-miR-148b	1	11.35026	28.26905	34.95855
hsa-miR-122a	1	11.06301	9.126085	16.51886
hsa-miR-146a	1	5.189159	14.2444	19.7232
hsa-miR-26b	1	5.824441	9.62672	14.56
hsa-miR-368	1	7.75968	10.30087	13.91249
hsa-miR-9*	1	7.64367	9.416519	13.2464
hsa-miR-19b	1	8.104035	10.48603	11.11664
hsa-miR-335	1	1.62836	5.84318	10.4418
hsa-miR-379	1	6.495094	6.488897	10.1394
hsa-miR-212	1	7.64367	10.5313	10.1193
hsa-miR-572	1	14.03861	8.195663	8.542398
hsa-miR-98	1	3.673534	6.434218	8.894021
hsa-miR-134	1	5.612838	6.579067	7.340769
hsa-let-7f	1	2.871265	4.92591	6.24624
Aberrantly expressed miRNAs				
has-miR-128a	1	2.27625	0.819059	1.129478
has-miR-128b	1	0.010958	1	0.878327
has-miR-135a	1	0.345547	0.76329	0.405171
has-miR-218	1	0.26019	1	1.19274
has-miR-124a	1	1	1	1
has-miR-20a	1	1.672148	0.497116	1.565041

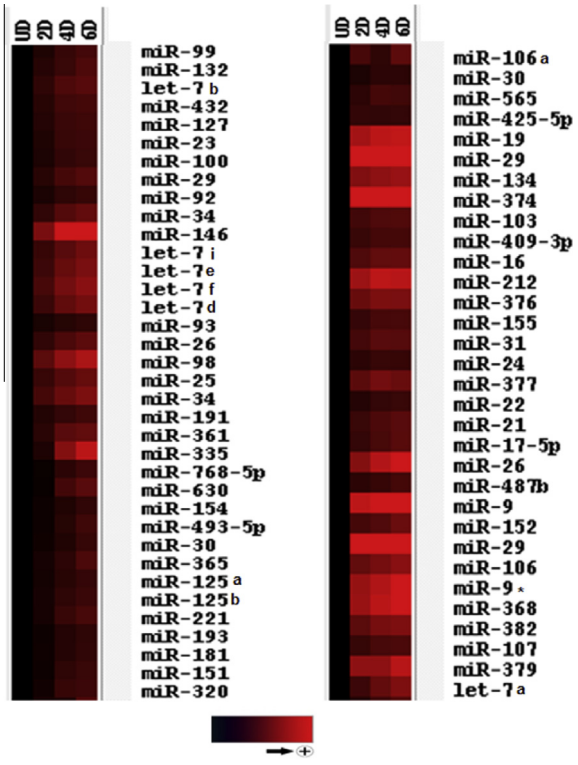

Fig. 2. Microarray-based microRNA expression analysis during neurogenesis in G2 cells. Clustering analysis represented relative expression levels of miRNAs at the neuronal differentiation stage in G2 cells. 68 miRNAs showing both a greater than twofold increase in expression between differentiation time points and a gradual increase expression pattern during progression of neuronal differentiation were selected and classified into four differentiation time point groups. Of the 68 miRNAs, several miRNAs including the miR-335, miR-26 and let-7 families showed distinct expression patterns at each stage of neuronal differentiation in G2 cells.

significantly repressed after neuronal differentiation of G2 cells, indicating no significant gradual increase in miR-124a expression during neurogenesis of G2 cells (Fig. 3B).

