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

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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 increased 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.
explore differential miRNA expression during neurogenesis 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 RNA was then 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 a 400-mer 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 nuclelease-free water using the mirVana™ 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 mirVana™ qRT-PCR primer set (Ambion, Austin, TX) and mirVana™ 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 (lactic acid) (PLLA) scaffold was kindly provided by S.J. Lee (Ewha Womans’ University, College of Pharmacy). PLLA scaffolds were fabricated from poly 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 complex was subcutaneously implanted into both thighs of nude mice (male BALB/c, 8 weeks). To acquire bioluminescence 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 normal 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).

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 [8, 19–21]. Similarly to the results of the microRNA microarray, real-time PCR measurement of miR-124a
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

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.
MicroRNAs expression profile during neurogenesis of G2 cells.

| miRNAs       | UD  | 2 D | 4 D | 6D  |
|--------------|-----|-----|-----|-----|
| 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 | 14.79546 |
| hsa-miR-29c  | 1   | 13.68596 | 19.7639  | 27.33652 |
| hsa-miR-9    | 1   | 10.60056 | 15.83843 | 26.63385 |
| 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-29b  | 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 | 11.1664 |
| 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  | 10.1993 |
| 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  | 6.26426 |

Table 1

List of up-regulated miRNAs during neurogenesis.

| miRNAs | Abundant expression in adult brain [27] | Up-regulated miRNA list during neurogenesis in ESC [5] |
|--------|----------------------------------------|--------------------------------------------------|
| miR-9  | Let-9                                  | Let-7b                                           |
| miR-9' | Let-7c                                 | Let-7g                                           |
| miR-100| miR-9'                                 | miR-124a                                         |
| miR-124a| miR-9'                               | miR-125a                                         |
| miR-125a| miR-124a                             | miR-132                                         |
| miR-125b| miR-125a                             | miR-132                                         |
| miR-135a| miR-125b                             | miR-132                                         |
| miR-16  | miR-125b                               | miR-132                                         |
| miR-218 | miR-124a                              | miR-132                                         |
| miR-30a | miR-124a                              | miR-132                                         |
| miR-98  | miR-124a                              | miR-132                                         |

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.

Table 2

List of up-regulated miRNAs during neurogenesis.

| miRNAs | Abundant expression in adult brain [27] | Up-regulated miRNA list during neurogenesis in ESC [5] |
|--------|----------------------------------------|--------------------------------------------------|
| miR-9  | Let-7a                                  | Let-7a                                           |
| miR-9' | Let-7b                                  | Let-7b                                           |
| miR-100| miR-9'                                 | miR-124a                                         |
| miR-124a| miR-9'                               | miR-125a                                         |
| miR-125a| miR-124a                             | miR-132                                         |
| miR-125b| miR-125a                             | miR-132                                         |
| miR-135a| miR-125b                             | miR-132                                         |
| miR-16  | miR-125b                               | miR-132                                         |
| miR-218 | miR-124a                              | miR-132                                         |
| miR-30a | miR-124a                              | miR-132                                         |
| miR-98  | miR-124a                              | miR-132                                         |

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 significantly repressed after neuronal differentiation of G2 cells, indicating no significant gradual increase in miR-124a expression during neurogenesis of G2 cells (Fig. 3B).
entrap implanted stem cells into the fibrous polymer scaffolds for in vivo imaging [23]. CMV/Gluc/miR-9_3/C2 PT or CMV/Gluc/3/C2 PT_miR-124a was co-transfected into 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/C2 PT, or CMV/Gluc/3/C2 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/C2 PT was observed in the right thigh at 1 day after neuronal differentiation due to the increase in expression of miR-9 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/C2 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/C2 PT miR-9 was dramatically and gradually reduced compared to that of CMV/Gluc/3/C2 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/C2 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. 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/C2 PT miR-9 (or CMV/Gluc/3/C2 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/C2 PT miR-9, due to the increase in expression of miRNA-9 during neuronal differentiation of G2 cells. CMV/Gluc/3/C2 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.)
**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 miRNA 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 retrovirial 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 neurally 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.

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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.jfo.2015.08.003.

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