RESEARCH ARTICLE

MicroRNA expression profiling in neurogenesis of adipose tissue-derived stem cells

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Abstract

Adipose tissue-derived stem cells (ADSCs) are one population of adult stem cells that can self renew and differentiate into multiple lineages. Because of advantages in method and quantity of acquisition, ADSCs are gaining attention as an alternative source of bone marrow mesenchymal stem cells. In this study, we performed microRNA profiling of undifferentiated and of neurally-differentiated ADSCs to identify the responsible microRNAs in neurogenesis using this type of stem cell. MicroRNAs from four different donors were analysed by microarray. Compared to the undifferentiation control, we identified 39–101 microRNAs with more than two-fold higher expression and 3–9 microRNAs with two-fold lower expression. The identified microRNAs were further analysed in terms of gene ontology (GO) in relation with neurogenesis, based on their target mRNAs predicted by computational analysis. This study revealed the specific microRNAs involved in neurogenesis via microRNA microarray, and may provide the basic information for genetic induction of adult stem cell differentiation using microRNAs.

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Introduction

Stem cells have the capacity to self renew and to differentiate into multiple lineages that can be largely divided into embryonic stem cells or adult stem cells (Passier and Mummery 2003). Embryonic stem cells have the greatest differentiation capability, however there are considerable challenges to the use of these cells for clinical application, including ethical limitations (Borge and Evers 2003). Adult stem cells have less potency, but higher availability than embryonic stem cells.

Adult stem cells can be obtained from a variety of adult tissues including brain, blood, muscle, skin, bone marrow, umbilical cord blood, amniotic fluid and adipose tissue (Clarke and Frisen 2001; Prockop *et al.* 2001; In 't Anker *et al.* 2004; Lu *et al.* 2006; Schaffler and Buchler 2007; Roh *et al.* 2008). Because of their capacity for differentiation into a variety of cell types including osteoblasts, chondrocytes, myocytes, adipocytes, and neural cells under certain conditions. (Musina *et al.* 2006; Chamberlain *et al.* 2007), adult stem cells are now in growing demand in various medical fields, including plastic surgery and regenerative medicine. Among these sources, adipose tissue-derived stem cells (ADSCs) are gaining importance due to their ease of acquisition in large numbers (Mizuno 2003; Pansky *et al.* 2007).

Due to the growth in the safe application of stem cell-based cell therapy towards medical treatment, it becomes important to understand more about stem cell biology. Emerging evidence indicates that microRNAs play a critical role in maintenance, differentiation and lineage commitment of stem cells, as they are found to be involved in various cellular and biological processes (Krichevsky *et al.* 2006; Foshay and Gallicano 2007).

MicroRNAs (miRNAs) are short (18–25 nucleotides) endogenous noncoding RNAs that post transcriptionally regulate gene expression (Mallory and Vaucheret 2004). miRNAs are generated in several processing steps. Primary miRNA (pri-miRNA) transcripts are produced in the cell nucleus by RNA polymerase II and then cleaved to precursor miRNAs (pre-miRNA) by RNase type III enzyme Drosha (Du and Zamore 2005; Zeng *et al.* 2005) The pre-miRNAs are then transported to cytoplasm where they become mature miRNA by another RNase type III enzyme Dicer (Gregory *et al.* 2006) The mature miRNA become a component of the RNA induced silencing complex (RISC), which can bind to

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3' untranslated regions (UTRs) of messenger RNAs through partial or complete base pairing (Tang *et al.* 2008). The major function of miRNAs is to silence the paired messenger RNAs by degradation or translation repression (Behm-Ansmant *et al.* 2006) Although miRNA expression profiling has been performed in embryonic stem cells, the basic information on their expression patterns in adult stem cells has not been fully established yet (Foshay and Gallicano 2007; Wang *et al.* 2008).

We have been interested in neurogenesis using ADSCs for future clinical application in neurodegenerative diseases. Hence, we attempted to investigate the importance of miRNA expression and function in ADSCs' biology by performing miRNA expression profiling in undifferentiated and neurally-differentiated ADSCs and identified the critical miRNAs in neurogenesis using the ADSCs by analysing the microarray data using computational software and web-derived databases.

Materials and methods

Isolation of ADSCs

ADSCs obtained from lipoaspirates of four different donors were washed extensively with sterile phosphate-buffered saline (PBS) to remove contaminating debris and red blood cells (Zuk *et al.* 2001; Raposio *et al.* 2007). The washed aspirates were treated with 0.075% collagenase (type I; Sigma-Aldrich, Missouri, USA) in PBS for 60 min at 37°C with gentle agitation, followed by inactivation with an equal volume of DMEM/10% foetal bovine serum (FBS). After centrifugation for 10 min at low speed, the cellular pellet was resuspended in DMEM/10% FBS and filtrated through a 100 μm mesh filter to remove debris. The filtrate was centrifuged as detailed above and plated onto conventional tissue culture plates in control medium (DMEM, 10% FBS, 1% antibiotic/antimycotic solution) and maintained at 37°C in a CO_2 incubator. The ADSCs utilized in this study underwent 3–5 passages. Each donor was fully informed and provided informed consent at the Kangbuk Samsung Hospital prior to study enrollment. The study protocols were approved by the institutional review board (IRB) of Kangbuk Samsung Hospital.

Neurogenesis of ADSCs

The ADSCs used in this study were differentiated into the neural lineage as previously established (Ashjian *et al.* 2003) Briefly, the cells were cultured for 2 weeks in neural induction media composed of control medium plus 5 μg/mL Insulin, 200 μM Indomethacin and 0.5 mM IBMX which was replaced every 2–3 days. The successful neurogenesis was confirmed by RT-PCR and immunocytochemistry for the neuron-specific markers including NF-M,

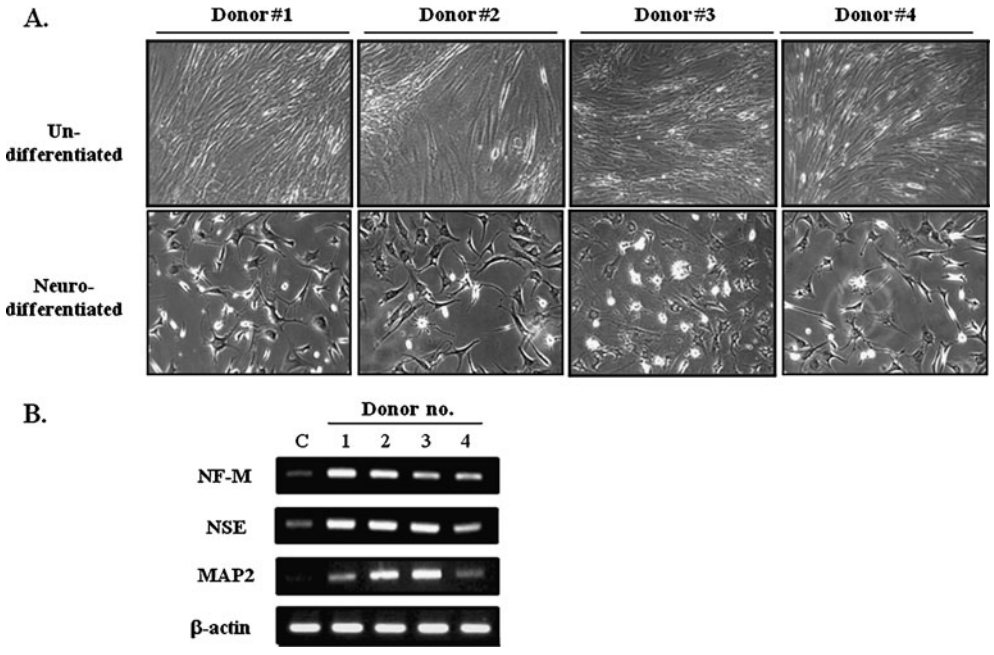

Figure 1. Neurogenesis of ADSCs from four different donors. (A) Microscopic observation of ADSCs that were undifferentiated or neurodifferentiated. The representative images were obtained at 100-fold magnitude. (B) RT-PCR for neuro-specific genes. RNAs were extracted from undifferentiated (C) and neurodifferentiated (donor # 1, 2, 3, 4) ADSCs. After reverse-transcription, PCR was performed using each gene-specific primers and then resolved in agarose gel. Images were obtained by gel documentation system.