3.4. In vitro and in vivo imaging of miR-9 and miR-124a during neuronal differentiation of G2 cells

To further verify a few neuron-specific miRNAs that are independent and dependent on neurogenesis of G2 cells, miR-9 and miR-124a were selected and their expression levels were assessed *in vitro* and *in vivo*. To image the endogenous gradual increase in expression of miR-9 and the lack of significant change in expression of miR-124a *in vitro* and *in vivo* during neuronal differentiation of G2 cells, two previously reported bioluminescent reporter gene vectors for imaging miRNA were selected [18,19]. Briefly, a Gaussia luciferase (Gluc) reporter vector was first designed having the following components (in order): a cytomegalovirus (CMV) promoter, an open reading frame of Gluc, 3 copies of perfectly complementary sequences of mature miR-9 (designated as CMV/Gluc/3×PT_miR-9) or mature miR124a (designated as CMV/Gluc/3×PT_miR-124a). When each bioluminescent miRNA reporter imaging system is bound by its cognate miRNA, the Gluc activity is repressed by the normal function of the miRNAs. To evaluate miR-9 and miR-124a expression patterns *in vitro* during neuronal differentiation of G2 cells, a firefly luciferase (Fluc) reporter gene regulated by a CMV promoter (designated as CMV/Fluc) was cotransfected with CMV/Gluc/3×PT_miR-9 or CMV/Gluc/3×PT_miR-124a into G2 cells for normalization of the study. Similar to results found with miRNA microarray and real-time PCR, the *in vitro* bioluminescence analysis showed that the Gluc activity of the CMV/Gluc/3×PT_miR-9 transfected into G2 cells had neuronal differentiation-dependent gradual repression due to the gradual increase in expression of miR-9 during neurogenesis of G2 cells (Fig. 3C). Meanwhile, the Gluc expression pattern of the CMV/Gluc/3×PT_miR-124a demonstrated a small increase rather than a decrease during neuronal differentiation of G2 cells. Also, the CMV/Gluc/3×PT_miR-124a vector was verified in the P19 cell system, which shows a gradual decrease in Gluc activity due to the gradual increase in miR-124a during neurogenesis of P19 cells [19]. Unlike G2 cells, P19 cells transfected with CMV/Gluc/3×PT_miR-124a vector displayed Gluc activity that continuously decreased during neuronal differentiation of P19 cells, a finding that is consistent with a gradual increase in miR-124a expression during the progression of neuronal differentiation of these cells (Supplementary Fig. 2).

For *in vivo* study, we used a PLLA scaffold, which can be utilized as a microenvironmental space, to support cell growth as well as to

Table 2
List of up-regulated miRNAs during neurogenesis.

Up-regulated miRNA list during neurogenesis in P19 cells [7]	Abundant expression in adult brain [27]	Up-regulated miRNA list during neurogenesis in ESC [5]
Let-7a	Let-7a	Let-7b
Let-7b	Let-7b	Let-7d
miR-9	Let-7c	Let-7g
miR-9*	miR-9	miR-124a
miR-100	miR-9*	miR-125a
miR-124a	miR-128	miR-135a
miR-125a	miR-132	miR-181
miR-125b	miR-125a	miR-22
miR-135a	miR-125b	miR-26
miR-16	miR-124a	miR-9
miR-218		miR-9*
miR-30a		
miR-98		

