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 MECPT2, 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
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% CO₂. Cells were plated at the concentration of 10⁵/cm² 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 medium 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 Mlu I 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 RARx gene upregulation in SH-SY5Y cells [18–20]. Though expression of CHAT, RARx and TH has been upregulated in presence of both DC and RA reagents but increased expression of CHAT and RARx 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
differentiation in SH-SY5Y cells as has been shown by many investigators.

**3.2. Altered micro RNA expressions in SH-SY5Y cells treated with DC and RA**

Role of miRNAs in neuronal development, neurogenesis, and maintenance of pluripotency has been reviewed recently [23,24]. Compiling data from various publications, we collected 99 miRNAs that had been reported to alter expression during neurogenesis/neuronal development and differentiated neurons (Supplementary Table S1A). Increased expression of 21 miRNA is reported in more than one publication; enhanced expression of miR-124, miR-125b and miR-9 is reported in 11, 10 and 7 publications respectively. Expression of miR-19a has been reported to decrease in 2 experiments and increase in 1 experiment. Result for 9 miRNA was opposite in two publications. Expression of other miRNAs was reported only once.

We have chosen 96 miRNAs from our earlier studies [15,25], bioinformatics analysis (http://miRNAMap.mbc.nctu.edu.tw) and

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**Fig. 1.** DC and RA induced neuronal differentiation in neuroblastoma SH-SY5Y cells. (A) Photomicrograph shows morphology of SH-SY5Y cells following treatment with DC and RA at day 7. SH-SY5Y cells were immunostained with neuronal progenitor marker NES (green) at day 0 (upper panel), neuronal marker TUBB3 (green) at day 7 in presence of DC (middle panel) and RA (lower panel). Nuclei were counterstained with DAPI (blue). Scale bar was 50 μm. The images were acquired by an apotome fluorescence microscope using a 20× objective lens. The percentage of neuron-like cells in each biological replicate was analyzed and averaged by at least 15 images; each image of 20× magnification typically captures 100–200 SH-SY5Y cells. Neurite outgrowths, staining positively for TUBB3 and longer than 30 μm are counted for DC and RA treated cells. Bar graph displayed number of neurites per cell and average length of neurite (≥30 μm) following the treatments with DC and RA. Very few control SH-SY5Y cells were continuously differentiated but their neurite outgrowths were not longer than 30 μm and their neurites were not positive for TUBB3. Neurites were manually counted using ImageJ and the NeuronJ plugin. (B) Expression of neuronal and progenitor markers by quantitative real-time PCR at day 3 and 7 of DC treatment. Expression of the genes was normalized with expression of beta-actin and compared with the values obtained at day “0”. (C) Expression of marker genes at day 3 and 7 of RA treatment. (D) BrdU incorporation, at day 3 and 7 by treatment with DC and RA (n = 4). Symbols * * * * represent statistical significance P < 0.05, P < 0.01 and P < 0.001 respectively. Error bars represent mean ± S.D. of data from three biological replicates for all experiments described above.
miR-432 increased in human and mouse cells during neuronal differentiation. Induction of neurite outgrowths in Neuro2A (mouse neuroblastoma cell line) cells after treatment with DC and RA has been observed. Expression of miR-432 was also increased in this cell line after treatment with RA.

Expression of miR-432 was also increased in the SH-SY5Y cell line after treatment with RA. The expression of miR-432 was normalized with the expression of miR-301. Error bars represent mean ± S.D. of three independent experiments. Symbols *, **, *** and **** represent statistical significance *P < 0.05; **P < 0.01, ***P < 0.001 and ****P < 0.0001 respectively.

