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MicroRNA expression profiling of NGF-treated PC12 cells revealed a critical role for miR-221 in neuronal differentiation

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ABSTRACT

MicroRNAs (miRNAs) are small non-coding RNAs that control protein expression through translational inhibition or mRNA degradation. MiRNAs have been implicated in diverse biological processes such as development, proliferation, apoptosis and differentiation. Upon treatment with nerve growth factor (NGF), rat pheochromocytoma PC12 cells elicit neurite outgrowth and differentiate into neuron-like cells. NGF plays a critical role not only in neuronal differentiation but also in protection against apoptosis. In an attempt to identify NGF-regulated miRNAs in PC12 cells, we performed miRNA microarray analysis using total RNA harvested from cells treated with NGF. In response to NGF treatment, expression of 8 and 12 miRNAs were up- and down-regulated, respectively. Quantitative RT-PCR analysis of 11 out of 20 miRNAs verified increased expression of miR-181a*, miR-221 and miR-326, and decreased expression of miR-106b*, miR-126, miR-139-3p, miR-143, miR-210 and miR-532-3p after NGF treatment, among which miR-221 was drastically up-regulated. Functional annotation analysis of potential target genes of 7 out of 9 miRNAs excluding the passenger strands (*) revealed that NGF may regulate expression of various genes by controlling miRNA expression, including those whose functions and processes are known to be related to NGF. Overexpression of miR-221 induced neuronal differentiation of PC12 cells in the absence of NGF treatment, and also enhanced neuronal differentiation caused by low-dose NGF. Furthermore, miR-221 potentiated formation of neurite network, which was associated with increased expression of synapsin I, a marker for synapse formation. More importantly, knockdown of miR-221 expression by antagomir attenuated NGF-mediated neuronal differentiation. Finally, miR-221 decreased expression of Foxo3a and Apaf-1, both of which are known to be involved in apoptosis in PC12 cells. Our results suggest that miR-221 plays a critical role in neuronal differentiation as well as protection against apoptosis in PC12 cells.

1. Introduction

MicroRNAs (miRNAs) are small non-coding RNAs of about 22–24 nucleotides in length and control protein expression through translational inhibition or mRNA degradation mainly by binding to the 3′-untranslated region (3′-UTR) of target mRNAs (Filipowicz et al., 2008). MiRNAs regulate diverse biological processes including development, proliferation, apoptosis and differentiation. In terms of differentiation, miRNAs have been shown to control differentiation of various types of cells such as neurons (Jing et al., 2011), adipocytes (Karbiener et al., 2011), myoblasts (Gagan et al., 2011) and osteoblasts (Eskildsen et al., 2011).

Neurotrophic factors play critical roles in neuronal development and survival as well as the maintenance of synaptic plasticity and connections, and thus have been considered as potential therapeutic targets for neurodegenerative disorders such as Alzheimer's diseases (AD). AD is characterized by cognitive decline, memory impairment and behavioral abnormalities due to progressive neuronal loss which is caused by extracellular amyloid

Abbreviations: miRNA, microRNA; 3′-UTR, 3′-untranslated region; AD, Alzheimer's diseases; NGF, nerve growth factor; MAPK, mitogen-activated protein kinase; DMEM, Dulbecco's modified Eagle's medium; HS, horse serum; FBS, fetal bovine serum; GEO, Gene Expression Omnibus; Foxo3a, forkhead box O3a; Apaf-1, apoptosis protease activator protein-1; KSR, kinase suppressor of Ras.

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plaques and intracellular neurofibrillary tangles. In addition, neurite dystrophy and synaptic dysfunction are another important pathological features associated with AD (Selkoe, 2002). Pure memory impairment observed in the earliest clinical stage of AD is ascribed to subtle alterations of synaptic efficacy prior to massive neuronal death. Since the hypofunction of the cholinergic system is linked to cognitive deficits (Bartus et al., 1982; Davies and Maloney, 1976; Whitehouse et al., 1982), and mature cholinergic neurons in the basal forebrain are highly dependent on the nerve growth factor (NGF) activity (Fischer et al., 1987; Hefti and Weiner, 1986), a clinical trial of NGF gene therapy was conducted. NGF delivery to the basal forebrain resulted in a mild but significant therapeutic benefit (Tuszynski et al., 2005).

Rat pheochromocytoma PC12 cells have been popularly used as a cell culture model of neurons (Greene and Tischler, 1976). When treated with NGF, PC12 cells extend neurites and form synapse-like structures and neurite network, differentiating into neuron-like cells, which is associated with increased expression of neuronal specific genes (Das et al., 2004). NGF binding to its receptor, TrkA, results in its dimerization (Jing et al., 1992) and autophosphorylation (Stephens et al., 1994). TrkA receptor activation by NGF induces neuronal differentiation through activation of the mitogen-activated protein kinase (MAPK) pathway (Cowley et al., 1994; Obermeier et al., 1994; Pang et al., 1995; Stephens et al., 1994; Traverse et al., 1992), and also protects against apoptosis via activation of the PI3-K/Akt signaling pathway (Yao and Cooper, 1995).