NSE, MAP2 and GFAP. Primers used for RT-PCR are as follows: NF-M, forward primer 5'-ggaggaagacatccaccggc-3' and reverse primer 5'-gccggtactcggcgatctct-3'; NSE, forward primer: 5'-atcgcgccagccctcatcag-3' and reverse primer: 5'-ttttccgtgtagccagcctt-3', MAP2, forward primer: 5'-tcagaggcaatgaccttacc-3' and reverse primer: 5'-gtggtaggctcttggtcttt-3'. Antibodies for immunocytochemistry were purchased from Millipore (Peenya, Bangalore) (anti-NSE and anti-NF-M antibody; Bangalore, India), SIGMA (St Louise, USA) (anti-MAP2 antibody) and DAKO (Carpinteria, USA) (anti-GFAP antibody).

RNA preparation and qualification

Total RNA was isolated from the cells with TRIZOL reagent (Invitrogen, Carlsbad, USA). Briefly, cells were lysed by suspension in TRIZOL solution and subsequent addition of chloroform. After centrifugation, the clear supernatant

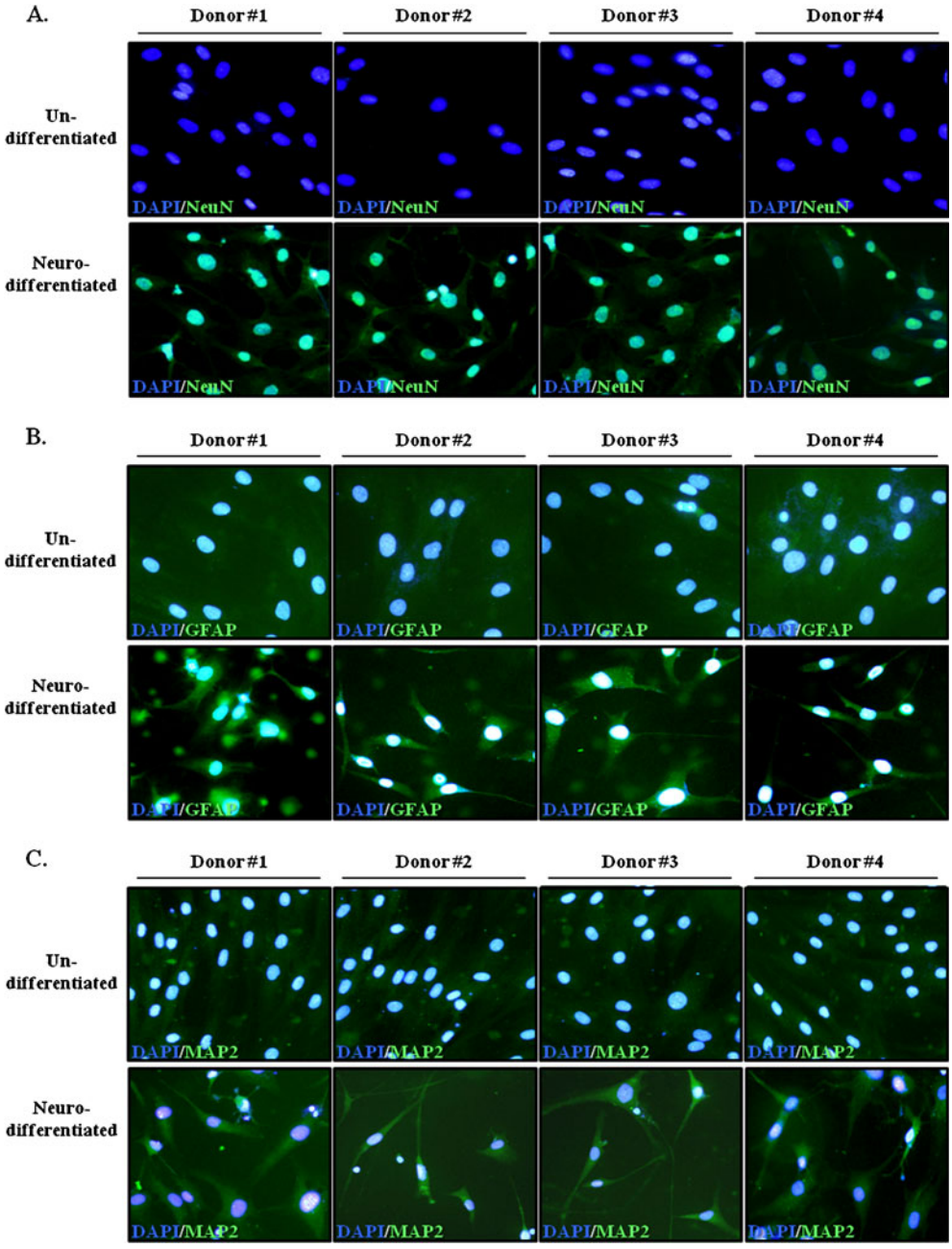

Figure 2. Verification for successful neurogenesis of the ADSCs. Immunocytochemistry was performed to confirm the successful neurogenesis of the four different ADSCs that were undifferentiated or neurodifferentiated. Cells were incubated with antibodies against NF-M (A), GFAP (B) or MAP2 (C), and subsequently Alexa488-tagged secondary antibodies (green colour). The images were obtained by fluorescence microscopy after counter staining with nuclear-staining dye DAPI (blue colour).

Table 1. MicroRNAs expressed in undifferentiated ADSCs.