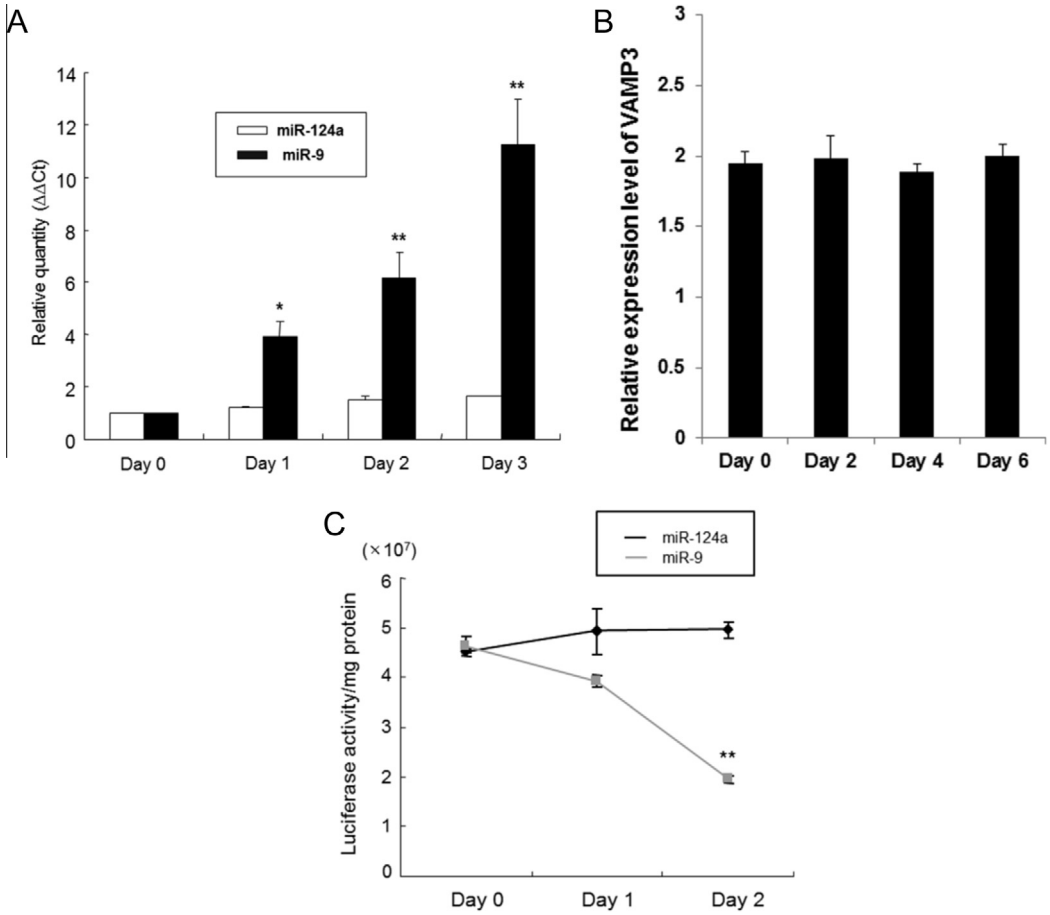

Fig. 3. *In vitro* validation of miR-9 and miR-124a expression patterns in neuronal differentiation of G2 cells. (A) Small RNA was extracted from G2 cells at 0, 2, 4, and 6 days after neuronal induction by the withdrawal of doxycycline. Quantitative RT-PCR analysis showed that the endogenous mature miR-9 expression level was gradually increased following the induction of neuronal differentiation of G2 cells. In contrast, there was no significant change in the expression level of mature miR-124a during neurogenesis. U6 snRNA was used as an internal control. Results are expressed as the mean (SD) of triplicate experiments. (B) qRT-PCR analysis was performed to assess the expression level of an miR-124a target gene using specific primers for VAMP3, a putative target gene for miR-124a. Similar expression patterns of VAMP3 were detected at each neuronal differentiation time point in G2 cells. (C) The constructed CMV/Gluc/3×PT_miR-9 (or CMV/Gluc/3×PT_miR-124a) plasmid vector was transiently transfected into undifferentiated G2 cells, and Gaussia luciferase activity was measured at each neuronal differentiation time point in G2 cells. A gradual decrease in the level of Gaussia luciferase was found in the treatment group of CMV/Gluc/3×PT_miR-9, due to the increase in expression of miRNA-9 during neuronal differentiation of G2 cells. CMV/Gluc/3×PT_miR-124a-treated G2 cells showed similar activity levels of Gaussia luciferase until 2 days after neuronal differentiation. ($^{*}P < 0.05$, $^{**}P < 0.01$).

