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Role of miRNAs in Neuronal Differentiation from Human Embryonic Stem Cell—Derived Neural Stem Cells

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Abstract

microRNAs (miRNAs) are important modulators in regulating gene expression at the post-transcriptional level and are therefore emerging as strong mediators in neural fate determination. Here, by use of the model of human embryonic stem cell (hESC)-derived neurogenesis, miRNAs involved in the differentiation from neural stem cells (hNSC) to neurons were profiled and identified. hNSC were differentiated into the neural lineage, out of which the neuronal subset was enriched through cell sorting based on select combinatorial biomarkers: CD15-/CD29^{Low}/CD24^{High}. This relatively pure and viable subpopulation expressed the neuronal marker β III-tubulin. The miRNA array demonstrated that a number of miRNAs were simultaneously induced or suppressed in neurons, as compared to hNSC. Real-time PCR further validated the decrease in levels of miR214, but increase in brain-specific miR7 and miR9 in the derived neurons. For functional studies, hNSC were stably transduced with lentiviral vectors carrying specific constructs to downregulate miR214 or to upregulate miR7. Manipulation of either miR214 or miR7 did not affect the expression of β III-tubulin or neurofilament, however miR7 overexpression gave rise to enhanced synapsin expression in the derived neurons. This indicated that miR7 might play an important role in neurite outgrowth and synapse formation. In conclusion, our data demonstrate that miRNAs function as important modulators in neural lineage determination. These studies shed light on strategies to optimize in vitro differentiation efficiencies to mature neurons for use in drug discovery studies and potential future clinical applications.

Keywords

microRNAs; Human neural stem cell; Neurogenesis; microRNA array

Introduction

Human embryonic stem cell (hESC) derived neural stem cells (hNSC) are self-renewing and multipotent to give rise to neuronal and glial lineages in vitro. These derived neurons hold

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great promise for cell replacement therapies for neurological diseases. However, how to efficiently drive differentiation of hNSC into mature neurons is still a great obstacle, which then needs a more comprehensive understanding of the regulatory networks that are involved.

Neurogenesis, the fundamental process for both embryonic neurodevelopment and adult brain plasticity, is initiated from neural stem cells to result in functional new neurons. The emerging concept is that microRNAs (miRNAs) play a central role in controlling the balance between stem cell self-renewal and fate determination by regulating the expression of stem cell regulators [1]. miRNAs are a group of small non-coding RNAs that modulate gene expression at the post-transcriptional level, thereby playing critical roles in a variety of biological processes. There are hundreds of human miRNAs cloned and/or identified and each is predicted to target tens or hundreds of different messenger RNAs. Many studies revealed that miRNAs are involved in neurogenesis, a highly orchestrated program of gene expression. Brain-specific miR124a and miR9 have been shown to affect neural lineage differentiation in mouse ESC-derived cultures [2]. Furthermore, miR132 was discovered to regulate the synaptic development, modulating dendritic growth, activity-induced spine growth and spine morphology [3, 4].

While most studies focus on examining a select number of miRNAs in the nervous system, several groups have performed the detailed profiling in immortalized cell lines, such as human NTera2/D1 [5] and mouse P19 [6]. However, no model could yet fully recapitulate the developmental regulation of human miRNAs that occurs in vivo. In this study we investigated this issue more closely by use of the platform of hNSC differentiation into neurons. By taking advantage of combined surface markers [7], derived neurons were enriched to exclude the contamination of other cell types. Through miRNA array analysis, we demonstrated the distinct miRNA profiles for both uncommitted hNSC and differentiated neurons. We also identified a functional role for miR7 in hNSC-derived neurogenesis. These studies have thus extended our understanding of the complex regulatory mechanisms of neural fate determination and neuronal maturation.