Donor #1	Donor #2	Donor #3	Donor #4
hur_1	hur_1	hur_1	hur_1
hur_2	hur_2	hur_2	hur_2
hur_6	hur_6	hur_6	hur_6
hur_4	hsa-miR-21	hsa-miR-21	hsa-miR-21
mr_1	hur_4	hur_4	hur_4
hsa-miR-21	mr_1	mr_1	mr_1
hsa-miR-29a	hsa-miR-29a	hsa-miR-29a	hsa-miR-29a
hsa-miR-145	hsa-miR-145	hsa-let-7a	hsa-miR-125b
hsa-miR-222	hsa-let-7a	hsa-miR-125b	hsa-miR-222
hsa-miR-125b	hsa-miR-125b	hsa-miR-199a*	hsa-let-7a
hsa-miR-22	hsa-let-7b	hsa-miR-22	hsa-miR-199a*
hsa-let-7a	hsa-miR-27a	hsa-miR-424	hsa-miR-22
hsa-miR-27a	hsa-let-7i	hsa-let-7b	hsa-let-7b
hsa-miR-24	hsa-miR-199a*	hsa-miR-222	hsa-let-7i
hsa-let-7i	hsa-miR-24	hsa-miR-24	hsa-miR-27a
hsa-miR-199a*	hsa-miR-143	hsa-miR-27a	hsa-miR-24
hsa-let-7b	hsa-miR-22	hsa-miR-23a	hsa-let-7f
hsa-miR-29b	hsa-miR-222	hsa-let-7f	hsa-miR-145
hsa-miR-143	hsa-miR-424	hsa-miR-145	hsa-miR-23a
hsa-miR-23a	hsa-miR-23a	hsa-let-7i	hsa-miR-29b
hsa-miR-424	hsa-miR-29b	hsa-miR-29b	hsa-miR-199a
hsa-let-7f	hsa-let-7f	hsa-miR-143	hsa-miR-143
hsa-miR-199a	hsa-miR-199a	hsa-miR-199a	hsa-miR-100
hsa-miR-31	hsa-let-7c	hsa-let-7c	hsa-miR-221
hsa-miR-214	hsa-miR-100	hsa-miR-26a	hsa-miR-424
hsa-miR-100	hsa-miR-31	hsa-miR-16	hsa-let-7c
hsa-miR-221	hsa-miR-214	hsa-miR-221	hsa-miR-16
hsa-let-7c	hsa-miR-26a	hsa-miR-100	hsa-miR-31
hsa-miR-16	hsa-let-7e	hsa-let-7e	hsa-miR-26a
hsa-miR-193b	hsa-miR-16	hsa-miR-214	hsa-let-7e
hsa-let-7e	hsa-miR-193b	hsa-miR-31	hsa-miR-214
hsa-miR-26a	hsa-miR-221	hsa-let-7d	hsa-let-7d
hsa-miR-494	hsa-let-7d	hsa-miR-494	hsa-miR-34a
hsa-let-7d	hsa-let-7g	hsa-miR-565	hsa-miR-193b
hsa-miR-565	hsa-miR-19b	hsa-miR-193b	hsa-miR-494
hsa-miR-19b	hsa-miR-125a	hsa-miR-23b	hsa-let-7g
hsa-miR-92	hsa-miR-565	hsa-let-7g	hsa-miR-19b
hsa-let-7g	hsa-miR-494	hsa-miR-19b	hsa-miR-125a
hsa-miR-15b	hsa-miR-15b	hsa-miR-638	hsa-miR-15b
hsa-miR-125a	hsa-miR-92	hsa-miR-15b	hsa-miR-324-3p
hsa-miR-320	hsa-miR-107	hsa-miR-130a	hsa-miR-107
hsa-miR-107	hsa-miR-130a	hsa-miR-125a	hsa-miR-193a
hsa-miR-324-3p	hsa-miR-23b	hsa-miR-34a	hsa-miR-92
hsa-miR-34a	hsa-miR-324-3p	hsa-miR-107	hsa-miR-130a
hsa-miR-130a	hsa-miR-34a	hsa-miR-199b	hsa-miR-199b
hsa-miR-376a	hsa-miR-320	hsa-miR-324-3p	hsa-miR-320
hsa-miR-193a	hsa-miR-103	hsa-miR-92	hsa-miR-23b
hsa-miR-20a	hsa-miR-193a	hsa-miR-103	hsa-miR-15a
hsa-miR-103	hsa-miR-20a	hsa-miR-320	hsa-miR-565
hsa-miR-23b	hsa-miR-376a	hsa-miR-370	hsa-miR-638
hsa-miR-638	hsa-miR-768-3p	hsa-miR-27b	hsa-miR-768-3p
hsa-miR-503	hsa-miR-638	hsa-miR-193a	hsa-miR-376a
hsa-miR-106a	hsa-miR-503	hsa-miR-503	hsa-miR-103
hsa-miR-106b	hsa-miR-106b	hsa-miR-15a	hur_5
hsa-miR-377	hsa-miR-27b	hsa-miR-376a	hsa-miR-106b
hsa-miR-19a	hsa-miR-106a	hsa-miR-10b	hsa-miR-29c
hur_5	hsa-miR-199b	hsa-miR-106b	hsa-miR-20a
hsa-miR-768-3p	hsa-miR-15a	hsa-miR-10a	hsa-miR-377
hsa-miR-127	hur_5	hsa-miR-768-3p	hsa-miR-370
hsa-miR-199b	hsa-miR-19a	hur_5	hsa-miR-146a
hsa-miR-29c	hsa-miR-377	hsa-miR-365	hsa-miR-30a-5p
hsa-miR-370	hsa-miR-99a	hsa-miR-20a	hsa-miR-365

Table 1 (*contd.*)

Donor #1	Donor #2	Donor #3	Donor #4
hsa-miR-27b	hsa-miR-29c	hsa-miR-377	hsa-miR-34b
hsa-miR-15a	hsa-miR-365	hsa-miR-29c	hsa-miR-155
hsa-miR-365	hsa-miR-30a-5p	hsa-miR-30a-5p	hsa-miR-127
hsa-miR-17-5p	hsa-miR-127	hsa-miR-26b	hsa-miR-19a
hsa-miR-155	hsa-miR-370	hsa-miR-19a	hsa-miR-26b
hsa-miR-136	hsa-miR-10b	hsa-miR-99a	hsa-miR-27b
ebv-miR-BART19	hsa-miR-17-5p	hsa-miR-195	ebv-miR-BART19
ebv-miR-BART13	hsa-miR-195	hsa-miR-127	hsa-miR-136
hsa-miR-30a-5p	ebv-miR-BART19	ebv-miR-BART13	hsa-miR-106a
hsa-miR-93	hsa-miR-93	hsa-miR-106a	hsa-miR-374
hsa-miR-10b	ebv-miR-BART13	hsa-miR-148a	hsa-miR-148a
hsa-miR-368	hsa-miR-26b	hsa-miR-152	hsa-miR-210
hsa-miR-30d	hsa-miR-136	hsa-miR-374	hsa-miR-99a
hsa-miR-99a	hsa-miR-155	hsa-miR-101	ebv-miR-BART13
hsa-miR-10a	hsa-miR-152	hsa-miR-136	hsa-miR-368
hsa-miR-25	hsa-miR-10a	hsa-miR-155	hsa-miR-503
hsa-miR-26b	hsa-miR-148a	hsa-miR-210	hsa-miR-152
hsa-miR-148a	hsa-miR-374	hsa-miR-30d	hsa-miR-101
hsa-miR-152	hsa-miR-30d	ebv-miR-BART19	hsa-miR-195
hsa-miR-34b	hsa-miR-101	hsa-miR-34b	hsa-miR-25
hsa-miR-20b	hsa-miR-368	hsa-miR-17-5p	hsa-miR-10a
hsa-miR-374	hsa-miR-331	hsa-miR-224	hsa-miR-30d
hsa-miR-331	hsa-miR-34b	hsa-miR-368	hsa-miR-17-5p
hsa-miR-140	hsa-miR-25	hsa-miR-25	hsa-miR-10b
hsa-miR-195	hsa-miR-140	hsa-miR-93	hsa-miR-196a
hsa-miR-409-3p	hsa-miR-218	hsa-miR-331	hsa-miR-93
hsa-miR-18a	hsa-miR-224	hsa-miR-218	hsa-miR-331
hsa-miR-186	hsa-miR-450	hsa-miR-188	hsa-miR-218
hsa-miR-224	hsa-miR-186	hsa-miR-575	hsa-miR-224
hsa-miR-218	hsa-miR-20b	hsa-miR-140	hsa-miR-140
hsa-miR-101	hsa-miR-210	hsa-miR-361	hsa-miR-186
hsa-miR-181b	hsa-miR-487b	hsa-miR-450	hsa-miR-361
hsa-miR-487b	hsa-miR-361	hsa-miR-513	hsa-miR-409-3p
hsa-miR-381	hsa-miR-99b	kshv-miR-K12-3	hsa-miR-574
hsa-miR-99b	hsa-miR-18a	hsa-miR-196a	hsa-miR-487b
hsa-miR-376b	hsa-miR-575	hsa-miR-186	hsa-miR-188
hsa-miR-575	hsa-miR-376b	hsa-miR-487b	hsa-miR-575
hsa-miR-188	hsa-miR-181a	hsa-miR-98	hsa-miR-202
hsa-miR-513	hsa-miR-409-3p	hsa-miR-20b	hsa-miR-20b
hsa-miR-185	hsa-miR-185	hsa-miR-185	kshv-miR-K12-3
hsa-miR-138	hsa-miR-202	hsa-miR-202	hsa-miR-212
hsa-miR-181a	hsa-miR-132	hsa-miR-574	hsa-miR-181b
hsa-miR-202	hsa-miR-181b	hsa-miR-493-5p	hsa-miR-513
hsa-miR-212	hsa-miR-151	hsa-miR-629	hsa-miR-185
hsa-miR-361	hsa-miR-493-5p	hsa-miR-99b	hsa-miR-493-5p
kshv-miR-K12-3	hsa-miR-196a	hsa-miR-212	hsa-miR-381
hsa-miR-379	hsa-miR-98	hsa-miR-33	hsa-miR-181a
hsa-miR-210	hsa-miR-379	NC1_00000215	hsa-miR-376b
hsa-miR-493-5p	hsa-miR-188	hsa-miR-130b	hsa-miR-99b
hsa-miR-151	hsa-miR-212	hsa-miR-409-3p	hsa-miR-137
hsa-miR-132	hsa-miR-574	hsa-miR-223	hsa-miR-98
hsa-miR-450	hsa-miR-223	hsa-miR-379	hsa-miR-379
hsa-miR-299-5p	hsa-miR-513	hsa-miR-30e-5p	hsa-miR-18a
hsa-miR-181d	hsa-miR-381	hsa-miR-339	hsa-miR-30e-5p
hsa-miR-574	hsa-miR-28	hsa-miR-630	NC1_00000215
hsa-miR-130b	hsa-miR-191*	hsa-miR-181b	hsa-miR-223
hsa-miR-223	hsa-miR-138	hsa-miR-381	hsa-miR-191
hsa-miR-98	kshv-miR-K12-3	hsa-miR-18a	hsa-miR-28
hsa-miR-17-3p	hsa-miR-17-3p	hsa-miR-28	hsa-miR-191*
hsa-miR-7	hsa-miR-130b	hsa-miR-376b	hsa-miR-138
hsa-miR-28	hsa-miR-181d	NC2_00106057	hsa-miR-299-5p