entrap implanted stem cells into the fibrous polymer scaffolds for *in vivo* imaging [23]. CMV/Gluc/miR-9_3×PT or CMV/Gluc/3×PT_miR-124a was co-transfected into 1×10^7 G2 cells with CMV/Fluc (an internal control for normalization), which supplied constant Fluc activity regardless of the presence of miRNAs or doxycycline, and the harvested G2 cells were incorporated into pre-wetted PLLA scaffolds. G2 cell/scaffold complex containing doxycycline in re-suspension buffer for maintenance of self-renewal function in G2 cells was surgically implanted into the left thigh of a mouse and G2 cell/scaffold complex without doxycycline for induction of neuronal differentiation of G2 cells was implanted into the right thigh of a mouse. Although transient transfection of each CMV/Fluc, CMV/Gluc/miR-9_3×PT, or CMV/Gluc/3×PT_miR-124a resulted in a luciferase signal that was reduced over time in the *in vivo* experiments, faster gradual regression of Gluc intensity from CMV/Gluc/miR-9_3×PT was observed in the right thigh at 1 day after neuronal differentiation of G2 cells due to the gradual increase in miR-9 expression during neurogenesis of G2 cells, compared with that of the left thigh, which was not induced to progress to *in vivo* neuronal differentiation (Fig. 4A). Meanwhile, the Gluc activity of CMV/Gluc/3×PT_miR-124a in both thighs of nude mice was not significantly different between the undifferentiated and differentiated G2 cell groups due to the fact that there was no significant increase in miR-124a during neuronal differentiation of G2 cells (Fig. 4B). As expected, *in vivo* Fluc signals of CMV/Fluc acquired by the IVIS 100 imaging device showed relatively similar bioluminescence signal patterns between the left and right thighs (Fig. 4A and B). The Gluc signal ratio between the undifferentiated and differentiated groups, as determined by quantitative region of interest (ROI) analysis, showed that the Gluc signal of CMV/Gluc/3×PT_miR-9 was dramatically and gradually reduced compared to that of CMV/Gluc/3×PT_miR-124a (Fig. 4C). An *in vivo* experiment regarding the changes in miR-124a expression levels during neurogenesis of P19 cells was also performed. CMV/Gluc/3×PT_miR-124a- and CMV/Fluc-cotransfected P19 cells were subcutaneously implanted into both thighs of mice. When neuronal differentiation of P19 cells by RA treatment was induced in the right thigh of mice, a gradually decreased luciferase signal was observed until 2 days after induction (Supplementary Fig. 3A). The fold-change in the ratios calculated from the ROI analysis compared between the undifferentiated and differentiated groups was higher for the differences in Gluc repression found after neuronal differentiation (Supplementary Fig. 3B). A similar cell survival pattern between two cell implantation groups was found by measuring the *in vivo* Fluc activity. To verify the neuronal differentiation pattern of G2 cells *in vivo*, G2 cell/PLLA scaffold from both thighs

