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AP2α transcriptional activity is essential for retinoid-induced neuronal differentiation of mesenchymal stem cells

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ABSTRACT

Pre-activation of the retinoid signaling pathway by all-trans retinoic acid facilitates neuronal differentiation of mesenchymal stem cells. Using protein/DNA based screening assays, we identified activator protein 2α as an important downstream target of all-trans retinoic acid. Although all-trans retinoic acid treatment significantly increased activator protein 2α transcriptional activity, it did not affect its expression. Inhibition of activator protein 2α with dominant-negative mutants reduced ATRA-induced differentiation of mesenchymal stem cells into neurons and reversed its associated functional recovery of memory impairment in the cell-based treatment of a hypoxic-ischemic brain damage rat model. Dominant-negative mutants of activator protein 2α inhibited the expression of neuronal markers which were induced by retinoic acid receptor β activation. All-trans retinoic acid treatment increased phosphorylation of activator protein 2α and resulted in its nuclear translocation. This was blocked by siRNA-mediated knockdown of retinoic acid receptor β. Furthermore, we found that retinoic acid receptor β directly interacted with activator protein 2α. In summary, the regulation of all-trans retinoic acid on activator protein 2α transcriptional activity was mediated by activation of retinoic acid receptor β and subsequent phosphorylation and nuclear translocation of activator protein 2α. Our results strongly suggest that activator protein 2α transcriptional activity is essential for all-trans retinoic acid-induced neuronal differentiation of mesenchymal stem cells.

1. Introduction

Mesenchymal stem cells (MSCs) derived from bone marrow are multipotent stromal cells that can be differentiated into a variety of cell types of different germ layers, including hepatocytes (from endoderm), osteoblasts, chondrocytes, and adipocytes (from mesoderm) (Jiang et al., 2002). Since MSCs are easy to isolate, grow, and have multi-potential and low immunogenicity, these progenitor cells are considered a reliable cell source in cell transplantation to replenish dying cells and regenerate damaged tissues (Chamberlain et al., 2007). MSCs can differentiate into neuronal cells (from ectoderm) in an appropriate induction environment in vitro and have been transplanted successfully in vivo to facilitate the recovery of learning and spatial memory impairments in a rat model of ischemia (Mahmood et al., 2004; Krabbe et al., 2005). These findings support the therapeutic potential of MSCs in neuronal damage and neurological diseases.

All-trans retinoic acid (ATRA), an active form of vitamin A, is known as an effective inducer of neuronal cell differentiation (Guan et al., 2001). Previously, we have shown that ATRA pretreatment of MSCs facilitates the differentiation of MSCs into neural cells in vitro, and that transplantation of ATRA pretreated MSCs in hypoxic ischemic brain damage (HIBD) neonatal rats promotes functional recovery of learning and memory (Liu et al., 2008; Bi et al., 2010). However, the molecular mechanism of ATRA-induced enhancement of neuronal differentiation of MSCs remains unclear.

Activator protein 2 (AP2) is a family of highly conserved DNA-binding transcription factors which is critically involved in cell proliferation, differentiation, and apoptosis and is required

Abbreviations: MSC, mesenchymal stem cells; ATRA, all-trans retinoic acid; MNM, modified neuronal induction medium; AP2α, activator protein 2α; dnAP2α, dominant negative deletion mutants of AP2α; ΔbHLH, deleted bHLH domain; ΔTAD, deleted TAD domain; GLuc, Gaussia luciferase; RAR, retinoic acid receptor; NSE, neuron specific enolase; MAP-2, microtubule-associated protein-2; GFAP, glial fibrillary acidic protein; Tuj1, neuron-specific class III beta-tubulin.

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in embryonic development and tumorigenesis (Hilger-Eversheim et al., 2000). It is known that ATRA can affect the expression of downstream targets through regulating transcription factors (Mark et al., 2006). From protein/DNA based screening assay, we found that in ATRA-induced neuronal induction of MSCs, the transcriptional activity of AP2 was significantly increased. The AP2 family is comprised of five isoforms, but only AP2α is expressed in MSCs. AP2α is highly expressed in neural crest cell lineages and plays an important role in their differentiation, development and emigration (Luo et al., 2003). Although AP2α has been previously shown to be retinoic acid-responsive (Liu et al., 2007), we did not observe any effect of ATRA on the gene expression of AP2α in MSCs. However, the transcriptional activity of AP2α itself was significantly up-regulated. In this study, we demonstrate that activation of AP2α plays an important role in ATRA-induced neuronal differentiation of MSCs and improves functional recovery of memory impairment in a cell-based treatment of the HIBD rat model. ATRA-stimulated AP2α transcriptional activity is mediated by RARβ and downstream phosphorylation and nuclear translocation of AP2α. These findings suggest that AP2α transcriptional activity may be a key regulatory point in ATRA-induced neuronal differentiation of MSCs.

