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MicroRNA-432 contributes to dopamine cocktail and retinoic acid induced differentiation of human neuroblastoma cells by targeting NESTIN and RCOR1 genes

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ABSTRACT

MicroRNA (miRNA) regulates expression of protein coding genes and has been implicated in diverse cellular processes including neuronal differentiation, cell growth and death. To identify the role of miRNA in neuronal differentiation, SH-SY5Y and IMR-32 cells were treated with dopamine cocktail and retinoic acid to induce differentiation. Detection of miRNAs in differentiated cells revealed that expression of many miRNAs was altered significantly. Among the altered miRNAs, human brain expressed miR-432 induced neurite projections, arrested cells in G0–G1, reduced cell proliferation and could significantly repress NESTIN/NES, RCOR1/COREST and MECP2. Our results reveal that miR-432 regulate neuronal differentiation of human neuroblastoma cells.

1. Introduction

MicroRNA (miRNA) belongs to a class of small non-coding single stranded RNA and regulates expression of protein coding genes. Mature miRNA is about 21–25 nucleotides long and is the final processed product of primary transcript (pri-miRNA) of miRNA locus. MiRNA generally binds with 3′-untranslated regions (3′-UTR) of the gene (target) in human. They suppress expression of targets either by degrading mRNA or repressing translation [1]. It has been predicted that miRNA may target most of human protein coding genes [2], although experimentally validated targets are limited. Only about 2500 genes in human have been experimentally validated [3–5]. Validated targets of miRNAs modulate various biological processes like neuronal differentiation [6,7], cell death [8], cell cycle and cell proliferation [9].

To maintain neuronal identity, expressions of genes are regulated by transcription factors and miRNAs. Transcription repressor REST and its co-repressor complex containing histone deacetylases, methyl CpG binding protein MECP2, and RCOR1 bind with a conserved 23-bp repressor element (RE1) to suppress the transcription of neuronal genes in non-neuronal cells. REST also recruits anti-neural factor like small C-terminal domain phosphatase1 (SCP1) at RE1. SCP1 is repressed in neuronal cells by miR-124 during neurogenesis. But miR-124 expression is absent in non-neuronal cells, even neuronal progenitor cells. A negative feedback loop operates between miR-124 and SCP1/REST pathway that decides neural or anti-neural behavior of the cell [10,11].

In spite of several evidences for altered miRNA expression during neuronal differentiation, it is expected that additional miRNAs may also be critical for neuronal identity. Expression of many miRNA alters during differentiation, but role of miRNA in inducing neuronal differentiation is mostly unknown.

Human neuroblastoma SH-SY5Y cells treated with dopamine cocktail (DC) and retinoic acid (RA), elicited neurite projections and differentiate into neuron-like cells. In search of miRNAs, whose expression could alter during neuronal differentiation, we identified that expression of 16 miRNAs were increased and 12 miRNAs were decreased in differentiated cells. We characterized in detail the novel miR-432, whose expression was increased after treatment with DC and RA. We also demonstrate that ectopic

Abbreviations: RA, retinoic acid; 3′-UTR, 3′-untranslated region; DAPI, 4′,6-diamidino-2-phenylindole; DC, dopamine cocktail; MECP2, methyl CpG binding protein 2; RCOR1, co-repressor 1 or COREST; TUBB3, beta III-tubilin; NES, NESTIN; CNR1, cannabinoid receptor type 1; MAP2, microtubule associated protein 2; SYT5, synaptotagmin V; BDNF, brain-derived neurotropic factor; CHAT, choline acetyl-transferase; RARα, retinoic acid receptor alpha; TH, tyrosine hydroxylase; miRNA, micro RNA; PTO, phosphorothioate modified anti-sense oligonucleotides

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expression of miR-432 induced neurogenesis, while inhibition of miR-432 reduced the neurogenesis. Besides, miR-432 could repress NESTIN, MECP2 and RCOR1. We also observed that miR-432 induced neurogenesis in human IMR-32 cells. This result shows that miR-432 may behave as a regulator in neuronal differentiation by regulating stem cell modulating genes in human neuroblastoma cells.

2. Materials and methods

2.1. Cell culture and differentiating conditions

Human derived neuroblastoma cells SH-SY5Y and IMR-32 were maintained in DMEM (HiMedia, India), 10% (v/v) FBS (Biowest, France), 1% (v/v) PS at 37 °C in humidified, 5% CO_2. Cells were plated at the concentration of $10^4/cm^2$ for neuronal differentiation.

We differentiated the SH-SY5Y and IMR-32 cells with the following cocktail: 10 ng/ml α-FGF, 250 μM IBMX, 200 nM TPA, 50 μM forskolin, 5 μM dopamine (Sigma) for 7 days as described earlier [12].