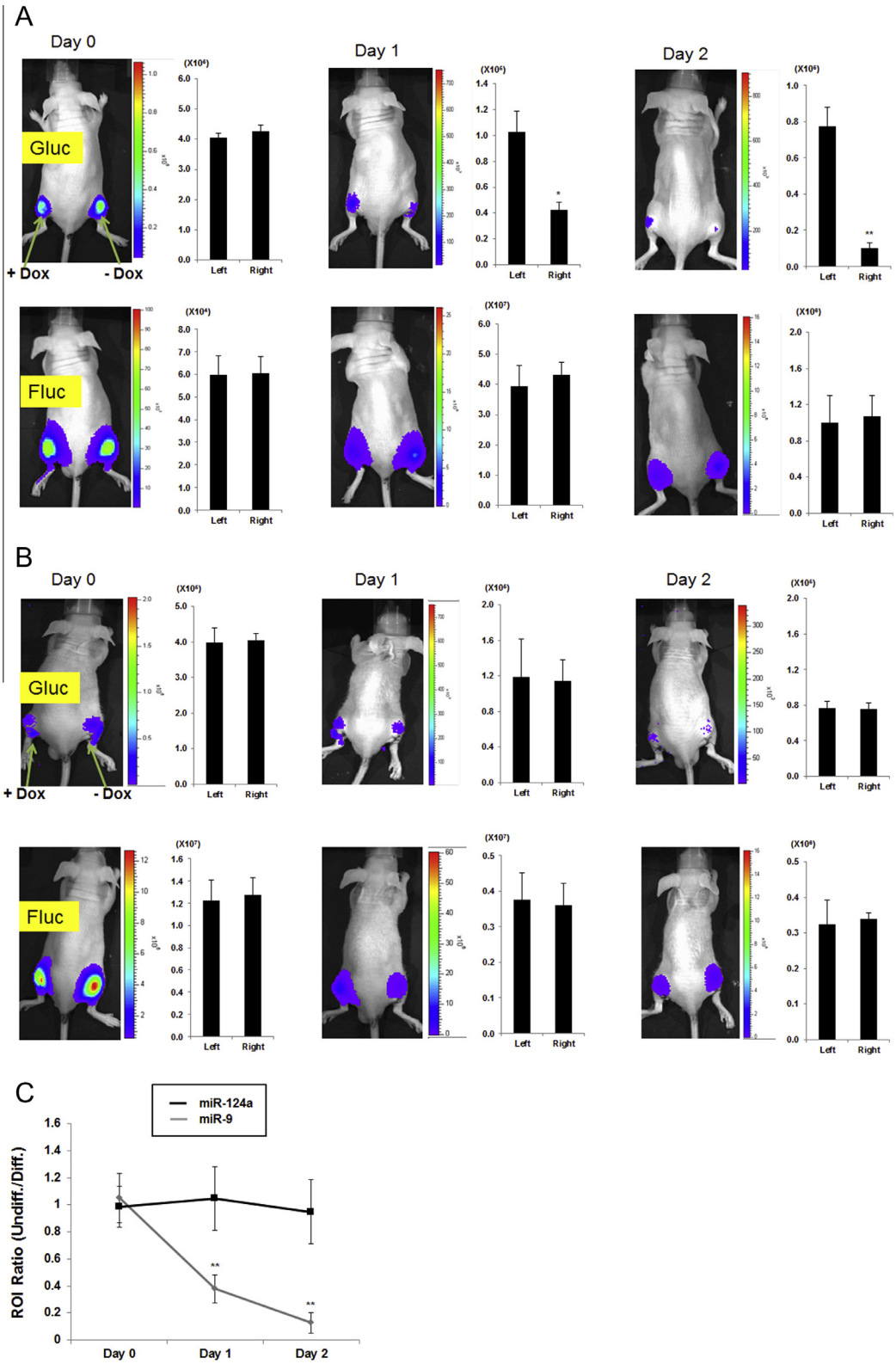

Fig. 4. *In vivo* monitoring of miR-9 (or miR-124a) expression levels during the progression of neuronal differentiation of G2 cells in nude mice. (A and B) CMV/Gluc/3×PT_miR-9 and CMV-Fluc (firefly luciferase vector regulated by CMV promoter) were co-transfected into undifferentiated G2 cells, and harvested G2 cells were incorporated into a pre-wetted PLLA scaffold. To induce neuronal differentiation, G2 cells were resuspended with PBS buffer without doxycycline. As soon as the G2 cell/PLLA complex was surgically implanted into the thighs of mice, the *in vivo* Gaussia luciferase signal was visualized and showed that a more strongly decreased signal was present in the group with induced neuronal differentiation in the right thigh of the mice, compared to that in the undifferentiated group (left thigh). In contrast, CMV/Gluc/3×PT_miR-124a-transfected G2 cells inside the PLLA scaffold in nude mice showed similar Gaussia luciferase signals between undifferentiated and differentiated G2 cells until 2 days after neuronal differentiation. Firefly luciferase signal was used to detect the survival of G2 cells for an in vivo normalization study. (C) Quantitative ROI data acquired from bioluminescence image results showed a continuously decreasing pattern for the ratio value between undifferentiated (Undiff.) and differentiated (Diff.) groups in CMV/Gluc/3×PT_miR-9-transfected G2 cells at 3 days, compared to that of CMV/Gluc/3×PT_miR-124a-transfected G2 cells ($^{*}P < 0.05$, $^{**}P < 0.01$).