2. Materials and methods

2.1. Chemicals

Unless indicated otherwise, all chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Cell culture and treatment

As previously described, primary rat mesenchymal stem cells were isolated from four-week old Sprague Dawley (SD) rat femurs and verified for the presence of MSC markers (Gong et al., 2011). Cells were cultured in complete Dulbecco's modified Eagle's medium/Ham's Nutrient Mixture F12 (DMEM/F12, Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO_2. Cells at a confluence of 90% were passaged. For the two-step neuronal induction, 1 μmol/L ATRA was added and maintained for 24 h. The medium was changed into modified neuronal induction media (MNM) composed of DMEM/F12, 100 units/ml penicillin, 100 μg/mL streptomycin, 1.6% DMSO, 160 μmol/L BHA, 20 mmol/L KCl, 1.6 mmol/L valproic acid, 8 μmol/L forskolin, 0.8 μmol/L hydrocortisone, and 4 μg/mL insulin for an additional 24 h. In the adenovirus-treated experimental groups, cells were infected with the indicated adenoviruses 48 h before induction.

2.3. Protein and DNA based screening assay

The screening assay was conducted by the KangChen Bio-tech Inc (Shanghai, China). Briefly, 1×10^7 MSCs were seeded in 100-mm tissue culture dishes (Corning, Lowell, CA, USA) and treated with ATRA for 24 h, with or without MNM culture for an additional 24 h. Nuclear proteins were extracted by using NE-PER Nuclear Protein Extraction Kit (Pierce, Rockford, IL, USA) and quantified with the BCA Protein Assay kit (Beyotime, Haimen, China). Biotin-labeled DNA binding probes were mixed with nuclear extract to form DNA/protein complexes, which were then passed through spin columns to remove unbound probes. The eluted bound probes were hybridized to a membrane which contained an array of 345 transcription factor consensus binding sequences (Spin Column version, Panomics, Freemont, CA, USA). After being washed, the DNA/protein array was incubated with HRP-conjugated streptavidin solution (Pierce) and visualized by using HRP Substrate Working Solution (Millipore, Billerica, MA, USA). The images of the chemiluminescent signal were captured using Syngene GBox Imaging System (Cambridge, UK) and quantitated with ScanAlyze software.

2.4. Electrophoretic mobility shift assay (EMSA)

EMSA was performed according to the manufacturer's instructions (LightShift Chemiluminescent EMSA Kit, Pierce). Nuclear proteins were prepared identically as in protein/DNA assay and incubated with biotin-labeled AP2 consensus oligonucleotides (5′-GATCGAACTGACCGCCCGCGGCCCGT-3′) in 20 μl binding reaction, containing: 10 mmol/L Tris-HCl pH 7.9, 0.5 mmol/L DTT, 100 mmol/L NaCl, 0.5 mmol/L EDTA, 10% glycerol, and 0.05% NP40. In cold competition experiments, a 10-fold molar excess of the appropriate unlabelled competitor or mutant oligonucleotides (5′-GATCGAACTGACCGCTTGCGGCCCGT-3′) were added to nuclear extract for 30 min at 4 °C. Samples were loaded onto a 6.5% polyacrylamide gel and separated in 0.5× TBE at 4 °C, followed by transfer to PVDF membrane. After crosslinked under commercial UV-light (Benchtop 3UV™ Transilluminator, Upland, CA, USA), the membrane was blocked and probed with HRP-conjugated streptavidin solution (Pierce) at room temperature for 30 min. The membrane was visualized by using HRP Substrate Working Solution (Millipore) and imaged with Syngene GBox Imaging System.

2.5. Gaussia luciferase reporter

As described previously, MSCs were transfected with homemade reporter vector pBGLuc-AP2-RE containing four copies of the AP2 consensus binding sequences upstream of Gaussia luciferase gene (GLuc) (He et al., 2011). The media of different treated cells were collected at the indicated time points, and the activity of GLuc was assessed by using the Gaussia Luciferase Assay Kit (New England Biolabs, Ipswich, MA, USA). Each assay condition was done in triplicate. The data were expressed as mean ± S.D.

2.6. Construction of adenoviruses expressing dominant negative deletion mutants of AP2α (dnAP2α)

Using pAd-AP2α plasmid containing AP2α full-length sequence as template, AP2α sequence with deleted bHLH domain (ΔbHLH) and deleted TAD domain (ΔTAD) were PCR amplified, respectively. Using the AdEasy system, two adenoviruses with dominant negative deletion mutants Ad-dnAP2α-ΔbHLH and Ad-dnAP2α-ΔTAD were constructed (He et al., 1998). MSCs were infected with Ad-dnAP2α-ΔbHLH and Ad-dnAP2α-ΔTAD to detect the effect of dominant-negative mutants on transcriptional activity of AP2α and neuronal differentiation.

2.7. RT-PCR and real-time PCR

Total RNA of different treated cells were isolated using an RNA Extraction Kit (Genemega Inc, USA). The cDNA was generated from 10 μg total RNA by reverse transcription with random primer and Superscript II reverse transcriptase (Takara, Japan), and then diluted five- to ten-times as PCR templates. Primer3.0 program was used to design PCR primers (usually 18–20mers) (Supplemental Table 1). The genes of interest were amplified by touch-down RT-PCR as follows: 94 °C for 20 s, 68 °C for 30 s, 70 °C for 20 s for 12 cycles, with 1 °C decrease per cycle, followed by 25–32 cycles at 94 °C for 20 s, 56 °C for 30 s, 70 °C for 20 s or real-time PCR with Bio-Rad protocol: 94 °C for 20 s, 56 °C for 20 s, 70 °C for 20 s, and reading plate for 40 cycles. All samples were normalized by GAPDH expression. RT-PCR products were resolved in 1.5% agarose gel electrophoresis. Based on primary data of real-time PCR Ct values, fold