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Cobalt chloride induces neuronal differentiation of human mesenchymal stem cells through upregulation of microRNA-124a

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ABSTRACT

Human mesenchymal stem cells (hMSCs) are known to have the capacity to differentiate into various cell types, including neurons. To examine our hypothesis that miRNA was involved in neuronal differentiation of hMSCs, $CoCl_2$, a hypoxia-mimicking agent was used to induce neuronal differentiation, which was assessed by determining the expression of neuronal markers such as nestin and Tuj1. Treatment of hMSCs with $CoCl_2$ led to increased expression of miR-124a, a neuron-specific miRNA. HIF-1α silencing and JNK inhibition abolished $CoCl_2$-induced miR-124a expression, suggesting that JNK and HIF-1α signals were required for the miR-124a expression induced by $CoCl_2$ in hMSCs. Overexpression of miR-124a or $CoCl_2$ treatment suppressed the expression of anti-neural proteins such as SCP1 and SOX9. Silencing of both SCP1 and SOX9 induced neuronal differentiation of hMSCs, indicating that suppression of miR-124a targets is important for $CoCl_2$-induced neuronal differentiation of hMSCs. Knockdown of HIF-1α or inhibition of JNK restored the expression of SCP1 and SOX9 in $CoCl_2$-treated cells. Inhibition of miR-124a blocked $CoCl_2$-induced suppression of SCP1 and SOX9 and abolished $CoCl_2$-induced neuronal differentiation of hMSCs. Taken together, we demonstrate that miR-124a is critically regulates $CoCl_2$-induced neuronal differentiation of hMSCs by suppressing the expression of SCP1 and SOX9.

1. Introduction

Mesenchymal stem cells (hMSCs) are also termed as stromal cells and isolated from a variety of tissues including bone marrow and adipose tissues. Owing to their capacity to differentiate into diverse cell types, including neuronal cells, hMSCs have been used in cell-based therapies to treat neurodegenerative diseases such as cerebral ischemia [1,2]. Recent reports indicate that hMSCs not only replace damaged neurons, but also supply protective neurotropic factors [3–5]. Hypoxia play pivotal roles in maintaining homeostasis and in regulating stem cell pluripotency [6]. Under hypoxic conditions, functions of hMSCs are regulated by several transcriptional factors, including the hypoxia-inducible factors (HIFs). Hypoxia-inducible factor-1α (HIF-1α) is the key regulator of cellular response to hypoxia by activating transcription of various genes involved in cellular metabolism, angiogenesis, metastasis/invasion and apoptosis. Cobalt chloride ($CoCl_2$) induces biochemical and molecular responses similar to those observed under hypoxic conditions; for example, treatment of PC12 cells with $CoCl_2$ leads to neurite outgrowth [7].

MicroRNAs (miRNAs) are small, single-stranded RNA molecules of 21–23 nucleotides in length. They fully or partially bind to their target mRNA and post-transcriptionally regulate their target genes by inducing decay of target mRNA or suppressing translation. Function of miRNA is essential for neural development and differentiation [8–10]. MiR-124a is preferentially expressed in neurons and is upregulated during neurogenesis. It has been recently found that miR-124a positively modulates the transitory progression of adult SVZ neurogenesis by repressing Sox9 [11]. Furthermore, miR-124a promotes neuronal differentiation by regulating nervous system-specific alternative splicing [12]. However, the potential role of miR-124a in neuronal differentiation of hMSCs has not been examined.

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In the study reported here, we examined the role of miRNAs in the neuronal differentiation of hMSCs. When treated with $CoCl_2$, hMSCs differentiated into neuron-like cells. These differentiated cells expressed neuronal markers such as such as doublecortin (DCX) and Tuj1. Treatment of hMSCs with $CoCl_2$ increased miR-124a expression in a HIF-1α and JNK-dependent manner. We demonstrate that miR-124a induces neuronal differentiation of hMSCs by suppressing SCP1 and SOX9. Furthermore, anti-miR-124a abolished $CoCl_2$-induced neuronal differentiation of hMSCs. Our results demonstrate for the first time that miR-124a is critical for the $CoCl_2$-induced neuronal differentiation of hMSCs.

2. Materials and methods

2.1. Cell culture and transfection

Human mesenchymal stem cells (hMSC, Lonza) were maintained at 37 °C, and 5% CO_2 in Mesenchymal Stem Cell Growth Medium (MSCGM, Lonza) supplemented with MSCGM SingleQuots (Lonza). All experiments were performed using cells of passages 5–8. Cells were passaged when the cultures reached 90% confluence and either used for experiments or redistributed to new culture plates. Cobalt chloride ($CoCl_2$) was from Sigma–Aldrich and JNK-specific inhibitor, SP600125 was purchased from Calbiochem (La Jolla, CA). For transfection, hMSCs were plated at a density of 5×10^5 cells/dish and were transfected with control or indicated siRNAs using Lipofectamine2000 (Invitrogen) according to the manufacturer's protocol. The siRNAs targeting HIF-1α(sc-44225), SCP1 (sc-37642), and SOX9 (sc-36533) were purchased from Santa Cruz Biotechnology. Control siRNA was synthesized by Genolution. Precursor miRNA-124a (pre-miR-124a) and antisense miR-124a (anti-miR-124a) were purchased from Ambion, and were respectively used for activation or inhibition of miRNA function.