The hypothesis that miR-432 may promote neuronal differentiation was directly addressed by transfecting pre-miR-432 cloned in vector U6 and empty vector U6 (control) into SH-SY5Y cell line. Expression of pre-miR-432 increased the expression of mature miR-432 as detected by real time PCR and stem-loop specific PCR primers (Supplementary Fig. S3A). It was observed that number of TUBB3 positive cells with neurite outgrowth significantly increased in cells expressing exogenous pre-miR-432 (Fig. 3A). Inhibition of miR-432 expression by treatment with phosphorothioate antisense mature miR-432 sequence (anti-miR-432) in cells expressing pre-miR-432 resulted in inhibition of increased neurite outgrowth (Fig. 3C). We also checked that expression of mature miR-432 expression was downregulated in presence of anti-miR-432 (Supplementary Fig. S3B). Inhibition of endogenous miR-432 by anti-miR-432 induced proliferation of SH-SY5Y cells as determined by BrdU assay (Supplementary Fig. S3C).

Exogenous expression of miR-432 in DC and RA treated cells significantly increased average neurite length by ~6 and ~3.5 μm respectively 4 days posttransfection. But ectopic expression of miR-432 in DC and RA treated cells does not significantly alter the number of neurites per cell. We also knocked down expression of miR-432 in presence of DC and RA by anti-miR-432, and assayed neurite outgrowth 4 days posttransfection. Knockdown of miR-432 does not reduce the number of neurites per cell but significantly reduced average length of neurite by ~7.5 μm in DC treated cells but only ~2.5 μm in RA treated cells (Supplementary Fig. S3D).

There are other endogenous factors that are necessary for induction and maintenance of neurite outgrowth in presence of DC and RA treated cells. We next measured the expression of neuronal markers CNR1, MAP2, SYT5, BDNF, CHAT, RARα and TH mRNA expression in miR-432 transfected SH-SY5Y cells (Fig. 3D). Expression of these genes was increased more than 3-fold in the presence of miR-432. These results show that enforced miR-432 expression promotes the neuron-specific protein expression in SH-SY5Y cells.
To exclude the possibility that neuronal differentiation property of miR-432 is cell line specific; we used another human neuroblastoma cell line IMR-32. We used DC and RA reagents to induce neuronal differentiation of IMR-32 cells. The TUBB3 positive neurite growth was observed at day 3 and we followed the neuronal differentiation process up to 7 days. During this process, we observed a continuous change in morphology marked by the appearance of neurite outgrowth (Supplementary Fig. S4A) as well as upregulation of neuronal markers and downregulation of progenitor markers was also observed (Supplementary Fig. S4B). Moreover, miR-432 expression was increased significantly during DC and RA treatment at day 3 and day 7 (Supplementary Fig. S4C). We measured expression of mature miR-432 after 48hrs of transfection in IMR-32 cell to determine its overexpression (Supplementary Fig. S4D). Ectopic expression of miR-432 also significantly induced TUBB3 positive neurites in IMR-32 cell compared to vector transfected cells. Notably, cotransfection of miR-432 and anti-miR-432 reduced TUBB3 positive neurones significantly in IMR-32 cells compared to miR-432 transfected IMR-32 cells (Supplementary Fig. S4E). So, the effect of miR-432 in neuronal differentiation of IMR-32 is quiet similar to SH-SY5Y cells.

3.4. MiR-432 expression inhibits cell proliferation and arrests SH-SY5Y cells at G0–G1 phase of the cell cycle

If miR-432 induces neuronal differentiation in SH-SY5Y cells, it is expected that miR-432 should inhibit the cell proliferation. Determination of cell proliferation by BrdU incorporation revealed that exogenous expression of pre-miR-432 and cotransfection of miR-432 and anti-miR-432. Error bars represent mean ± S.D. of three biological independent experiments. Symbols *, ** and *** represent statistical significance P < 0.05; P < 0.01 and P < 0.001 respectively.