Materials & Methods

Cell Culture and Differentiation

The H9 hESC line was grown on irradiated mEFs in H9 medium consisting of 80 % knockout DMEM/F12, 20 % knockout serum replacement, 4 ng/ml basic fibroblast growth factor, 1 mmol/l glutamax, 0.1 mmol/l β -mercaptoethanol and 1 % nonessential amino acid solution. To derive hNSC, H9 colonies were detached by use of collagenase IV (1 mg/ml) and then cultured in suspension as EBs in H9 medium minus bFGF for 1 week. Embryoid bodies (EBs) were then cultured for an additional 2 days in suspension in neural induction media containing knockout DMEM/F12 with Glutamax, NEAA, N2 (1 %) and bFGF (20 ng/ml). After 6–7 days attachment on matrigel-coated petri-dishes, numerous neural rosettes were formed and were manually dissected out for further dissociation into single hNSC by use of accutase. The hNSC population was expanded in neural induction medium plus 0.1 % B27 and 10 ng/ml EGF on polyornithine/ laminin coated dishes.

Neuronal differentiation from hNSC was performed in the presence of ascorbic acid (0.2 mM), BDNF (20 ng/mL), GDNF (20 ng/mL) and cyclic-AMP (0.5 mM) in neurobasal medium plus 1 % B27 for 4–5 weeks.

Cell Sorting

Differentiated cells were harvested and stained on ice with fluorochrome-conjugated monoclonal antibodies specific for CD15 FITC, CD24 PE and CD29 APC (BD Biosciences, San Jose, CA). Propidium iodide was added to exclude dead cells prior to analysis and sorting using a BD InFlux sorter (Becton Dickinson (BD) Biosciences) equipped with a 100um nozzle at 17.1 psi using a low sample differential pressure (1 lb, 18.2 psi). Acquisition gates were set using Spigot software (BD Biosciences) and the appropriate unstained and single-color compensation control samples. Post-sort data analysis was performed using FlowJo version 7.6.1 (Treestar, Inc., Ashland, OR).

Immunofluorescence

Cells were fixed with 4 % paraformaldehyde and permeabilized by use of 0.2 % Triton-X. After blocking in 10 % goat serum, cells were incubated with the following primary antibodies at 4°C overnight: rabbit anti-Sox1 (Millipore, Billerica, MA, AB15766), mouse anti-nestin (R&D, Minneapolis, MN, MAB1259), mouse anti-Tyrosine Hydroxylase (TH) (Millipore, MAB318), and rabbit anti-TuJ1 (Abcam, Cambridge, MA, ab24629). Then, the appropriate fluorescence-labeled secondary antibodies were applied. Nuclei were counterstained with DAPI (Vector labs, Burlingame, CA, H1200). Images were captured on a Nikon fluorescence microscope.

Realtime PCR

Total RNA was extracted with RNeasy Mini kit (Qiagen, Valencia, CA). Reverse transcription was done with the QuantiTect Reverse Transcription Kit (Qiagen). Quantitative PCR was carried out using SYBR Green Master Mix Kit (Applied Biosystems, 4309155) and ABI 7300 instrument (Applied Biosystems, Carlsbad, CA) according to the manufacturer's instructions. Primers for β III-tubulin, TH, synapsin and neurofilament were designed using SciTools Software (Integrated DNA Technologies Coralville, Iowa).

miRNA validation qPCR was performed by a service provider (LC Sciences, Houston, Texas) by use of TaqMan miRNA assay system (Applied Biosystems). Data were normalized to the endogenous control genes RNU24. MiRNA of human fetal neuron was purchased from ScienCell Research Laboratories (#1527).

To confirm the overexpression of miR7 and knockdown of miR214 in transduced NSCs, a QuantiMir RT kit (System Biosciences, Mountain view, CA) was used according to the manufacturer's protocol. The forward primers for miR7 and miR214 were 5'-TGGAAGACTAGTGAATTTGTTGT-3' and 5'-ACAGCAGGCACAGACA GGCAGT-3', respectively.