Table 1 (*contd.*)

Donor #1	Donor #2	Donor #3	Donor #4
hsa-miR-339	hsa-miR-7	hsa-miR-572	hsa-miR-450
hsa-miR-33	hsa-miR-299-5p	hsa-miR-299-5p	hsa-miR-151
hsa-miR-134	hsa-miR-33	hsa-miR-191*	hsa-miR-342
hsa-miR-191*	hsa-miR-191	hsa-miR-151	hsa-miR-339
hsa-miR-342	hsa-miR-342	hsa-miR-324-5p	hsa-miR-33
hsa-miR-154*	hsa-miR-339	hsa-miR-181a	hsa-miR-130b
hsa-miR-422b	hsa-miR-30e-5p	hsa-miR-342	NC2_00106057
hsa-miR-410	hsa-miR-148b	hsa-miR-663	hsa-miR-17-3p
hsa-miR-191	hsa-miR-154*	hsa-miR-191	hsa-miR-422b
hsa-miR-484	NC1_00000215	hsa-miR-197	hsa-miR-181d
hsa-miR-411	hsa-miR-30c	hsa-miR-17-3p	hsa-miR-484
hsa-miR-630	hsa-miR-484	hsa-miR-138	hsa-miR-7
NC2_00106057	hsa-miR-324-5p	hsa-miR-452	hsa-miR-154*
hsa-miR-425-5p	hsa-miR-410	NC2_00092197	hsa-miR-30c
hsa-miR-154	hsa-miR-422b	hsa-miR-425-5p	hsa-miR-134
hsa-miR-148b	hsa-miR-432	hsa-miR-181d	hsa-miR-196b
hsa-miR-30e-5p	hsa-miR-154	hsa-miR-137	hsa-miR-154
hsa-miR-324-5p	hsa-miR-425-5p	hsa-miR-497	hsa-miR-324-5p
hsa-miR-299-3p	hsa-miR-542-3p	hsa-miR-542-3p	hsa-miR-425-5p
hsa-miR-432	hsa-miR-452	hsa-miR-30b	hsa-miR-148b
hsa-miR-382	hsa-miR-134	hsa-miR-30c	hsa-miR-584
NC1_00000215	hsa-miR-197	hsa-miR-148b	hsa-miR-197
hsa-miR-495	hsa-miR-497	hsa-miR-768-5p	NC2_00092197
hsa-miR-431	hsa-miR-630	hsa-miR-484	hsa-miR-132
hsa-miR-196b	hsa-miR-30b	hsa-miR-196b	hsa-miR-296
hsa-miR-452	hsa-miR-411	hsa-miR-411	hsa-miR-495
hsa-miR-671	hsa-miR-663	hsa-miR-671	hsa-miR-660
hsa-miR-137	hsa-miR-382	hsa-miR-432	hsa-miR-30b
hsa-miR-509	hsa-miR-296	hsa-miR-132	hsa-miR-411
hsa-miR-590	hsa-miR-196b	hsa-miR-7	NC2_00122731
hsa-miR-197	hsa-miR-768-5p	NC2_00122731	hsa-miR-32
hsa-miR-663	NC2_00106057	hsa-miR-154	hsa-miR-299-3p
hsa-miR-296	hsa-miR-431	hsa-miR-590	hsa-miR-630
hsa-miR-572	hsa-miR-299-3p	hsa-miR-410	hsa-miR-590
hsa-miR-768-5p	hsa-miR-590	hsa-miR-660	hsa-miR-671
hsa-miR-30c	hsa-miR-493-3p	hsa-miR-134	hsa-miR-663
hsa-miR-455	hsa-miR-660	hsa-miR-495	hsa-miR-625
hsa-miR-146a	hsa-miR-495	hsa-miR-296	hsa-miR-452
hsa-miR-625	hsa-miR-671	NC1_00000197	hsa-miR-500
hsa-miR-301	hsa-miR-572	hsa-miR-32	hsa-miR-493-3p
hsa-miR-30b	hsa-miR-190	hsa-miR-422b	hsa-miR-410
hsa-miR-564	hsa-miR-509	hsa-miR-190	hsa-miR-382
hsa-miR-345	hsa-miR-542-5p	hsa-miR-382	hsa-miR-572
hsa-miR-362	NC2_00092197	hsa-miR-542-5p	hsa-miR-190
hsa-miR-660	hsa-miR-455	hsa-miR-454-3p	hsa-miR-432
hsa-miR-30e-3p	hsa-miR-32	hsa-miR-299-3p	hsa-miR-509
hsa-miR-190	hsa-miR-301	hsa-miR-509	hsa-miR-629
hsa-miR-128a	hsa-miR-146b	hsa-miR-301	hsa-miR-768-5p
hsa-miR-149	hsa-miR-625	hsa-miR-602	hsa-miR-142-3p
hsa-miR-146b	hsa-miR-801	hsa-miR-198	hsa-miR-532
hsa-miR-532	hsa-miR-532	hsa-miR-532	hsa-miR-497
hsa-miR-602	hsa-miR-137	hsa-miR-146a	hsa-miR-146b
hsa-miR-652	hsa-miR-181c	hsa-miR-500	hsa-miR-128b
hsa-miR-128b	hsa-miR-146a	hsa-miR-765	hsa-miR-181c
hsa-miR-584	hsa-miR-362	hsa-miR-601	hsa-miR-455
hsa-miR-198	hsa-miR-149	hsa-miR-149	hsa-miR-557
hsa-miR-500	hsa-miR-500	hsa-miR-362	hsa-miR-345
hsa-miR-769-5p	hsa-miR-602	hsa-miR-770-5p	hsa-miR-652
hcmv-miR-UL70-3p	hsa-miR-629	hsa-miR-564	hsa-miR-602
hsa-miR-373*	hsa-miR-770-5p	hsa-miR-652	hsa-miR-126
hsa-miR-452*	hsa-miR-564	hsa-miR-181c	hsa-miR-149

Table 1 (*contd.*)