To exclude the possibility that neuronal differentiation property of miR-432 is cell line specific; we used another human neuroblastoma cell line IMR-32. We used DC and RA reagents to induce neuronal differentiation of IMR-32 cells. The TUBB3 positive neurite outgrowth was observed at day 3 and we followed the neuronal differentiation process up to 7 days. During this process, we observed a continuous change in morphology marked by the appearance of neurite outgrowth (Supplementary Fig. S4A) as well as upregulation of neuronal markers and downregulation of progenitor markers was also observed (Supplementary Fig. S4B). Moreover, miR-432 expression was increased significantly during DC and RA treatment at day 3 and day 7 (Supplementary Fig. S4C). We measured expression of mature miR-432 after 48hrs of transfection in IMR-32 cell to determine its overexpression (Supplementary Fig. S4D). Ectopic expression of miR-432 also significantly induced TUBB3 positive neurites in IMR-32 cell compared to vector transfected cells. Notably, cotransfection of miR-432 and anti-miR-432 reduced TUBB3 positive neurones significantly in IMR-32 cells compared to miR-432 transfected IMR-32 cells (Supplementary Fig. S4E). So, the effect of miR-432 in neuronal differentiation of IMR-32 is quiet similar to SH-SY5Y cells.
level of p27 protein, a known marker of cell cycle arrest at G0–G1 [32], was upregulated in cells expressing pre-miR-432 after 48hr of transfection than vector transfected cells and cotransfection with miR-432 and anti-miR-432 (Fig. 4C). Decreased incorporation of BrdU in DNA, cell cycle block at G0–G1 and increased expression of p27 by exogenous expression of miR-432 and inhibition of such changes by anti-miR-432 show that miR-432 inhibits cell growth in a condition where it induces neurogenesis/neuronal differentiation in SH-SY5Y cells.

3.5. Mir-432 targets NES, RCOR1/COREST and MECP2 in SH-SY5Y cells

In order to investigate the neuronal differentiating property of miR-432, we have searched three prediction tools (Microcosm, TargetScan Release 5.2 and RNAhybrid) to identify the neuronal differentiation associated target(s) of miR-432. As shown in Fig. 5A, miR-432 has two putative binding sites in 3’UTR of NES gene (predicted by Microcosom, RNAhybrid algorithm), one site in 3’UTR of RCOR1 and one pairing in 3’UTR of MECP2 gene (predicted by TargetScan 5.2 and RNAhybrid algorithm). MECP2 [33], has two isoforms and the longer transcript is expressed in brain [34]. Interestingly miR-432 has binding sites in this longer transcript (NM_004992). To examine the potential interaction between miR-432 and these three target genes, 3’UTR of these genes containing binding sites of miR-432, was cloned into pMIR-REPORT luciferase vector. Luciferase activity of 3’UTR of these genes has been significantly reduced in presence of miR-432 but such repression rescued after transfection of anti-miR-432 (Fig. 5B–D). Furthermore, expression of NES, MECP2 and RCOR1 mRNA and protein level was also reduced in presence of miR-432 significantly. But this reduction was rescued in presence of cotransfection of pre-miR-432 and anti-miR-432 (Fig. 5E and F). Overexpression of miR-132 mimic induced the expression of mature miR-132 in SH-SY5Y cells (data not shown) and it inhibited MECP2 expression in RNA and protein level (Supplementary Fig. S5A and S5B). Thus, both miR-132 and miR-432 repress MECP2 expression in SH-SY5Y cells.