miRNA Microarray

Total RNA containing miRNAs was isolated from sorted cells by use of the miRNeasy kit (Qiagen). The microarray assay was then performed by a service provider (LC Sciences). The assay started from 2 µg total RNA sample, where the total RNA strands were 3'-extended with a poly(A) tail. An oligonucleotide tag was then ligated to the poly(A) tail for later fluorescent Cy3 dye staining. Hybridization was performed overnight on a µParaflo[®] microfluidic chip, on which each detection probe consisted of a chemically modified nucleotide coding segment that was complementary to a target microRNA (obtained from miRBase version 15, including 1,090 unique mature human miRNAs, <http://microrna.sanger.ac.uk/sequences>) and a spacer segment of polyethylene glycol to extend the coding segment away from the substrate. Hybridization images were collected using a laser scanner (GenePix 4000B, Molecular Device) and digitized using Array-Pro image analysis software (Media Cybernetics). Data were analyzed by first subtracting the background and then normalizing the signals using a LOWESS filter10 (Locally-weighted Regression).

Generation of Lentiviral Transduced hNSC Lines

Lenti-miR miR7 precursor, miRZip anti-miR-214 and pGreenPuro scramble hairpin control constructs with cop-GFP as a reporter (System Biosciences) were packaged separately into lentiviruses by transfection of 293 T cells. hNSC were then stably transduced with miR7 precursor, anti-miR214 or scrambled control, at a multiplicity of infection of 20. The percentage of positive expressing cells was analyzed by FACS on a Beckman Coulter FC500.

Statistical Analysis

All data were expressed as means ± SE. One-way ANOVA followed by Newman-Keuls multiple comparison tests was carried out to test for differences between the mean values within the same study. A difference of $p < 0.05$ was considered significant.

Results

Directed Differentiation of H9 to hNSC and Neurons

hNSC were differentiated from H9 through embryoid body (EB) formation and rosette isolation according to previous studies [9]. One week after EB attachment, numerous clusters of columnar cells that formed rosettes appeared (Fig. 1a), resembling the early neural tube. Those rosette structures were then manually dissected out under dissecting microscopy to eliminate contamination by non-neural cells. The isolated rosettes were dissociated into single hNSC by accutase, and then expanded as a monolayer adherent cell culture (Fig. 1b). Immunofluorescence analysis showed that the derived hNSC stained positive for the neuroepithelial markers Sox-1 and nestin (Fig. 1c). Under the defined culture conditions, hNSC could be continued up to 20 passages and maintained to be a homogenous population through multiple freeze-thaw cycles. This is consistent with previous report that hNSC derived from human embryonic stem cells are self-renewing over the long-term [10]. After 4 weeks of directed differentiation, hNSC gave rise to neurons positive for β III-

tubulin (Fig. 1e), among which some expressed the dopaminergic neuron marker tyrosine hydroxylase (TH, Fig. 1f).

Enrichment of Neuronal Subset from the Differentiation Culture

The differentiated culture from hNSC was a mixed preparation in which the non-neural cells would certainly mask the results of RNA analyses. Therefore it was crucial to enrich the neuronal subpopulation for miRNA profiling. There are several reports on the identification of cell-surface markers for the isolation of neural lineage [7, 8]. Here we adopted one of such strategies for selection [7] and further miRNA array analysis. As shown in Fig. 2a, FACS analysis identified a CD15-/CD29^{Low} fraction that occupied around 14 % of the PI negative population. CD15 is a marker for immature neuroepithelial cells, while CD29 was found to label rosette structures. Then combined with CD24 (heat-stable antigen), an antigen that increases through in vitro development from hESC toward the neuronal differentiation, the subset of CD15-/CD29^{Low}/CD24^{High} cells were isolated. This sorting strategy gave rise to a near-pure, viable, neuronal culture, demonstrated by positive staining with β III-tubulin (Fig. 2b and c). Gene comparisons between hNSC and this enriched neuronal culture showed significantly higher expression of β III-tubulin and synapsin after the flow-based enrichment (Fig. 2d).