Donor #1	Donor #2	Donor #3	Donor #4
hsa-miR-629	hsa-miR-126	hsa-miR-146b	hsa-miR-564
ebv-miR-BART20-3p	hsa-miR-557	hsa-miR-126	hsa-miR-560
hsa-miR-491	hsa-miR-652	hsa-miR-557	hsa-miR-452*
hsa-miR-560	hsa-miR-30e-3p	hsa-miR-801	hsa-miR-373*
hsa-miR-32	hsa-miR-335	hsa-miR-584	
hsa-miR-124a	hsa-miR-345	hsa-miR-455	
hsa-miR-126	hsa-miR-198	hsa-miR-373*	
	hsa-miR-601	hsa-miR-345	
	hsa-miR-128b	hsa-miR-452*	
	hsa-miR-452*	hsa-miR-124a	
	hsa-miR-128a		
	hsa-miR-560		
	hcmv-miR-UL70-3p		
	hsa-miR-373*		
	hsa-miR-584		
	hsa-miR-124a		

a, b, *, probes for different regions of the same miRNA; 5p, 5' side of stem-loop sequence; 3p, 3' side of stem loop sequence.

was transferred to a new tube and mixed with isopropanol, followed by another round of centrifugation. The pellet was washed with 75% ethanol solution and then centrifuged following the same procedures as stated earlier. The final pellet was air-dried and suspended in RNase-free water. The quality of the isolated RNA was evaluated using Agilent's 2100 Bioanalyzer system (Santa Clara, CA, USA), as RNA integrity number (RIN) through migration pattern by electrophoretic trace and peak pattern by electropherogram. RNA with RIN above 7 was utilized in the microarray analysis.

Microarray analysis

To control and test RNAs, the synthesis of target miRNA probes and hybridization were performed using Agilent's miRNA Labelling Reagent and Hybridization kit (Agilent Technology, Santa Clara, USA) according to the manufacturer's instructions. Briefly, each 100 ng of total RNA 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. Dephosphorylated RNA was ligated with pCp-Cy3

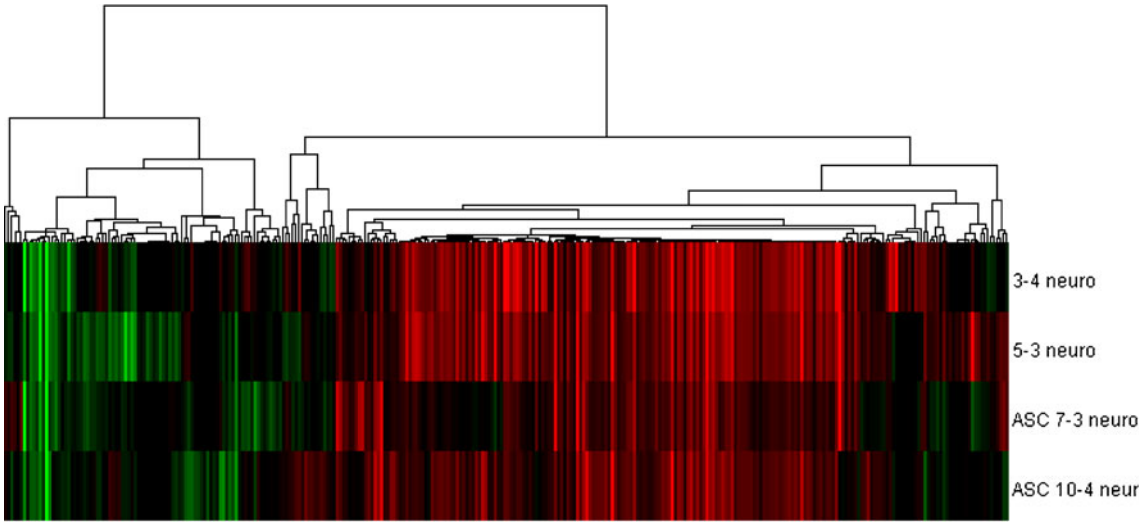

Figure 3. Unsupervised hierarchical clustering of miRNAs. The expression levels of miRNAs were compared between undifferentiated and neurodifferentiated groups, upregulated miRNAs by neurogenesis were shown as red and downregulated miRNAs by neurogenesis were shown as green.

Table 2. miRNAs upregulated or downregulated at over 2 fold after neurogenesis of ADSCs.

