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

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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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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