2.2. Western blot analysis

For Western blot analysis, hMSCs were lysed with RIPA buffer containing protease inhibitors and phosphatase inhibitors (Roche). Protein concentrations were determined by Bradford assay. Lysates were subjected to SDS–polyacrylamide gels electrophoresis and transferred to PVDF membranes (Millipore). Membranes were blocked by incubating overnight with 5% skim milk. Following incubation with the appropriate primary and secondary antibodies, bands were visualized with an enhanced chemiluminescence (ECL, Amersham). Antibodies for SCP1 and SOX9 were purchased from R&D Systems. Antibody for β-actin was obtained from Santa Cruz.

2.3. Quantitative real-time PCR (RT-qPCR)

To determine the level of mRNA and miRNA, total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized using Superscript III Reverse Transcriptase (Invitrogen) and RT-qPCR was performed using power SYBR® Green PCR Master Mix (Applied Biosystems). The primers used in this study were as follows: *SCP1*, forward (5′-AAG CCG GGG CAT CCT CCA CT-3′) and reverse (5′-CTG GGC CTT GGC CTC AGG GA-3′); *SOX9*, forward (5′-CCC TTC GTG GAG GAG GCG GA-3′) and reverse (5′-GGC CTG CAG CGC CTT GAA GA-3′); and *β-actin*, forward (5′-TAA GGA GAA GCT GTG CTA CG-3′) and reverse (5′-TGA AGG TAG TTT CGT GGA TG-3′). β-Actin mRNA was used for normalization. The level of miR-124a was determined using stem loop-specific RT primer and TaqMan PCR Master Mix (Applied Biosystems) and was normalized against the level of U6 snRNA.

2.4. Immunohistochemical analysis

The expression level of neuronal markers, nestin and Tuj1, was determined by immunohistochemical analysis. Briefly, hMSCs were washed with PBS, fixed in 4% paraformaldehyde for 15 min, and permeabilized with PBS containing 0.2% Triton X-100 for 10 min. After blocking with 2% bovine serum albumin, they were incubated with anti-nestin or anti-Tuj1 antibody for 2 h and then incubated with Alexa Fluor® 568-conjugated anti-mouse secondary antibody (red, for nestin) or Alexa Fluor® 488-conjugated anti-rabbit secondary antibody (green, for Tuj1) (Molecular Probes) for 1 h. Nuclei were stained by applying DAPI for 30 min. The fluorescence was visualized under a confocal microscope.

2.5. Statistical analysis

Statistically significance ($P < 0.05$) of the result was analyzed using independent-sample *t*-test. Data are expressed as mean ± SEM, and represents that from 3 to 5 independent experiments.

3. Results

3.1. $CoCl_2$ induced neuronal differentiation of human mesenchymal stem cells

Human mesenchymal stem cells (hMSCs) are multipotent stromal cells having a capacity to differentiate into various cell types including neuron. Tuj1 has been known as neuron-specific class III β-tubulin and DCX is reported to be expressed by neuronal progenitor cells or immature neuron. To investigate whether treatment of $CoCl_2$ induces neuronal differentiation, hMSCs were cultured in presence of 100 μM $CoCl_2$. After 72 h incubation, morphology of hMSCs was examined under a microscope and expression of neuronal markers such as doublecortin (DCX) and Tuj1 was analyzed by Western blot. hMSCs treated with $CoCl_2$ displayed neuron-like morphological characteristics including neurite outgrowth. Treatment of hMSCs with $CoCl_2$ dramatically induced the expression of HIF-1α and neuronal markers such as DCX and Tuj1 (Fig. 1A), suggesting that hMSCs differentiated into neuronal cells under hypoxic condition. Immunohistochemical analysis confirmed that $CoCl_2$ treatment induced neuronal differentiation of hMSCs (Fig. 1B).

3.2. $CoCl_2$ increased expression of miR-124a through HIF-1α and JNK signals

Expression of miRNAs is tightly controlled during neural development, and is essential for neuronal differentiation. To examine if miRNAs were involved in $CoCl_2$-induced neuronal differentiation, hMSCs were treated with $CoCl_2$ and the expression of miR-124a was analyzed. As expected, treatment of hMSCs with $CoCl_2$ induced HIF-1α expression within 3 h. In company with HIF-1α induction, the level of miR-124a was significantly increased in $CoCl_2$-treated hMSCs (Fig. 1C). MiR-124a is known to be preferentially expressed in neurons and is upregulated during neurogenesis. It was also reported to promote neuronal differentiation by suppressing the expression of PTBP, a global repressor of alternative splicing, which triggers brain-specific alternative pre-mRNA splicing [12]. To elucidate the detail mechanism, the role of HIF-1α and JNK signal in $CoCl_2$-induced miR-124a expression was investigated. First, we examined whether HIF-1α induction was involved in $CoCl_2$-induced miR-124a expression. hMSCs were transfected with control (CTRL) or HIF-1α siRNA; 48 h post-transfection, cells were treated with DMSO or $CoCl_2$ for 3 h. The level of HIF-1α and miR-124a was determined by Western blot and RT-qPCR, respectively. Treatment