miRNA Array Between hNSC and Neuronal Derivatives

miRNAs were then profiled by use of the μ Paraflo[®] microfluidic chip technology (LC sciences). Analysis indicated distinct miRNA expression patterns in hNSC and enriched H9-Derived Neurons (H9-DN). Clustered heat maps were used to classify miRNAs into two groups: miRNAs that were significantly expressed in H9-DN ($p < 0.01$, Fig. 3a) and miRNAs that were significantly expressed in hNSC ($p < 0.01$, Fig. 3b). Consistent with previous reports that let-7 miRNAs stabilize the differentiated cell fate, the let-7 family was robustly induced in differentiated H9-DN, as compared to the uncommitted hNSC. More importantly, the well-known brain-specific miRNAs, miR124, miR125 and miR9, showed an increased expression in H9-DN. Among these 23 neural phenotype-associated miRNAs, the induction of miR7 was observed and validated by real-time PCR, with a 60 fold increase in H9-DN as compared to the levels obtained from hNSC. This increase was even larger with miR9, which had 200 times upregulation in H9-DN, as compared to hNSC (Fig. 3c). Interestingly, miR7 expression in H9-DN was higher than that in human fetal brain neurons (F-DN), whereas miR9 expression was lower.

Our analysis also identified 22 miRNAs that were expressed at significantly higher levels in hNSC than in the differentiated stage, including miR214, which was reported to be involved in the neurite outgrowth during the differentiation of neuroblastoma cells [11]. In contrast, we detected a consistent down-regulation of miR214 in both derived and fetal neurons in comparison to undifferentiated hNSC (Fig. 3c).

Overexpressing miR7/Inhibiting miR214

Given that the expression of miR7 and miR214 changed remarkably in the process of hNSC differentiation, we further investigated how manipulating miR7/miR214 expression affects neuronal gene expression so as to assign a function to them. hNSC were stably transduced

with a GFP tagged lentiviral miR7 construct designed to overexpress miR7 or an miRZip-214 shRNA construct designed to inhibit miR214. 72 h later (Fig. 4a–c), realtime PCR demonstrated a successful up-regulation of miR7 and miRZip-214 shRNA in the transduced cells. The latter reflects the anti-miR214 expression level, which competitively binds endogenous miR214 and inhibits its function (System Biosciences, mountain view, CA). Since the inhibition is not caused by degradation of endogenous miR214, the expression level of endogenous miR214 did not decrease (data not shown). We then further differentiated the transduced hNSC into neurons, which remained continuously positive for the GFP reporter. As shown in Fig. 4d, FACS confirmed the percentage of GFP + cells in the cultures after 4 weeks of differentiation: 70–90 % for scramble- and miR7-transduced, and 40 % for the anti-miR214-transduced culture.

Effects of Manipulating miR7/miR214

At the same time, neuronal gene expression was detected by qPCR. Interestingly, the overexpression of miR7 throughout the differentiation process led to increased synapsin gene expression (Fig. 5b), a key player in neurite outgrowth and synapse formation during development [12]. However, no changes were found in the expression levels of β III-tubulin or neurofilament (Fig. 5a and c). By use of the online database of TargetScan (www.Targetscan.org) and Ingenuity pathway analysis (Ingenuity Systems, Redwood, CA), we performed the canonical pathway analysis of predicted targets for miR7 (Table 1). The analysis revealed that many targeted genes are involved in cell mobility, cell cycle/growth regulation and brain development. On the other hand, inhibition of endogenous miR214 by MiRZip shRNA, which was found to be rich in the hNSC stage, did not cause enhanced expression of neuronal related genes (Fig. 5).

Discussion

MiRNAs are shown to be extensively involved in all stages of central nervous system development and function, due to their important characteristics of fine-tuning of multiple targets. To extend our understanding of the role of miRNAs in lineage determination during neuronal differentiation from neural stem cells, we have compared the expression profiles of miRNAs involved in this process. Our results revealed a very dynamic and defined expression pattern of miRNAs for each stage. Beyond the significant changes of well-studied miR9, miR124 and miR125, other miRNAs such as miR7 also showed neuron-specific expression. Overexpression of miR7 in hNSC significantly increased the synaptic protein expression in the derived neurons.