was isolated 2 days after bioluminescence image acquisition and fixed with 4% formaldehyde solution. Immunofluorescence staining using antibodies to Oct4 (a stem cell marker) and Neuro-D (a neuronal marker) showed that a high expression level of Oct4 was found in the cytoplasm of undifferentiated G2 cells isolated from the left thigh, whereas the Oct4 expression level was decreased after neuronal differentiation of G2 cells isolated from the right thigh (Supplementary Fig. 4A). In addition, the Neuro-D expression level was significantly increased in G2 cells isolated from the right thigh compared to G2 cells isolated from the left thigh (Supplementary Fig. 4B). These results indicated the successful neuronal induction of G2 cells *in vivo*.

4. Discussion

Recently, a wide range of miRNAs controlling developmental timing, such as the process of neuronal differentiation, have been found in vertebrates as well as invertebrates [24–26]. The global miRNA expression profiles found during neurogenesis have been explored using a host of technological approaches, including microarray analysis [7,27,28]. Representative neuronal-specific miRNAs such as miR-124a and miR-9 identified by microarray are gradually up-regulated during neuronal development by suppressing a variety of nonneuronal genes. In this study, G2 cells, which conditionally express the v-myc oncogene with tetracycline for maintenance of their self-renewal ability, were utilized due to the simple manipulation of G2 cells to generate a neuronal lineage. Although the cellular characteristics of recently developed G2 human neural stem cells were reported during the process of neuronal differentiation [13], the molecular characteristics of G2 cells, including global mRNA and miRNA expression analysis, were not well investigated. In our study, overall miRNA expression profiles of G2 cells after removal of doxycycline showed a strong correlation with the normal miRNA expression pattern of brain tissue. Several known neuron-specific miRNAs including miR-9, miR-9*, miR-132, miR-125a and miR-125b showed gradually increased expression levels after neuronal differentiation of G2 cells. Meanwhile, neuron-specific miRNAs including miR-124a, miR-135a, miR-218, miR-20a, miR-128a and miR-128b had aberrant miRNA expression profiles during neuronal differentiation of G2 cells, showing no significant increases in miRNA expression after neuronal differentiation of G2 cells. Compared with other reported neuronal progenitor cells, G2 cell showed aberrant expression of these neuron-specific miRNAs, suggesting G2 cell is not efficient as a model system for neuronal differentiation. The aberrant miRNA expression might have resulted from the modification of these cells by retroviral transduction of v-myc oncogene. Further studies are required to verify this. *In vitro* and *in vivo* studies of G2 cells using bioluminescent miRNA reporter imaging systems verified the abnormal expression of miR-124a during neuronal differentiation of G2 cells. Meanwhile, *in vitro* and *in vivo* studies of P19 cells demonstrated that miR-124a expression was normally and gradually increased during neurogenesis of P19 cells. These data indicate that neurogenesis of G2 cells is independent of miR-124a. These results imply that G2 cells during neurogenesis have an miRNA expression pattern that is distinct from other cells capable of differentiating into neuronal lineages, including P19 cells and NT2 cells (human teratocarcinoma cells). MiR-124a is one of the essential and high expressed miRNAs during neuronal differentiation. However, the miR-124a-independent neuronal differentiation of G2 cells may demonstrates its altered cell nature in gene expression by retroviral transduction of v-myc oncogene.