We also differentiated neuroblastoma cells with RA as published elsewhere [13]. Next day after seeding, cells were incubated with RA (sigma) at a final concentration of 10 μM. The differentiating mediums also contain 10% B27 growth supplement (Invitrogen) for both cases. All treatments were performed in dark.

2.2. Flow cytometry

Proportion of SH-SY5Y cells at different phases of cell cycle was determined using the procedures described earlier [14].

2.3. RNA preparation

Total RNA was extracted from cultured cells using Trizol Reagent (Invitrogen, USA) according to manufacturer's protocol. RNA samples were quantitated using Biophotometer (Eppendorf, Germany).

2.4. Quantitative real-time reverse transcription PCR (qRT-PCR) for mRNA and microRNAs

Methods used for detection of miRNA expression using stem loop specific primers and expression of protein coding genes using gene specific primers were similar to that has been published earlier [15,16]. List of primers for cloning pre-miR-432, 3′-UTR of the target genes and gene expression studies and their ensemble ID are shown in Supplementary Table S2A. The miR-132 mimic (ambion) was commercially obtained.

2.5. Immunocytochemistry

The method for immunocytochemistry was as described elsewhere [14] except the secondary anti body goat anti-mouse IgG-Alexa 488, 1:300, Invitrogen, USA was used. Neurite number was quantified using published method [17].

2.6. Western blot analysis

Western blot analysis was carried out using standard methods described earlier [15]. List of antibodies and their sources are shown in Supplementary Table S2B.

2.7. Cell proliferation assay

Cell proliferation assay was determined by measuring the incorporation of BrdU into DNA following the protocol provided by the manufacturer of the KIT (Calbiochem, Cat. No. QIA58) and described earlier [14].

2.8. Luciferase reporter assays

The predicted recognition site of miR-432 at 3′-UTR of NES, MECP2 and RCOR1 were cloned into the pmiR-Report luciferase vectors between the *Hind III* and *MluI* site, immediately 3′ downstream of the Renilla luciferase gene as described earlier [15].

2.9. Image processing

Images of histograms, Western blots and cell cycle distributions were prepared with the help of Adobe photoshop CS2 software.

2.10. Statistical analysis

Data are presented as mean ± S.D. of three independent experiments. Statistical significance of differences between groups was determined by Student's unpaired *t*-test with the help of Graphpad Software, QuickCalcs, (http://www.graphpad.com/quickcalcs/index.cfm) when two groups were present.

3. Results

3.1. Differentiation of SH-SY5Y cells by dopamine cocktail (DC) and retinoic acid (RA) treatment

Neurite outgrowth was apparent after 3-day with DC and RA treatment in SH-SY5Y cells. Though the number of roots was not significantly altered at day 7 but the frequency and length of neurites increased with the duration of chemical treatment. At day 7, >70% of cells exhibited at least 1 neurite that extended in length more than 1 cell-body from cell periphery. Treated cells expressed neuronal markers TUBB3 but showed low level of expression of progenitor marker NES at day 7. Similarly, control SH-SY5Y cells expressed NES at day 0 to day 7 but did not express TUBB3 at day 7 (Fig. 1A and Supplementary Fig. S1A). Expression (mRNA level) of additional neuronal markers (CNR1, MAP2, SYT5, BDNF, CHAT, RARα and TH) was increased whereas mRNA expression of neural progenitor markers POU5F1, NES and NANOG was decreased significantly at day 3 and day 7 after DC (Fig. 1B) and RA (Fig. 1C) treatment. DC induced adrenergic phenotype that was associated with TH gene upregulation, whereas RA induced cholinergic phenotype that has been marked by CHAT and RARα gene upregulation in SH-SY5Y cells [18–20]. Though expression of CHAT, RARα and TH has been upregulated in presence of both DC and RA reagents but increased expression of CHAT and RARα was higher in presence of RA compared to TH expression. Increased expression of TH in RA treated cells was also reported earlier as TH was regulated by Retinoic acid receptor [21]. Similarly increased expression of TH was higher in presence of DC compared to RA.

Western blot analysis also revealed that NES protein expression decreased at day 7 while the expression of TUBB3 was elevated at day 7 after treatment with DC and RA (Supplementary Fig. S1B). We also tried to find out the status of two other neurodevelopment associated proteins MECP2 and RCOR1 in DC and RA treated cells. RCOR1 protein expression was reduced at day 7 of DC and RA treated cells but this reduction was less compared to NES protein expression in DC and RA treated cells. But MECP2 expression was increased at day 7 of DC and RA treatment (Supplementary Fig. S1C) as reported earlier [22]. Expression of progenitor markers and neuronal markers correlated with neurite outgrowth. Further, incorporation of BrdU in DNA, a measure for cell proliferation, was reduced significantly by treatment of DC and RA at day 3 and day 7 (Fig. 1D). Thus, treatment with DC and RA induce neuronal