The in vitro neurogenesis in our study initiated from self-renewable hNSC and resulted in differentiated neurons, resembling embryonic neurodevelopment. The directed neuronal differentiation by use of a combination of growth factors, however, gave rise to a mixed population, though mainly composed of neuronal derivatives, which is a big barrier for clinical application. Therefore for the first time, in the current study cell sorting was performed in derived cultures according to the neuronal surface biomarkers prior to miRNA array analysis, in order to exclude artifacts from other cell types. This neuronal enrichment is indispensable for the miRNA profile to be precisely neuron-specific. Groups of miRNAs

were found to be co-induced at each stage of this transition process including hNSC self-renewal, fate specification and neuronal integration. Given the fine-tuning, multi-targeting and combinatorial modulating properties of miRNAs, this implies a highly complex regulatory network during neurogenesis.

In this study, we focused on two miRNAs, miR7 and miR214, whose expression changes were opposite during the transition from hNSC to neurons. miR7 is one of the most highly conserved animal miRNAs and is specifically expressed in neurosecretory cells of the vertebrate brain and in homologous cells of the annelid nervous system [13]. Recently miR-7 has been characterized as a tumor suppressor in several human cancers [14, 15] and was reported to suppress α -synuclein-mediated cell death [16]. Interestingly, a prominent increase of miR7 was observed in hNSC-derived neurogenesis in the current studies. Early overexpression of miR7 in hNSC enhanced synaptic protein expression in derived neurons, indicating an important role of miR7 in synaptic formation [12]. In support of this notion, the top two enriched biocategories for miR7 using the Ingenuity pathways analysis software were “Behavior” and “Nervous system Development and Function” [17]. Canonical pathways further revealed that many targeted genes are involved in synaptic plasticity (Table 1). However, such overexpression did not lead to increased neuronal gene expression. It is also noted that miR7, unlike miR9, was only mildly increased in fetal neurons. Therefore, miR7 might be involved in neurogenesis by targeting synaptic formation and modulating development in a stage/situation-limited way. This notion is supported by the report that miR-7 is essential for stable gene expression and cell fate determination in the face of environmental fluctuation during neural development in *Drosophila* [18].

miR214 is one of the cell cycle-related miRNAs and is up-regulated in tumor cells [19, 20]. It is also reported to be highly expressed in fast cycling, early embryonic retinal progenitors [21]. Consistently, we revealed extremely high expressions of miR214 in hNSCs, as compared to differentiated neurons. This led to our prediction that miR214 might act as an opposite partner of let-7, whose overexpression led to increased neural differentiation from adult mouse NSCs, whereas knockdown resulted in enhanced proliferation of NSCs [22]. However, functional inactivation of miR214 in our study did not facilitate neurogenesis, unlike its role in supporting the last steps of mature neuron generation in the retina [21]. This suggests that miR214 may be more involved in maintaining the proliferation of hNSC than switching the cell fate to maturing neuron. It is interesting to note that during differentiation of mouse primary cortical neurons, mouse embryonic stem cells and development of mouse brain cortex, the changes in expression of miR7 and miR214 were consistent with our findings [11]. However, during the RA-induced neuroblastoma differentiation investigated in the same study [11], miR214 was up-regulated and miR7 was down-regulated. This discrepancy caused by different models emphasizes the necessity to test multiple in vitro platforms for in vivo development.

It is noted that the epigenetic and transcriptional patterns of the pluripotent state can render iPSC/ESC lines different neural differentiation potential [23, 24]. Another study further demonstrated that low expression of miR371–3 can predict a higher neural differentiation propensity of a given iPSC line [25]. This not only confirmed the role of miRNAs in neural differentiation, but also, implied the complexity of the neural fate determination, a multiple

step and dimension developmental process. Therefore more comprehensive comparisons by addition of the ESCs into the study of miRNA during neurogenesis is warranted for the future study.

In summary, our studies demonstrate that the miRNA expression profile is distinct for the different stages of neuronal differentiation and that specific miRNAs can function as regulators of neurogenesis. These data shed light on strategies to optimize in vitro differentiation efficiencies to mature neurons for use in drug discovery studies and potential future clinical applications.