Donor #1		Donor #2		Donor #3		Donor #4	
miRNA ID	Neu/Con	miRNA ID	Neu/Con	miRNA ID	Neu/Con	miRNA ID	Neu/Con
Upregulated							
hsa-miR-452*	12.3649	hsa-miR-124a	22.3409	hsa-miR-124a	12.8188	hsa-miR-373*	8.99201
hsa-miR-629	12.2515	hsa-miR-335	10.29985	hsa-miR-345	7.047919	hsa-miR-126	7.35977
ebv-miR-BART20-3p	11.18480	hsa-miR-373*	9.718501	hsa-miR-584	6.86552	hsa-miR-149	6.12764
hsa-miR-126	11.00000	hsa-miR-452*	9.038787	hsa-miR-132	6.157643	hsa-miR-132	6.09768
hsa-miR-32	11.00000	hsa-miR-560	7.817356	hsa-miR-452*	6.029531	hsa-miR-452*	5.9293
hsa-miR-124a	8.99336	hsa-miR-629	7.001485	hsa-miR-146b	5.475309	hsa-miR-560	4.72855
hsa-miR-500	8.868321	hsa-miR-345	6.522692	hsa-miR-181a	4.335888	hsa-miR-564	4.46107
hsa-miR-30c	8.591849	hcmv-miR-UL70-3p	6.4893	hsa-miR-801	4.234988	hsa-miR-345	4.23609
hsa-miR-452	7.954802	hsa-miR-584	5.95749	ebv-miR-BART19	4.001035	hsa-miR-324-5p	4.21623
hsa-miR-30b	7.114367	hsa-miR-137	5.812335	hsa-miR-30c	3.868032	hsa-miR-191	3.70129
hsa-miR-532	6.994533	hsa-miR-452	5.726561	hsa-miR-191	3.727272	hsa-miR-452	3.68101
hsa-miR-191	6.761363	hsa-miR-126	5.512305	hsa-miR-455	3.597304	hsa-miR-663	3.58290
hsa-miR-660	6.522535	hsa-miR-210	5.106823	hsa-miR-373*	3.594157	hsa-miR-181a	3.48405
hsa-miR-560	6.406269	hsa-miR-630	4.604335	hsa-miR-126	3.493665	hsa-miR-30c	3.25191
hsa-miR-584	6.320209	hsa-miR-34a	4.548903	hsa-miR-564	3.018522	hsa-let-7i	3.16957
hsa-miR-324-5p	5.823898	hsa-miR-128a	4.508485	hsa-miR-452	2.909913	hsa-miR-148b	3.08372
hsa-miR-663	5.741501	hsa-miR-128b	4.507167	hsa-miR-324-5p	2.902999	hsa-miR-103	2.93546
hsa-miR-198	5.38616	hsa-miR-191	4.385028	hsa-miR-768-3p	2.87438	hsa-miR-33	2.86461
hsa-miR-769-5p	5.381649	hsa-miR-324-5p	4.30642	hsa-miR-148b	2.787305	hsa-miR-30b	2.82572
hsa-miR-190	5.299296	hsa-miR-34b	4.002358	hsa-miR-491	2.50978	hsa-miR-602	2.79583
hsa-miR-149	5.241024	hsa-miR-660	3.744209	hsa-miR-30a-5p	2.388547	hsa-miR-768-5p	2.72545
hsa-miR-491	5.15773	hsa-miR-32	3.722026	hsa-miR-30b	2.379695	hsa-miR-107	2.68212
hcmv-miR-UL70-3p	5.15466	hsa-miR-30c	3.70265	hsa-miR-557	2.362832	hsa-miR-630	2.65980
hsa-miR-345	5.146942	hsa-miR-181a	3.681028	hsa-miR-342	2.336154	hsa-miR-652	2.60935
hsa-miR-373*	5.10286	hsa-miR-30b	3.567784	hsa-let-7i	2.304572	hsa-miR-34a	2.55219
hsa-miR-34a	4.814482	hsa-let-7i	3.519226	hsa-miR-560	2.301989	hsa-miR-185	2.52091
hsa-miR-30e-5p	4.673209	hsa-miR-422b	3.507341	hsa-miR-103	2.284109	hsa-miR-768-3p	2.49794
hsa-miR-34b	4.639537	hsa-miR-663	3.480477	hsa-miR-181c	2.231021	hsa-miR-574	2.49607
hsa-miR-148b	4.638073	hsa-miR-196a	3.408277	hsa-miR-320	2.202201	hsa-miR-197	2.49528
hsa-miR-101	4.569371	hsa-miR-198	3.356802	hsa-miR-422b	2.179526	hsa-miR-101	2.48669
hsa-miR-137	4.536166	hsa-miR-532	3.340554	hsa-miR-149	2.150473	hsa-miR-629	2.42244
hsa-miR-128b	4.421787	hsa-miR-149	3.282256	hsa-miR-34a	2.147344	hsa-miR-320	2.410985
hsa-miR-374	4.360524	hsa-miR-500	3.252840	hsa-miR-500	2.113932	hsa-miR-125b	2.36336
hsa-miR-422b	4.259643	hsa-miR-374	3.214476	hsa-miR-494	2.107529	hsa-miR-146b	2.34493
hsa-miR-10a	4.243414	hsa-miR-574	3.192512	hsa-miR-34b	2.073393	hsa-miR-34b	2.29725
hsa-miR-181a	4.169982	hsa-miR-101	3.155423	kshv-miR-K12-3	2.072254	hsa-miR-584	2.27556
hsa-miR-197	4.134848	hsa-miR-107	3.028175	hsa-miR-107	2.031978	hsa-miR-196a	2.23804
hsa-miR-574	4.036704	hsa-miR-148b	3.027027	hsa-miR-146a	2.020711	hsa-miR-572	2.22798
hsa-miR-630	3.997406	hsa-miR-224	2.988696	hsa-miR-532	2.020271	hsa-miR-30a-5p	2.19691
hsa-miR-30a-5p	3.788593	hsa-miR-30e-3p	2.963207			hsa-miR-181c	2.18760
hsa-let-7i	3.728025	hsa-miR-103	2.95928			hsa-miR-98	2.18565
hsa-miR-572	3.721510	kshv-miR-K12-3	2.957622			hsa-miR-425-5p	2.18087
hsa-miR-768-3p	3.504502	hsa-miR-601	2.937058			hsa-miR-565	2.16676
hsa-miR-103	3.490409	hsa-miR-572	2.926896			hsa-miR-24	2.14789
hsa-miR-362	3.485242	hsa-miR-30e-5p	2.912391			hsa-let-7g	2.13755
hsa-miR-224	3.401972	hsa-miR-10a	2.886910			hsa-miR-23a	2.12465
hsa-miR-107	3.400869	hsa-miR-638	2.859239			hsa-miR-125a	2.10804
hsa-miR-98	3.394763	hsa-miR-98	2.769848			hsa-let-7d	2.08312
hsa-miR-26a	3.331323	hsa-miR-15a	2.754295			hsa-let-7e	2.07814
hsa-miR-148a	3.305013	hsa-miR-30a-5p	2.729208			hsa-miR-152	2.06899
hsa-miR-210	3.283549	hsa-miR-185	2.692396			hsa-let-7a	2.06242
hsa-miR-15a	3.178056	hsa-miR-25	2.665933			hsa-miR-32	2.04561
hsa-let-7g	3.171610	hsa-miR-197	2.662608			hsa-miR-25	2.01883
hsa-let-7d	3.163391	hsa-miR-125b	2.640156			hsa-miR-30d	2.01789
hsa-miR-652	3.152711	hsa-let-7g	2.639784			hsa-miR-532	2.01505
hsa-miR-146b	3.151944	hsa-miR-22	2.622234			hsa-miR-221	2.01049
hsa-miR-361	3.129899	hsa-miR-365	2.600721				
hsa-miR-602	3.123897	hsa-miR-768-3p	2.598262				
hsa-let-7e	3.073295	hsa-miR-33	2.579403				

Table 2 (*contd.*)

Donor #1		Donor #2		Donor #3		Donor #4	
miRNA ID	Neu/Con	miRNA ID	Neu/Con	miRNA ID	Neu/Con	miRNA ID	Neu/Con
hsa-miR-342	3.034777	hsa-miR-564	2.577174				
hsa-miR-638	2.942289	hsa-miR-193a	2.559637				
hsa-miR-199a	2.927726	hsa-miR-26b	2.555959				
hsa-let-7a	2.919707	hsa-miR-342	2.552469				
hsa-let-7f	2.909311	hsa-miR-148a	2.552165				
hsa-miR-331	2.9054	hsa-miR-199a	2.541623				
hsa-miR-26b	2.898149	hsa-miR-652	2.537624				
hsa-miR-21	2.817319	hsa-miR-106b	2.525400				
hsa-miR-125b	2.797019	hsa-miR-30d	2.497665				
hsa-miR-185	2.786142	hsa-miR-21	2.485808				
hsa-miR-30d	2.700738	hsa-miR-16	2.477186				
hsa-let-7c	2.676802	hsa-miR-152	2.463894				
hsa-miR-25	2.674456	hsa-miR-26a	2.448325				
hsa-miR-125a	2.649787	hsa-let-7d	2.434028				
hsa-miR-152	2.621993	hsa-let-7e	2.422249				
hsa-miR-16	2.586751	hsa-let-7a	2.405793				
hsa-let-7b	2.575221	hsa-let-7f	2.397403				
hsa-miR-425-5p	2.559289	hsa-miR-320	2.367259				
hsa-miR-28	2.55221	hsa-miR-361	2.312166				
hsa-miR-365	2.543642	hsa-let-7c	2.312142				
hsa-miR-128a	2.534724	hsa-miR-370	2.276301				
hsa-miR-146a	2.516203	hsa-let-7b	2.238897				
hsa-miR-106b	2.496998	hsa-miR-331	2.231834				
hsa-miR-132	2.470434	hsa-miR-188	2.204851				
hsa-miR-199a*	2.443915	hsa-miR-199a*	2.186400				
hsa-miR-188	2.441890	hsa-miR-29c	2.158105				
hsa-miR-186	2.426049	hsa-miR-602	2.157494				
hsa-miR-484	2.413653	hsa-miR-190	2.152054				
hsa-miR-370	2.412335	hsa-miR-425-5p	2.141620				
hsa-miR-575	2.370684	hsa-miR-28	2.132881				
hsa-miR-10b	2.354013	hsa-miR-125a	2.118136				
hsa-miR-33	2.332419	hsa-miR-146a	2.118039				
kshv-miR-K12-3	2.329550	hsa-miR-455	2.098571				
hsa-miR-22	2.318712	hsa-miR-186	2.087319				
hsa-miR-513	2.299014	hsa-miR-196b	2.080946				
hsa-miR-301	2.289068	hsa-miR-132	2.07247				
hsa-miR-30e-3p	2.271584	hsa-miR-575	2.059546				
hsa-miR-193a	2.252327	hsa-miR-100	2.032472				
hsa-miR-196b	2.231798	hsa-miR-770-5p	2.016630				
hsa-miR-99a	2.173747						
hsa-miR-590	2.024366						
hsa-miR-339	2.001859						
Downregulated							
hsa-miR-138	0.121478	hsa-miR-503	0.155579	hsa-miR-138	0.090909	hsa-miR-503	0.11639
hsa-miR-503	0.137202	hsa-miR-431	0.167739	hsa-miR-503	0.111841	hsa-miR-138	0.33211
hsa-miR-431	0.199448	NC2_00092197	0.187425	hsa-miR-431	0.292740	hsa-miR-154*	0.49797
hsa-miR-154*	0.261160	hsa-miR-138	0.386866	hsa-miR-424	0.384771		
hsa-miR-376b	0.384067	hsa-miR-154*	0.402828	hsa-miR-154*	0.426307		
hsa-miR-7	0.442085	hsa-miR-424	0.424680				
hsa-miR-424	0.493339	hsa-miR-376b	0.471871				
		hsa-miR-7	0.488960				
		NC1_00000215	0.493839				