The advent of molecular imaging technology enables us to visualize the gene expression pattern or implanted stem cells based on a reporter gene system *in vivo* [29,30]. A recently developed miRNA imaging system using an optical reporter gene provides a powerful tool for non-invasive monitoring of an miRNA expression levels in cancer or neuronal differentiation [18,31,32]. In this study, the *in vivo* miRNA imaging system successfully monitored the unique miRNA expression patterns from neuronally differentiated G2 cells. CMV/Fluc was cotransfected with CMV/Gluc/3×PT_miR-9 or CMV/Gluc/3×PT_miR-124a as an internal control to normalize Gluc activities. CMV/Fluc was designed to be controlled by only CMV promoter to express constant Fluc activity irrespective of the presence or absence of miR-9, miR-124a, and other substrates. Constant Fluc activity confirmed the stable localization of implanted G2 cells with PLLA scaffold throughout the 2 days of *in vivo* neuronal differentiation without cell death or damage of transfected vectors. The constant Fluc signal further demonstrated the decrease in Gluc signal of CMV/Gluc/3×PT_miR-9 or similar Gluc signal of CMV/Gluc/3×PT_miR-124a between undifferentiated and differentiated G2 cells was resulted only from the endogenous miR-9 or miR-124a.

Bioinformatics approaches based on miRNA microarray analysis providing miRNA information on a large scale can be proven through the evidence of real experimental data and eventually be translated into an *in vivo* monitoring system for clinical application. Therefore, the integrated research strategy ranging from a bioinformatics approach to a non-invasive *in vivo* study using an animal model could help researchers to provide complementary information of high quality and with better reliability relative to neuronal development and contribute to the better understanding of brain development and neuronal differentiation in the field of stem cell biology.

Authors' contributions

S.U. Kim, D.S. Lee, and S. Kim designed the experiment. J. Lee, D. W. Hwang, Y.S. Lee, H. Heo performed the experiments. S.U. Kim, D. S. Lee, B.A. Ali, A.A. Al-Khedhairy, S. Kim analyzed the data. J. Lee, D. W. Hwang, and S. Kim wrote the paper.

Acknowledgements

This work was supported by the Bio & Medical Technology Development Program of the National Research Foundation (NRF) funded by the Korean government (MEST) (No. 2013R1A2A2A01068140), the Next-Generation BioGreen 21 program (# PJ010002), Rural Development Administration and a grant of the Korean Health Technology R&D Project, Ministry of Health & Welfare (HI14C3297), and the Visiting Professor Program (VPP) at King Saud University, Kingdom of Saudi Arabia.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fob.2015.08.003.

References

[1] Ambros, V. (2004) The functions of animal microRNAs. Nature 431, 350–355.
[2] Wienholds, E. and Plasterk, R.H. (2005) MicroRNA function in animal development. FEBS Lett. 579, 5911–5922.
[3] Schratt, G. (2009) Fine-tuning neural gene expression with microRNAs. Curr. Opin. Neurobiol. 19, 213–219.
[4] Christensen, M. and Schratt, G.M. (2009) MicroRNA involvement in developmental and functional aspects of the nervous system and in neurological diseases. Neurosci. Lett. 466, 55–62.
[5] Krichevsky, A.M., Sonntag, K.C., Isacson, O. and Kosik, K.S. (2006) Specific microRNAs modulate embryonic stem cell-derived neurogenesis. Stem Cells 24, 857–864.
[6] Smirnova, L., Gräfe, A., Seiler, A., Schumacher, S., Nitsch, R. and Wulczyn, F.G. (2005) Regulation of miRNA expression during neural cell specification. Eur. J. Neurosci. 21, 1469–1477.