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Abbreviations

hESC	Human embryonic stem cells
hNSC	hESC derived neural stem cells
H9-DN	hESC (H9 line) derived neurons
EB	Embryoid body

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Fig. 1. Derivation of hNSC and neurons from hESC. **a** Neural rosettes formed in plated EBs; **b** Dissociated hNSC from rosettes cultured as an adherent monolayer; **c** hNSC were positive for Sox-1 and nestin; **d** hNSC can differentiate into neurons labeled by β III-tubulin (**e**); **f** some expressed the dopaminergic neuron marker tyrosine hydroxylase (TH). Scale bars, 100 μ m

Fig. 2.

Analysis of neuronal subpopulations derived from hNSC. **a** The CD15⁻/CD29^{Low}/CD24^{High} population (H9-DN) was sorted and after replating, the H9-DN subset generated viable, pure neuronal cultures that expressed β -III tubulin (**b** and **c**). qPCR confirmed significantly higher expressions of β -III tubulin and the synapse marker synapsin in the H9-DN subset, as compared to hNSC (**d**). * $p < 0.05$ and ** $p < 0.01$ compared to hNSC. Scale bars, 100 μ m

Fig. 3. miRNA array heat maps (**a** and **b**). Normalized log₂ miRNA expression for miRNAs that showed significant differences ($p < 0.01$) between groups were listed according to the color scale (GEO accession GSE30572, reviewer link <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=dbmjfwsiaosckhk&acc=GSE30572>). **a** miRNAs primarily expressed in H9-DN; **b** miRNAs more expressed in hNSC. **c** Expression profiles of miR214, miR7 and miR9 were validated by qPCR. F-DN, human fetal neurons. * $p < 0.05$, ** $p < 0.01$ compared to hNSC; # $p < 0.05$, ## $p < 0.01$ compared to H9-DN

Fig. 4.

Overexpression of miR7 or downregulation of miR214 during neuronal differentiation. **a** miR7, anti-miR214 or scramble construct-transduced hNSC were positive for GFP and remained green through differentiation into neurons; qPCR demonstrated upregulation of miR7 (**b**) and anti-miR214 construct (miRZip-214, **c**) in hNSC; **d** FACS results confirmed 40–90 % GFP + cells among the derived neuronal population. Scale bar, 100 μ m

Fig. 5. Effects of miR7 overexpression or miR214 inhibition on neuronal differentiation. qPCR showed expression of β III-tubulin (a), synapsin (b) and neurofilament (c) induced by the manipulation of the two miRNAs; S, scramble

Table 1

Selected canonical pathways of the targets of miR7

Ingenuity canonical pathways	−log (p value)	Molecules
Glioma signaling	3.33E00	RAF, RB1, CALM3, CAMK2D, PIK3CD, EGFR
Synaptic long term potentiation	1.65E00	RAF1, CALM3, CAMK2D, PLCB1
Integrin signaling	1.43E00	MYLK, RAF1, PAK2, PIK3CD, ACTC1
Actin cytoskeleton signaling	1.25E00	MYLK, RAF1, PAK2, PIK3CD, ACTC1
Axonal guidance signaling	1.8E00	RAF1, SEMA4D, PAK2, GLI3, PLCB1, FIGF, PIK3CD
Huntington's disease signaling	1.16E00	SP1, PLCB1, PIK3CD, SNCA, EGFR
Neuropathic pain signaling in dorsal horn neurons	1.12E00	CAMK2D, PLCB1, PIK3CD
Calcium signaling	1.04E00	CALM3, CAMK2D, ACTC1, CAMKK2
Parkinson's signaling	8.03E-01	SNCA
Neurotrophin/TRK signaling	8E-01	RAF1, PIK3CD
Cyclins and cell cycle regulation	7.64E-01	RAF1, RB1
Sonic hedgehog signaling	6.05E-01	GLI3
GABA receptor signaling	4.08E-01	GABRA6
Synaptic long term depression	3.74E-01	RAF1, PLCB1