The numbers indicate the ratio that was obtained by dividing the signal intensity (expression level) of each probe after neurogenesis (Neu) by that before neurogenesis (Con). Therefore, the more regulated, the upper located at the list.
a, b, *, probes for different regions of the same miRNA; 5p, 5' side of stem-loop sequence; 3p, 3' side of stem loop sequence.

mononucleotide and purified with MicroBioSpin 6 columns (Bio-Rad, Gurgaon, India). After purification, labelled samples were resuspended with Gene Expression blocking reagent and Hi-RPM Hybridization buffer, followed by boiling for 5 min at 100°C and 5 min chilling on ice. Finally, denatured labelled probes were pipetted onto assembled

Agilent's miRNA microarray (15 K) and hybridized for 20 h at 55°C with 20 RPM rotating in Agilent Hybridization oven (Agilent Technology, USA). The hybridized microarrays were washed as the manufacturer's washing protocol (Agilent Technology, USA).

Data acquisition and analysis

The hybridized images were scanned using Agilent's DNA microarray scanner and quantified with Feature Extraction Software (Agilent Technology, Palo Alto, USA). All data normalization and selection of fold-changed genes were performed using GeneSpringGX 7.3 (Agilent Technology, Palo Alto, USA). The averages of normalized ratios were calculated by dividing the average of normalized signal channel intensity by the average of normalized control channel intensity (Lopez-Romero *et al.* 2010) Functional annotation of genes was performed according to Gene Ontology™ Consortium by GeneSpringGX 7.3 (http://www.geneontology.org/index.shtml).

MicroRNA target predictions and gene ontology (GO)

The human targets of the differentially expressed miRNAs were predicted using public websites such as miRBase Targets (http://microrna.sanger.ac.uk/targets/v5). The GO of the predicted targets was analysed using functional items on http://www.geneontology.org.

Results and discussion

Identification of miRNAs expressed in undifferentiated ADSCs

Although miRNAs are expected to play critical roles in the biological processes of adult stem cells including self renewal and differentiation, there is very little information about them. We performed the human miRNA microarray (see table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/; gene expression omnibus accession number GSE15290), using the extracted RNA from ADSCs of four different donors which were undifferentiated and differentiated into neural lineage. Neurogenesis was

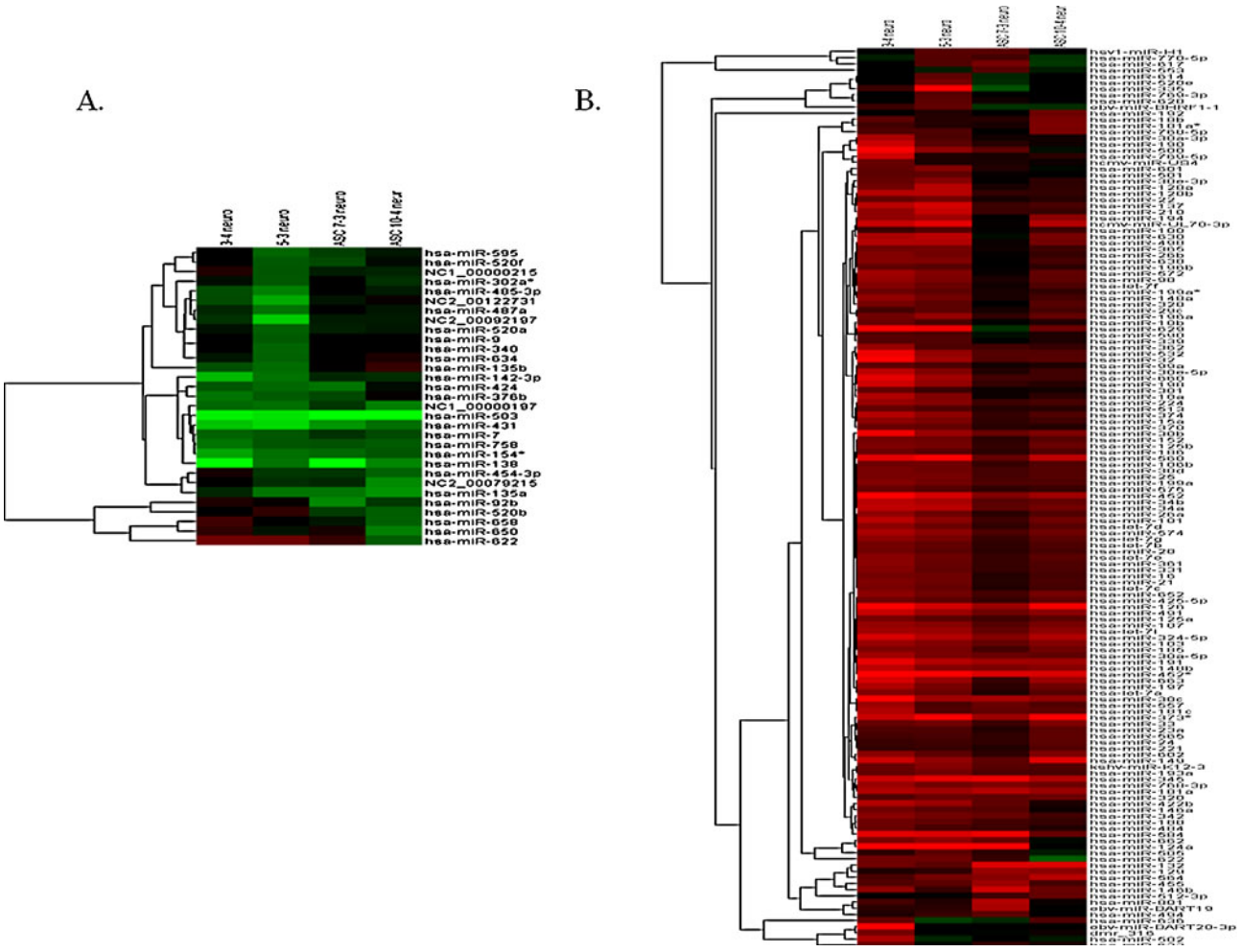

Figure 4. Supervised hierarchical clustering of miRNAs. Supervised clustering was performed for downregulated (A) or upregulated (B) miRNA at over two-fold by neurogenesis.

followed by the method previously reported (Ashjian *et al.* 2003) and verified by microscopic observation and also RT-PCR (figure 1), followed by immunocytochemistry for neurogenic markers (figure 2). The specificity of neurogenesis was confirmed by performing RT-PCR for adipocyte-specific genes (PPARγ, Adiponectin, LPL) (see figure 1 in electronic supplementary material).

First, we analysed the miRNA expression profile of the undifferentiated ADSCs. Of the 550 probes, about 190–200 miRNAs were found to be statistically significant on analysis, and the results are listed in table 1 according to their expression level. The comparison between the donors revealed the similar miRNA expression profiling, which have the high expression of mir-29a, mir-125b, let-7a, mir-27a, mir-24, and mir-222 in common. This analysis suggests that undifferentiated ADSCs express the unique set of miRNAs to maintain the stem cell characteristics.

Identification of miRNAs expressed in differentiated ADSCs into neural lineage

Then, we compared the miRNA profiling of the undifferentiated ADSCs with that of the differentiated ADSCs. The unsupervised hierarchical clustering analysis showed that the ADSCs of four donors had the very similar expression patterns for a specific miRNA (figure 3), indicating that ADSCs commonly express a specific set of miRNAs for neurogenesis.