4. Discussion

Dopaminergic and cholinergic neuron loss is associated with different neurodegenerative diseases [35,36]. Hence, we used two reagents DC and RA that are known to induce dopaminergic and cholinergic neurons respectively [37–40]. MiRNAs that are altered in presence of two reagents may have some therapeutic roles for treatment of neurodegenerative diseases. Hence, we differentiated our cells with these two reagents to strengthen our findings. Increased expression of CNR1, MAP2, SYT5, BDNF, CHAT, RARα and TH markers for neuronal cells [30] and decreased expression of neuronal progenitor markers POU5F1, NES and NANOG were observed in DC and RA treated cells. In addition, inhibition of cell proliferation was observed in such condition. This result shows DC and RA treatment induces neuronal differentiation in neuroblastoma cell lines. Among 16 miRNAs, whose expression was increased at day 7 by treatment with both the agents, increased expression of let-7a, miR-100, miR-124, miR-125b, miR-134, miR-15b, miR-16, miR-132, miR-125a, miR-137, miR-9, miR-9* and miR-34a in differentiated neuron has been reported earlier (Supplementary Table S1A). We report here increased expression of miR-126,
miR-127 and miR-432 in differentiated neurons. Among these, we identified that miR-432 induced neurite outgrowth as evident from increased expression of neuronal markers, decreased expression of neuronal progenitor markers, inhibition of cell proliferation and cell cycle arrest in miR-432 over expressing cells. Moreover expression of TH gene was induced more compared to expression of CHAT and RARα in presence of miR-432 in SH-SY5Y cells. So, miR-432 may induce expression of adrenergic phenotype more compared to cholinergic phenotype. Knockdown of miR-432 in SH-SY5Y cells couldn’t block neurite outgrowth, but decrease average length of neurite more in DC treated cells than RA treated cells. Role of other miRNAs whose expression was altered remains to be found out.

NES protein is a microtubule skeletal protein that is expressed during the early neurogenesis but is almost absent in mature neural cells. MiR-125b target NES gene in neural stem cell and it can target NES gene in SH-SY5Y cells also (Data not shown). Downregulation of NES gene after DC and RA treatment may be due to the combined effect of miR-432 and miR-125b. As miR-432 and miR-125b could target this protein and thus expression of miR-432 and miR-125b could be a marker for neuronal differentiation.

Expression of MECP2, another target of miR-432 is spatiotemporally regulated throughout human life as its loss or gain of function causes multiple neurodevelopment related disorders. Loss of MECP2 function observed in Rett syndrome patients (R106W or T158M) reduced dendritic and axonal length. BDNF expression was regulated by wild type MECP2. MECP2 is targeted by miR-132 and miR-483-5p in human brain. In our experiments, miR-132 and miR-432 suppress the expression of MECP2 in SH-SY5Y cells and expression of miR-132 and miR-432 were upregulated in DC and RA treated cells at day 7. However, the expression of their target gene MECP2 was also upregulated at day 7 of DC and RA treated cells. Elevated level of MECP2 cannot be solely explained from the increased expression of miR-132 and miR-432 in DC and RA treated cells. MECP2 is a predicted target of most of the miRNAs altered in differentiated cells (data not shown). Thus MECP2 in our experimental condition might be
regulated by several miRNAs. Transcription factor NF-kB that was upregulated in RA treated cells induced the expression level of MEC2P during neuronal differentiation [50,51]. Factors other than miR-132 and miR-432, may account for the observed increase in the expression of miR-432 in DC and RA treated cells. Thus, miR-432 might not modulate neuronal differentiation by targeting MEC2P in SH-SY5Y cells.

RCOR1, target of miR-432, co-operating with transcriptional repressor REST and other transcription regulators silences expression of neuronal genes in non-neuronal cells and neural stem cells (NSCs) [52]. Recently, it has been shown that a large number of genes might be regulated by REST independent of REST. RCON1 occupies upstream to genes without REST binding sites in NSC but not in neuronal cells [53,54]. Expression of miR-9/miR-9*, miR-124 and miR-123 are regulated by REST. RCON1 and REST are target of miR-9* and miR-9 respectively [55]. It is also known that miR-124 and miR-9 target BAFFs and involve in dendritic outgrowth [7]. Downregulation of RCON1 after DC and RA treatment can be explained by corresponding increase in miR-432 expression.

In summary, we have shown here that expression of 16 miRNAs was increased and 12 miRNAs was decreased in differentiated SH-SY5Y cells compared to control neuroblastoma SH-SY5Y cells. Possibly, this is the first report to show that human brain enriched miR-432 [28] is involved in neuronal differentiation by targeting neurogenesis related genes NES and RCON1.

**Conflict of interest**

The authors declare no conflict of interest.

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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.febslet.2014.03.015](http://dx.doi.org/10.1016/j.febslet.2014.03.015).

**References**