[7] Sempere, L.F., Freemantle, S., Pitha-Rowe, I., Moss, E., Dmitrovsky, E. and Ambros, V. (2004) Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. Genome Biol. 5, R13.
[8] Makeyev, E.V., Zhang, J., Carrasco, M.A. and Maniatis, T. (2007) The microRNA miR-124 promotes neuronal differentiation by triggering brain-specific alternative pre-mRNA splicing. Mol. Cell 27, 435–448.
[9] Wu, J. and Xie, X. (2006) Comparative sequence analysis reveals an intricate network among REST, CREB and miRNA in mediating neuronal gene expression. Genome Biol. 7, R85.
[10] Packer, A.N., Xing, Y., Harper, S.Q., Jones, L. and Davidson, B.L. (2008) The bifunctional microRNA miR-9/miR-9* regulates REST and CoREST and is downregulated in Huntington's disease. J. Neurosci. 28, 14341–14346.
[11] Liu, C. and Zhao, X. (2009) MicroRNAs in adult and embryonic neurogenesis. Neuromol. Med. 11, 141–152.
[12] Kim, S.U. (2004) Human neural stem cells genetically modified for brain repair in neurological disorders. Neuropathology 24, 159–171.
[13] Kim, K.S. et al. (2011) Self-renewal induced efficiently, safely, and effective therapeutically with one regulatable gene in a human somatic progenitor cell. Proc. Natl. Acad. Sci. U.S.A. 108, 4876–4881.
[14] Krichevsky, A.M., King, K.S., Donahue, C.P., Khrapko, K. and Kosik, K.S. (2003) A microRNA array reveals extensive regulation of microRNAs during brain development. RNA 9, 1274–1281.
[15] Babak, T., Zhang, W., Morris, Q., Blencowe, B.J. and Hughes, T.R. (2004) Probing microRNAs with microarrays: tissue specificity and functional inference. RNA 10, 1813–1819.
[16] Yang, N. et al. (2008) MicroRNA microarray identifies Let-7i as a novel biomarker and therapeutic target in human epithelial ovarian cancer. Cancer Res. 68, 10307–10314.
[17] Hohjoh, H. and Fukushima, T. (2007) Marked change in microRNA expression during neuronal differentiation of human teratocarcinoma NTera2D1 and mouse embryonal carcinoma P19 cells. Biochem. Biophys. Res. Commun. 362, 360–367.
[18] 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.
[19] 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.
[20] Lagos-Quintana, M., Rauhut, R., Yalcin, A., Meyer, J., Lendeckel, W. and Tuschl, T. (2002) Identification of tissue-specific microRNAs from mouse. Curr. Biol. 12, 735–739.
[21] 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.
[22] Conaco, C., Otto, S., Han, J.J. and Mandel, G. (2006) Reciprocal actions of REST and a microRNA promote neuronal identity. Proc. Natl. Acad. Sci. U.S.A. 103, 2422–2427.
[23] Hwang, D.W. et al. (2008) Real-time in vivo monitoring of viable stem cells implanted on biocompatible scaffolds. Eur. J. Nucl. Med. Mol. Imaging 35, 1887–1898.
[24] Hallam, S.J. and Jin, Y. (1998) Lin-14 regulates the timing of synaptic remodelling in *Caenorhabditis elegans*. Nature 395, 78–82.
[25] Lagos-Quintana, M., Rauhut, R., Meyer, J., Borkhardt, A. and Tuschl, T. (2003) New microRNAs from mouse and human. RNA 9, 175–179.
[26] Johnston, R.J. and Hobert, O. (2003) A microRNA controlling left/right neuronal asymmetry in *Caenorhabditis elegans*. Nature 426, 845–849.
[27] Miska, E.A. et al. (2004) Microarray analysis of microRNA expression in the developing mammalian brain. Genome Biol. 5, R68.
[28] Wheeler, G., Ntounia-Fousara, S., Granda, B., Rathjen, T. and Dalmay, T. (2006) Identification of new central nervous system specific mouse microRNAs. FEBS Lett. 580, 2195–2200.
[29] Massoud, T.F. and Gambhir, S.S. (2003) Molecular imaging in living subjects: seeing fundamental biological processes in a new light. Genes Dev. 17, 545–580.
[30] Sun, N., Lee, A. and Wu, J.C. (2009) Long term non-invasive imaging of embryonic stem cells using reporter genes. Nat. Protoc. 4, 1192–1201.
[31] Lee, J.Y., Kim, S., Hwang, D.W., 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.
[32] 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.