We screened the miRNAs according to the level of expression change by neurogenesis. As compared to miRNAs that were unchanged or not significantly changed at below 2-fold by neurogenesis (see table 2 in electronic supplementary material), table 2 displays the summarized list of miRNAs that were upregulated or downregulated at over 2-fold by neurogenesis. It was interesting that the number of the upregulated miRNAs (39–101 miRNAs) was much higher than the number of the downregulated miRNAs (3–9 miRNAs). As with the miRNA expression profiling of undifferentiated ADSCs, the miRNA profiling changed by the differentiation that showed the common expression regulation pattern in four donors. As shown in figure 4, some miRNAs were upregulated (miR-452, miR-126, miR-560, miR-373, miR-584, miR-149, miR-191, miR-30c etc.) and some miRNAs were downregulated (miR-138, miR-503 and miR-154). To validate the microarray data, we performed real-time PCR for the several top changing miRNA that were differentially expressed during neurogenesis (figure 5). These results demonstrated clearly the different miRNA profiling before and after differentiation, suggesting that the combination of those upregulated or downregulated miRNAs may promote the neurogenic differentiation.

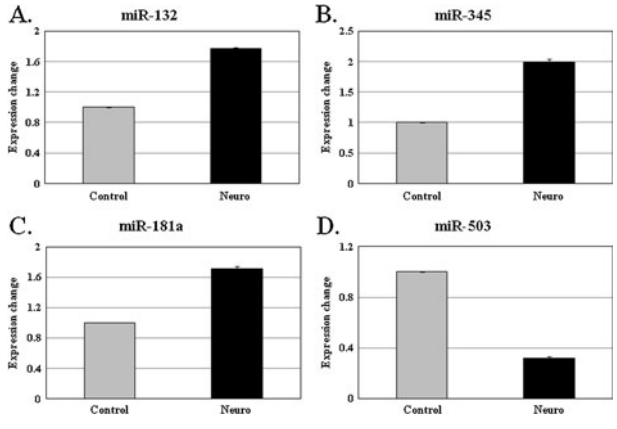

Figure 5. Measurement of the top changing miRNAs by real-time RT-PCR. Among miRNAs that were differentially expressed during neurogenesis, representative several miRNAs were analysed by real-time PCR using relative quantification method. Data were normalized with a house-keeping gene *RNU48*, and were presented as fold change for each miRNA in 'neuro' sample relatively calculated by 'control' sample.

Assortment of miRNAs specifically involved in development of neuron cells

We performed the chemically-induced neurogenesis method to generate all the neural cells including neurons, astrocytes and oligodendrocytes. Thus, we examined whether a specific combination of miRNAs is involved in the generation of each cell type. For this purpose, we predicted the target genes for each miRNA listed in table 2 using the Sanger database (http://microrna.sanger.ac.uk/targets/v5). Then, we assorted the predicted target genes using the GO website (amigo; http://www.geneontology.org) by matching them with genes classified according to ontology. First, we obtained the list of miRNAs that are expected to be involved in neuron generation (Ontology search term was 'neuron morphogenesis during differentiation' and 'regulation of neurogenesis') (table 3). Interestingly, most of the top-ranked miRNAs in table 2 belong to this category (eg. miR-126, miR-373, miR-584, miR-149, miR-191, miR-30c etc.). Some of the miRNAs in this table were well-known miRNAs that are involved in other differentiation processes, such as chondrogenesis (miR-663 and miR-638) and adipogenesis (miR-103 and miR-107) (Wilfred *et al.* 2007; Lakshmipathy and Hart 2008). In addition, the downregulated miR-503 was previously found to be involved in pancreas development (Joglekar *et al.* 2007).

Assortment of miRNAs specifically involved in development of glial cells

We made a list of miRNAs that are expected to participate in the generation of astrocytes and oligodendrocytes (ontology search term was 'regulation of astrocyte differentiation' and 'oligodendrocyte differentiation') in table 4. Two miRNAs, miR-560 and miR-452, were among the top-ranked miRNAs in table 2.

Finally, we selected miRNAs that we predicted would be involved in generation of both neuron cells and glial cells

Table 3. miRNAs specifically related to neuron cell generation.

Upregulated	Downregulated
hsa-miR-373*	hsa-miR-503
hsa-miR-584	hsa-miR-154*
hsa-miR-126	
hsa-miR-191	
hsa-miR-30c	
hsa-miR-132	
hsa-miR-149	
hsa-miR-148b	
hsa-miR-663	
hsa-miR-34b	
hsa-miR-137	
hsa-let-7i	
hsa-miR-30b	
hsa-miR-630	
hsa-miR-103	
hsa-miR-768-3p	
hsa-miR-107	
hsa-miR-572	
hsa-miR-101	
hsa-miR-660	
hsa-miR-32	
hsa-miR-652	
hsa-miR-500	
hsa-miR-564	
hsa-miR-638	
hsa-miR-198	
hsa-miR-30d	
hsa-miR-210	
hsa-miR-98	
hsa-let-7g	
hsa-let-7e	
hsa-let-7d	
hsa-miR-557	
hsa-miR-152	

Table 4. miRNAs specifically related to glial cell generation.

Astrocyte-specific
hsa-miR-345
hsa-miR-629
hsa-miR-10a
hsa-miR-26a
Oligodendrocyte-specific
hsa-miR-560
hsa-miR-452
hsa-miR-224
hsa-miR-602
hsa-miR-25
hsa-miR-106b

Table 5. miRNAs related to neuron and glial cell generation.

hsa-miR-324-5p
hsa-miR-181a
hsa-miR-34a
hsa-miR-197
hsa-miR-185
hsa-miR-320
hsa-miR-125b

(table 5). In this list, we noticed that miR-324-5p and miR-181a were among the top-ranked miRNAs in table 2. As both, neuron cells and surrounding glial cells should co-operate to accomplish their biological functions, we propose that miRNAs in this list may provide an important role in clinical and therapeutic aspects for the treatment of neuron-degenerative diseases.

In this study, we used the neurogenesis method developed by Ashjian *et al.* (2003) for generation of neuro-progenitor cells using ADSCs (Ashjian *et al.* 2003) We assumed that there might be some variations in miRNA profiling according to neurogenesis method, by method and by stem cell origin. Nevertheless, this study provides the first clue that a unique set of miRNAs play a role in specific differentiation of ADSCs.

A recent study reported the miRNA expression profiles of human embryonic stem cells that were undifferentiated and differentiated into endodermal lineage. This study also proposes the possibility of a potential role for specific miRNAs in endodermal differentiation of hESC (Tzur *et al.* 2008). Another study found that the modulation of a single miRNA could promote the formation of adipocytes from precursor cells (Esau *et al.* 2004) Consistent with those studies, our study provides attention to the fact that stem cells can be differentiated to neural cells by a single or combined treatment of the miRNA(s) revealed in this study.

Most of the miRNAs in this study have not been previously reported in relationship to neurogenesis. Among the miRNAs distinctly-regulated in neuron cells, miR-373, miR-126, and miR-30 are only known to be related to tumour cell biogenesis, such as metastasis stimulation or suppression, and structural change of the extra-cellular matrix (Duisters *et al.* 2009; Huang *et al.* 2008; Tavazoie *et al.* 2008). These miRNAs are the first to be reported towards the neurogenesis of ADSCs, and may play significant roles in regulating neurogenic differentiation using these cells. Several miRNAs including miR-532, miR-574, miR-124, miR-342 and miR-33 were not found in the GO database related to neurogenesis and cannot be sorted by miRNAs to any of the GO categories.

Since there is a possibility of some changes to the results if a different normalization protocol is used, we need further studies to confirm the vast scale of information touched upon in this study. Nevertheless, this study suggests the specific set of miRNAs that may possess a potential role in neurogenesis and thereby provides a possibility about a new strategy using miRNAs in clinical application of ASCs for neurodegenerative diseases by demonstrating the unique set of miRNAs in undifferentiated and neurally-differentiated ADSCs.

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