In the present study, we performed miRNA microarray analysis and identified NGF-regulated miRNAs in PC12 cells. Among miRNAs up- and down-regulated by NGF treatment, we focused on miR-221 whose expression was robustly increased in response to NGF, and examined its effects on neuronal differentiation and expression of apoptotic genes. Our results suggested that miR-221 plays a critical role in neuronal differentiation as well as protection against apoptosis in PC12 cells.

2. Materials and methods

2.1. Cell culture and NGF treatment

Rat pheochromocytoma PC12 cells were obtained from RIKEN Cell Bank (Tsukuba, Ibaraki, Japan) and cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum (HS, Invitrogen, Carlsbad, CA, USA), 5% fetal bovine serum (FBS, MP Biomedicals, Irvine, CA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C under a humidified atmosphere of 5% CO_2. Cells seeded on PolyD-lysine-coated plates were treated with murine NGF-7S (Invitrogen) in DMEM containing 1% HS and 0.5% FBS, and the medium was changed every other day.

2.2. MiRNA microarray analysis and data processing

Cells were treated with 100 ng/ml NGF for 0, 12, 24 and 48 h. Total RNA containing miRNA was extracted from cells using the miRNeasy Mini Kit (Qiagen, Hilden, Germany). One hundred nanograms of total RNA were labeled using the Agilent miRNA Complete Labeling and Hybridization Kit (Agilent Technologies, Santa Clara CA, USA) according to the manufacturer's instructions. The labeled RNA was hybridized to the Agilent Rat miRNA Microarray Release 10.1 (Agilent Technologies) in a rotating hybridization oven at 10 rpm for 20 h at 65 °C. After hybridization, the microarrays were washed according to the manufacturer's instructions and scanned on an Agilent DNA Microarray Scanner with the Scan Control software (Agilent Technologies). The resulting images were processed, and raw data were collected using the Agilent Feature Extraction software. Expression data were analyzed using GeneSpring GX 11 (Agilent Technologies). The signal intensity of each probe was normalized by a percentile shift, in which each value was divided by the 75th percentile of all values in its array. Normalized expression values were used for further analysis. For pair-wise comparison analysis, only the probes which have present expression flags in at least one condition were considered. The microarray data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through the GEO Series accession number GSE32122 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE32122). Lists of predicted effectors of NGF-regulated miRNAs were generated using the TagetScan program (http://www.targetscan.org). The list was analyzed using the Ingenuity Pathways Analysis software (Ingenuity Systems, Redwood, CA, USA).

2.3. Quantitative reverse transcription-PCR

Total RNA was extracted using the miRNeasy Mini Kit (Qiagen). Target miRNA was reverse transcribed to cDNA by a gene specific primer using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). TaqMan MicroRNA Assay was then performed with Premix Ex Taq (Takara, Shiga, Japan) using the Thermal Cycler Dice Real Time System (Takara). The relative quantification value of the target miRNA, normalized to a control, U6 RNA, was calculated by the comparative Ct method.

2.4. Transfection

Cells were transfected with rno-miR-221 precursor, pre-miR miRNA precursor negative control #2, miR-221 antagomir or anti-miR miRNA inhibitor negative control #1 (Ambion, Austin, TX, USA) using Lipofectamine 2000 (Invitrogen) in Opti-MEM I reduced-serum medium for 4 h. Then, culture medium was changed to normal medium.

2.5. Quantification of neuronal differentiation

Neurite outgrowth was examined 6 days after NGF treatment, because PC12 cell differentiation and neurite growth have been shown to reach a plateau after 6 days in culture (Das et al., 2004). Phase contrast microscopic pictures were taken using a digital microscopy, BZ-8100 (Keyence, Osaka, Japan). Cells extending at least one neurite with a length longer than the diameter of the cell body were assessed as differentiated cells. The neurite length was quantified by tracing the neurite using BZ-Analyzer software (Keyence). One hundred cells from each optical field were examined. For each group, six optical fields from three independent experiments, each in duplicate, were analyzed.

2.6. Western blot analysis

Antibodies against Apaf-1 and β-actin were purchased from Millipore (Bedford, MA, USA) and Sigma (St. Louis, MO, USA), respectively. Anti-Foxo3a and -GAPDH antibodies were from Cell Signaling Technology (Beverly, MA, USA). Cells were lysed in RIPA buffer (10 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 0.1% sodium deoxycholate) containing protease inhibitor cocktails (Sigma). Cell lysates were separated by SDS–PAGE and transferred onto PVDF membranes. After blocking in 5% skim milk, membranes were incubated with a primary antibody and then incubated with a horseradish peroxidase-conjugated secondary antibody (GE Healthcare, Piscataway, NJ, USA). The immunoreactive proteins were visualized using the ECL Plus Western blotting detection reagents (GE Healthcare) and the